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The localization of the local anesthetic tetracaine in phospholipid vesicles: A fluorescence quenching and resonance energy transfer study

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Abstract

Fluorescence quenching and resonance energy transfer have been used to determine the localization of the local anesthetic tetracaine in vesicles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) as a function of both temperature and ionic strength. The fluorescence behaviour of tetracaine in vesicles can be attributed to its different partition coefficients in acid and basic solution, in gel phase and fluid phase vesicles, respectively. Using both steady-state and time-resolved fluorescence measurements we show that a saturable binding rather than a partitioning model holds for the interaction of tetracaine with gel phase bilayers. The relative quenching efficiencies of the series of *n*-AS dyes depend on the phase state of the bilayer and suggest a deeper incorporation of tetracaine in fluid phase than in gel phase membranes. Resonance energy transfer measurements support the view that tetracaine is incorporated predominantly in the region of the 9-AS chromophore in DMPC-bilayers. © 1997 Elsevier Science Ireland Ltd.

Keywords: Tetracaine; Fluorescence quenching; Resonance energy transfer; 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC)

1. Introduction

It is known that local anesthetics exert their pharmacological action by blocking the sodium channels of nerve axonal membranes (Roth, 1979; Roth and Miller, 1986). However, it is still a matter of debate whether this blocking is the result of a direct anesthetic–protein interaction or

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due to a perturbation of the lipid matrix surrounding the protein (Seeman, 1972; Strichartz, 1973; Trudell et al., 1973; Lee, 1976; Vanderkooi et al., 1977; Ueda et al., 1977; Trudell, 1977; Boulanger et al., 1981; Ueda and Kamaya, 1984; Buchet et al., 1986; Franks and Lieb, 1987; Beurer and Galla, 1987; Seelig, 1987). The first theories are based on the hypothesis that proteins have non-specific hydrophobic binding centers for anesthetics (Adade et al., 1987; Butterworth and Strichartz, 1990). A correlation between the affinity for membrane proteins and the anesthetic potency has been reported (Garcia-Martin and Gutiérrez-Merino, 1986).

As for the interaction with the lipid phase the observation of the increase in surface area (Seelig, 1987) and fluidity (Papahadjopoulos et al., 1975) as well as the influence on the lipid phase transition (Vanderkooi et al., 1977) supported the role of structural properties of the lipid matrix in their mode of action. In addition, the local anesthetics could interact with the lipid–protein interface, affecting the enzymatic properties of the proteins indirectly (Saeki et al., 1979).

A variety of biophysical approaches has been used to study structural and thermodynamic aspects of the interaction of amphiphilic drugs with membrane systems, including electron spin resonance (Butler et al., 1973), X-ray diffraction (Coster et al., 1981), high resolution magnetic resonance (Cerbon, 1972), deuterium nuclear magnetic resonance (Boulanger et al., 1980, 1981; Kelusky and Smith, 1983, 1984; Smith and Butler, 1985; Auger et al., 1988a, 1989) and high pressure infrared spectroscopy (Auger et al., 1988b, 1990). All these studies suggest a partial incorporation of the anesthetic into the bilayer. Moreover, it has been shown by ^{2}H NMR studies that the location of the anesthetic and its effects on the orientational and motional properties of the lipid depend on the charge of the local anesthetic and the lipid as well as the molecular structure of the lipid studied. Obviously, the precise orientation and position of a local anesthetic within the bilayer is important if a mechanistic description of the anesthetic action is desired.

It is a matter of debate whether the local anesthetic shows a saturable binding to lipid bilayers or if the process should be described by a partition coefficient. In the first case which is typical for the binding of extrinsic membrane proteins the interaction may be described by a Langmuir isotherm

$$v = \frac{nK_L[LA]}{1 + K_L[LA]} \quad (1)$$

where v is the amount of bound species, [LA] is the concentration of the free species, K_L is the association constant and n is the maximum number of binding sites per lipid molecule. If a non-saturable process is assumed, Henry's law can be used, where K_P is a partition coefficient for the anesthetic between the lipid and the water phase.

$$v = K_p[LA] \quad (2)$$

Yet, the kind of interaction for uncharged tetracaine is not exactly known.

Fluorescence spectroscopy provides another useful technique for the study of local anesthetic–membrane interaction (Sikaris and Sawyer, 1982; Continho et al., 1990). The series of n-(9-anthroyloxy) fatty acids (n-AS, where $n = 2$, 3, 6, 9, 12 with the chromophore attached to stearic acid and $n = 16$ in case of palmitic acid) provides a useful set of membrane probes, as they are located at a graded series of depth (Thulborn and Sawyer, 1978; Thulborn et al., 1978, 1979) in the bilayer. They are effectively quenched by local anesthetics of the amine type and can yield information about the precise localization of the quenching groups and thus the position of the anesthetic molecule within the bilayer.

In this work we report the influence of temperature and ionic strength on the localization of the local anesthetic tetracaine (Fig. 1) in vesicles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), using fluorescence quenching of the n-AS and resonance energy transfer from tetracaine as complementary meth-

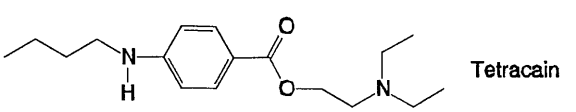

Fig. 1. Molecular structure of tetracaine.

ods. We show that static quenching has to be considered in order to reveal the binding behaviour of tetracaine to gel phase vesicles, which seems to follow a saturable binding. The localization of tetracaine depends on ionic strength and is shown to be different in gel and fluid phase vesicles, with a deeper incorporation of tetracaine in the liquid crystalline phase.

2. Materials and methods

DMPC and tetracaine were obtained from Fluka. The series of *n*-AS dyes was supplied by Molecular Probes. All other compounds were of the highest purity commercially available. Large unilamellar vesicles (LUVET) were prepared by the extrusion method of MacDonald et al. (1991) in a borate-phosphate-citrate buffer (Küster, 1985). For the quenching experiments, lipid–dye mixtures (100:1) were dried under a nitrogen stream and then overnight in vacuum to remove traces of solvent. The appropriate buffer was added to a concentration of 5 mM lipid. The mixture was vortexed at a temperature above the lipid phase transition and extruded through polycarbonate filters (Nucleopor; 100 nm pore size) using a LiposoFast extruder (Avestin).

Fluorescence spectra were recorded using 10 × 10 mm quartz cuvettes in an Aminco Bowman II spectrometer. Temperature was controlled within 0.2°C. Emission spectra were corrected for instrument response.

Fluorescence decays were measured using commercial single photon counting equipment (Edinburgh Instruments 199 S) using a nitrogen flashlamp for excitation. Data acquisition and analysis has been performed as described elsewhere, using a least-squares iterative reconvolution technique (Marquard Levenberg algorithm) (Hof et al., 1989). For tetracaine in solution, the decays were monoexponential with maximum standard deviations of less than 3% for several independent measurements. The decays of the AS-dyes near the emission maximum could be well described by a biexponential decay law (Hutterer et al., 1997). For the Stern–Volmer plots mean decay times were calculated according to

$$\langle\tau\rangle = (A_1\tau_1 + A_2\tau_2)/100 \quad (3)$$

where τ_1, τ_2 are the decay times and A_1, A_2 the corresponding amplitudes. Both lifetimes are quenched with comparable efficiency. A detailed comparison of the decay behaviour of the AS-dyes at different emission wavelengths in phosphatidylcholine vesicles and isotropic solvents of different viscosity has been given elsewhere (Hutterer et al., 1997).

2.1. Fluorescence quenching

The dynamic quenching of a fluorophore in its excited state in free solution is described by the Stern–Volmer equation

$$\frac{\Phi_0}{\Phi} = 1 + K_{SV}[Q] = \frac{\tau_0}{\tau} \quad (4)$$

where Φ_0 and Φ, τ_0 and τ are the yield and lifetime in the absence and presence of quencher Q, respectively. K_{SV} is the collisional quenching constant which is equal to $k_q\tau_0$, where k_q is the apparent rate constant for the collisional quenching process. If static quenching also occurs a modified form of Eq. (4) holds (Eftink and Ghiron, 1976)

$$\frac{\Phi_0}{\Phi} = (1 + K_{SV}[Q])\, e^{V[Q]} \quad (5)$$

where V is the static quenching constant. Several models have been proposed to describe the significance of V (Smoluchowski, 1917; Frank and Wawilow, 1931; Boaz and Rollefson, 1950; Moon et al., 1965). The contribution of static quenching leads to an upward curvature compared to the plot of purely dynamic quenching (Eftink and Ghiron, 1976). As the lifetimes are not affected by static quenching, the pure dynamic contribution can be obtained from a plot of τ_0/τ versus $[Q]$ (Chance et al., 1975). If a fluorophore in a phospholipid dispersion is bound exclusively to the bilayer phase or is fluorescent only in this phase, quenching will depend on the effective concentration of quencher in the lipid phase $\langle Q\rangle$. The distribution of quencher between the aqueous and the lipid phase may be described either by a partition coefficient, a saturable binding or a com-

bination of partitioning and quenching (Blatt et al., 1986). $\langle Q \rangle$ is then given by

$$\langle Q \rangle = \langle Q_P \rangle + \langle Q_B \rangle = V_L K_P [Q_A] + \frac{s K_B [Q_A]}{1 + K_B [Q_A]} \quad (6)$$

where Q_A is the concentration of quencher in the aqueous phase, $\langle Q_P \rangle$ and $\langle Q_B \rangle$ are the average numbers of partitioned and bound quenchers per lipid structure, respectively, K_P is the partition coefficient, K_B the binding constant and s the number of binding sites. While a Stern–Volmer plot using the total quencher concentration remains linear (in the absence of static quenching) (Thulborn, 1981), saturable binding of the quencher leads to negative deviations from linearity. If both $\langle Q_P \rangle$ and $\langle Q_B \rangle$ contribute by static and dynamic quenching, the Stern–Volmer equation may be written as

$$\frac{\Phi_0}{\Phi} = \left[1 + \frac{K_{SV,P} \langle Q_P \rangle + K_{SV,B} \langle Q_B \rangle}{V_L} \right] \times e^{V(\langle Q_P \rangle + \langle Q_B \rangle)/V_L} \quad (7)$$

Quenching titrations were carried out by adding aliquots of anesthetic stock solution directly to the fluorescence cuvette. Relative quenching efficiencies have been determined plotting either I_0/I (which equals Φ_0/Φ if the shape of the fluorescence spectra do not change by quenching) or τ_0/τ versus $[Q]$.

2.2. Resonance energy transfer

The energy transfer efficiency E is defined as the ratio of the transfer rate k_T to the sum of the rates of all deactivation processes:

$$E = \frac{k_T}{k_T + k_F + k_{nr}} \quad (8)$$

where k_F is the rate of the fluorescence decay and k_{nr} the sum of all other non-radiative processes. E was obtained from the relative quantum yields of the donor determined in the presence and absence of acceptor

$$E = 1 - \frac{\Phi}{\Phi_0} \quad (9)$$

where Φ is the quantum yield in the presence and Φ_0 in the absence of acceptor. Values of Φ_0/Φ were obtained by integration of the tetracaine emission spectra between 336–372 nm (corresponding to the wavelengths of half maximum intensity) to avoid contributions of *n*-AS emission. *n*-AS dyes were added to vesicles containing tetracaine from a concentrated EtOH-solution and the mixture was allowed to equilibrate for 30 min to ensure complete uptake of the probes. All energy transfer measurements were done at 15°C.

3. Results

3.1. Fluorescence properties of tetracaine

Tetracaine in buffer has an absorption maximum at 310 nm. As expected for a dye molecule containing both a donor and an acceptor group in the *para* position of an aromatic system, its emission is dependent on the polarity of the environment. While an emission maximum of 366 nm was found for tetracaine in buffer, this value was considerably blue shifted in lipid vesicles (λ_{em} = 351 nm). In buffer, λ_{em} is independent of pH and temperature; the decay time is short ($\sim$330 ps). Because of the higher quantum yield in vesicles (i.e. DMPC-LUVET) compared to buffer the fluorescence intensity increases strongly with lipid concentration. Lower fluorescence intensities were obtained in acidic solution (pH 5.5), where tetracaine is protonated, than in basic solution (pH 9.0), as shown in Fig. 2. This result reflects the higher partition coefficient reported for tetracaine at pH 9.0 compared to pH 5.0 for neutral membranes (Boulanger et al., 1980; Okahata and Ebato, 1991). The position of the emission maximum is also dependent on lipid concentration and pH. For higher vesicle concentrations, the emission maximum λ_{em} is observed at 351 nm; it increases with decreasing lipid concentration. This shift is more pronounced in acidic solution where tetracaine has a lower affinity for the lipid phase. The lower the lipid concentration and the pH value the more tetracaine molecules remain in the buffer causing the red-shift in the emission maximum and the decrease in fluorescence intensity.

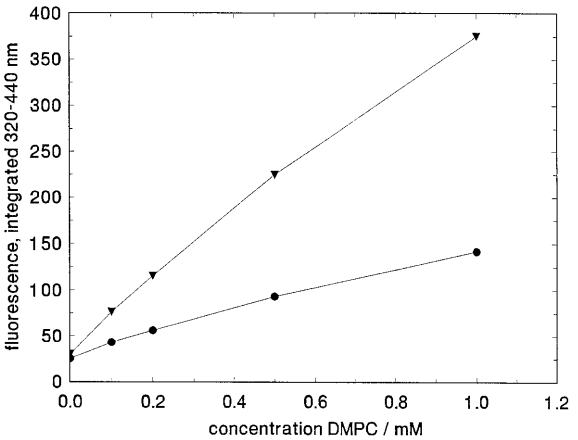

Fig. 2. Integrated fluorescence intensities (320–440 nm) of tetracaine (150 μM) in DMPC vesicles at $T = 15$°C and pH = 5.8 (circles) and pH = 9.0 (triangles).

The temperature dependence of the fluorescence intensity of tetracaine (150 μM) in DMPC (1 mM) was determined in both acidic (pH 5.0) and basic (pH 9.0) solution. An increase in the fluorescence intensity was observed from 15 to 25°C for both pH values (Fig. 3a). Above the phase transition of DMPC the intensity decreased continuously. This behaviour is different from that of most other frequently employed membrane probes like 1,6-diphenyl-1,3,5-hexatrien (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatrien (TMA-DPH), 6-palmitoyl-2-[[trimethylammoniumethyl]methylamino]naphthalene chloride (Patman), 6-propionyl-2-(dimethylamino)-naphthaline (Prodan) and the *n*-AS which show a continuous decrease in fluorescence intensity with increasing temperature (Hutterer et al., 1996). In contrast to the fluorescence intensity the decay times of tetracaine in DMPC decrease smoothly with increasing temperature from 0.80 ns at 15°C to 0.50 ns at 45°C with the steepest decrease in the region of the phase transition (Fig. 3b).

3.2. The location of tetracaine as a function of temperature and ionic strength as determined by fluorescence quenching of n-AS

The series of *n*-AS was chosen for this study because they could be selectively excited at 358 nm where tetracaine does not absorb and because they are located at a defined depth of the bilayer. While in fluid solutions quenching processes are mostly dynamic, a contribution of static quenching has to be taken into account in vesicle systems (Blatt et al., 1986; Stubbs and Williams, 1992). For pure collisional quenching Stern–Volmer plots of I_0/I or τ_0/τ versus quencher concentration $[Q]$ should yield identical results; thus, contributions of static quenching are revealed by different slopes of both plots. A plot of I_0/I and τ_0/τ, respectively, for 12-AS in DMPC at pH 9.0 both in the gel and in the liquid crystalline phase is given in Fig. 4. For both temperatures, the decrease of the intensities is much more pronounced than in the mean lifetimes, providing evidence for a contribution of static quenching. The quenching efficiencies are higher in the liquid crystalline phase monitored by both I_0/I and τ_0/τ; however, the decrease of the lifetimes is larger in the liquid phase than in the gel phase. This behaviour suggests a considerably larger contribution of the collisional quenching above the phase transition temperature T_c.

For the interaction of local anesthetics with lipid membranes both a partitioning process and a saturable binding have been proposed. In the case of an adsorption process (incomplete binding of the quencher) a negative deviation from a linear Stern–Volmer plot results while contributions of static quenching yield positive deviations for I_0/I. In order to detect a possible negative deviation due to incomplete binding of quencher in the presence of static quenching the decay times of the *n*-AS dyes were measured as a function of tetracaine concentration. The results are shown in Fig. 5 for 15°C (a) and 45°C (b), respectively. The plots of τ_0/τ versus quencher concentration at 15°C show large negative deviations from linearity, suggesting a saturable binding of tetracaine to the gel phase bilayer. In the liquid crystalline phase at 45°C quenching is much stronger; how-

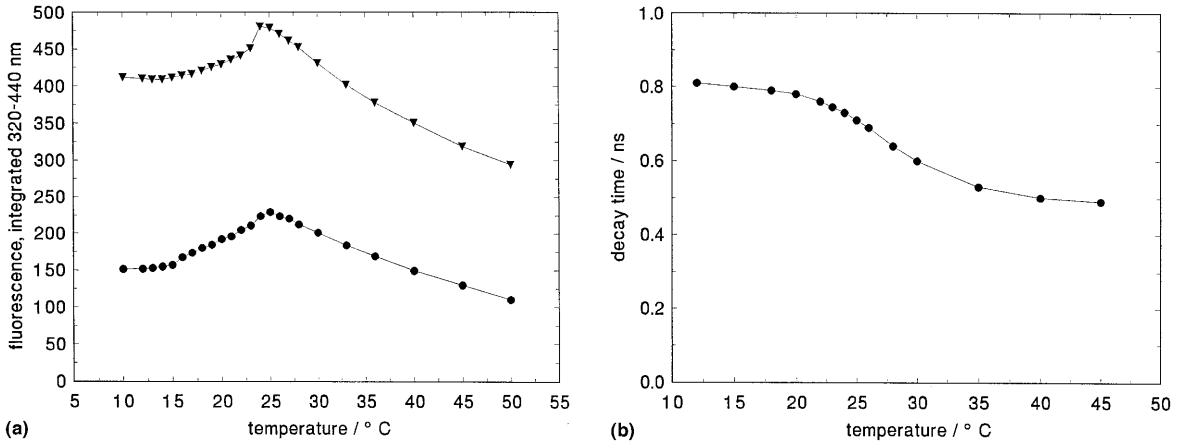

Fig. 3. Temperature dependence of (a) integrated fluorescence intensities at pH = 5.8 (circles) and pH = 9.0 (triangles) and (b) fluorescence lifetimes at pH = 9.0 of tetracaine in DMPC vesicles; $\lambda_{ex} = 316$ nm, $\lambda_{em} = 351$ nm.

ever, smaller negative deviations are observed for all dyes. The latter result shows that the binding of tetracaine is considerably facilitated if the lipid matrix is in the fluid phase.

The quenching efficiencies are different within the series of *n*-AS for both examined temperatures. In the gel phase the relative quenching efficiencies are in the order 12-AS > 16-AP > 9-AS > 3-AS > 6-AS, while in the fluid phase the order is 16-AP ≫ 12-AS ≫ 9-AS > 3-AS > 6-AS. Thus, the localization of tetracaine must be assumed to depend on the phase state of the bilayer. Above the phase transition tetracaine seems to be incorporated deeper in the membrane. For both temperatures 6-AS is quenched least of all *n*-AS dyes. Two possible explanations might be given for that behaviour: Either, there is a second localization of tetracaine close to the headgroup region (besides the preferred one in the bilayer interior) or another process contributes to the quenching of 3-AS. This could be quenching by an increased amount of water molecules in the upper membrane region due to the incorporation of the local anesthetic.

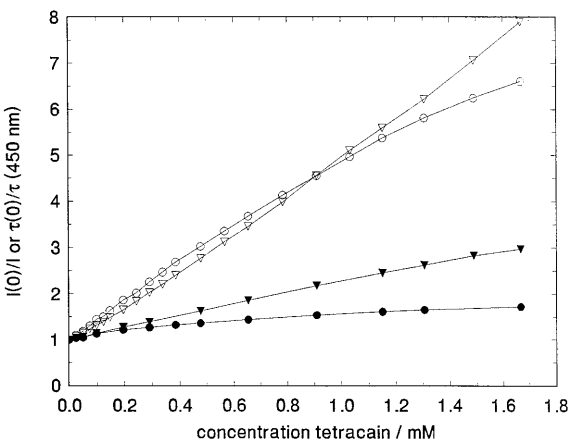

Fig. 4. Decay times (filled symbols) and fluorescence intensities (open symbols) at 15°C (circles) and 45°C (triangles) as a function of tetracaine concentration for 12-AS in DMPC (1 mM); pH = 9.0, 0.1 M NaCl, $\lambda_{ex} = 358$ nm, $\lambda_{em} = 450$ nm.

In order to investigate the influence of temperature and ionic strength on the quenching behaviour (i.e. the localization of tetracaine), fluorescence intensities of the series of *n*-AS were determined at different tetracaine concentrations. As the relative quenching rates of the *n*-AS were independent of the concentration of tetracaine, quenching profiles, showing the relative quenching efficiency as a function of the position of the AS chromophore could be obtained. In the gel phase (15°C) small negative deviations from linear Stern–Volmer behaviour are observed (Fig. 6) at both high (0.1 M NaCl, (A)) and low (without NaCl, (B)) ionic strength despite contributions of static quenching, suggesting a saturable binding. At high ionic strength the efficiency of quenching follows the order 12-AS ≫ 16-AP ≫ 9-AS ≫ 3-

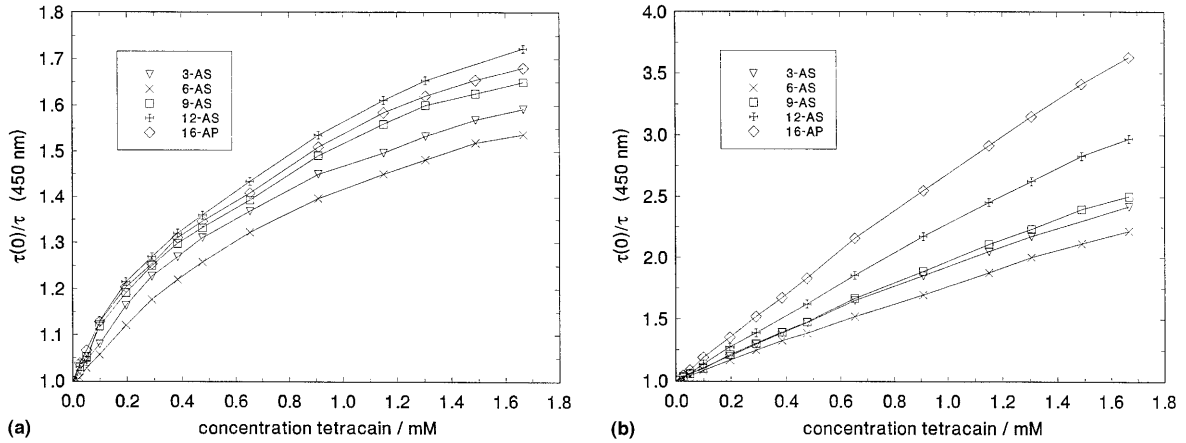

Fig. 5. Behaviour of the decay times of the AS-dyes with increasing tetracaine concentration in DMPC vesicles (1 mM); 10 μM n-AS, 0.1 M NaCl, $\lambda_{ex} = 358$ nm, $\lambda_{em} = 450$ nm; (a) $T = 15$°C, (b) $T = 45$°C.

AS > 6-AS. Without NaCl the quenching is somewhat lower and another profile is obtained. Efficiency is now in the order 16-AP > 12-AS ≫ 9-AS ≫ 3-AS > 6-AS (Fig. 7). Smaller differences are observed in the inner bilayer region, i.e. for 9-, 12-AS and 16-AP.

In the liquid crystalline phase (45°C) negative deviations from linearity are no longer observed; the plots of I_0/I versus quencher concentration are nearly linear. A comparison with the plot of τ_0/τ (Fig. 5) shows, however, that the binding of the local anesthetic still shows some saturation, although less than in the gel phase. The positive deviation caused by the contribution of static quenching compensates the negative one due to incomplete incorporation of tetracaine. At low ionic strength positive deviations from linearity are visible for 9-, 12-AS, 16-AP and less for 3- and 6-AS, showing the importance of static quenching. The order of relative quenching efficiencies is the same for high and low ionic strength, respectively: 16-AP ≫ 12-AS ≫ 9-AS > 3-AS > 6-AS. However, the differences in quenching efficiencies are somewhat larger at high ionic strengths, suggesting a more distinct localization of tetracaine. Compared to the gel phase, a deeper incorporation of tetracaine in the fluid phase can be assumed. This effect is more pronounced at high than at low ionic strength.

3.3. The localization of tetracaine as determined by Förster energy transfer measurements

Because of the favourable overlap of the emission spectrum of tetracaine with the n-AS absorption spectra, a radiationless energy transfer from the local anesthetic to the anthranoyloxy chromophore should be possible. It is assumed that the relative orientation of the chromophores of tetracaine and the AS dyes in the membrane are the same for all members of the n-AS series; thus, the transfer efficiency is a function of the distance between the molecules only.

Energy transfer experiments ($\lambda_{ex} = 320$ nm) were done for the n-AS in DMPC (1 mM) at pH 9.0 in the presence of 150 μM tetracaine and 0.1 M NaCl at 15°C. In order to exclude a trivial emission–reabsorption process, some decay times of tetracaine in the presence of n-AS were determined. In all cases a decrease in decay times was observed, as expected for a radiationless transfer. Two representative data sets for 2-AS and 16-AP, respectively, are shown in Fig. 8. As the absorption spectra of all n-AS are identical (Continho et al., 1990) the spectral overlaps between donor and acceptor are also identical. Thus, from these experiments, values of relative quantum yields Φ_0/Φ could be obtained as a function of the acceptor (n-AS) concentration. The spectra were integrated between 336 and 373 nm (wavelengths of half

maximum intensity) in order to avoid a contribution from *n*-AS fluorescence. In Fig. 9 plots of the energy transfer efficiencies calculated according to Eq. (9) versus the concentration of the respective *n*-AS are given. The qualitative behaviour is similar for 2-, 6-, 9- and 12-AS, but not for 16-AP which yields a much higher slope and approaches saturation earlier.

4. Discussion

In the present study, we reinvestigated the fluorescence behaviour of the local anesthetic tetracaine in model membranes and determined the influence of temperature and ionic strength on fluorescence quenching of the series of *n*-AS using both steady-state and time-resolved fluorescence.

The fluorescence intensity of tetracaine has been shown to depend strongly on the lipid concentration due to the large differences in quantum yields of tetracaine in buffer compared to the lipid phase. Lower fluorescence intensities were obtained in acidic solution in agreement with the previously determined partition coefficients (Boulanger et al., 1980) which are considerably smaller for the charged tetracaine in neutral lipids. The red-shift in the emission maximum with decreasing lipid concentration is also in agreement with

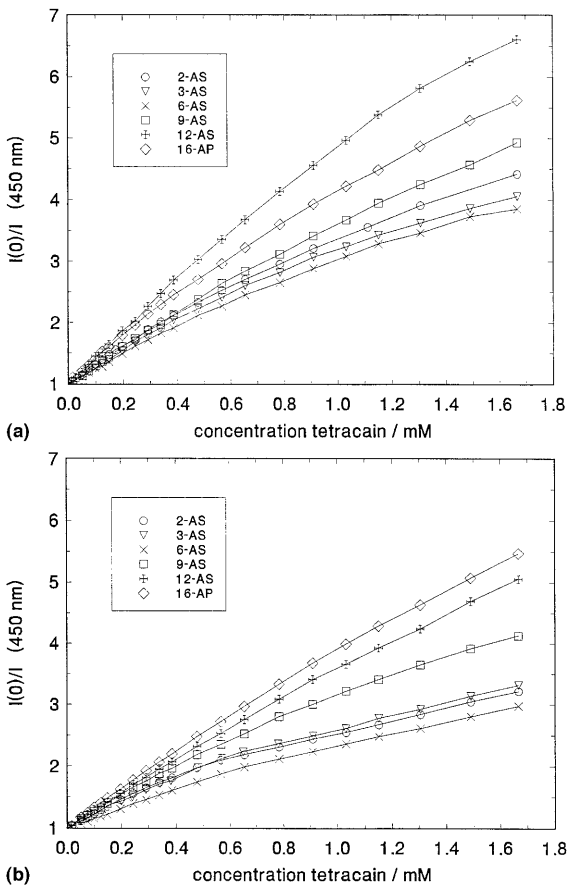

Fig. 6. Behaviour of the fluorescence intensities of the AS-dyes with increasing tetracaine concentration in DMPC vesicles (1 mM); 10 μM *n*-AS, $\lambda_{ex} = 358$ nm, $\lambda_{em} = 450$ nm; $T = 15$°C, pH = 9.0; (a) at high ionic strength (0.1 M NaCl), (b) low ionic strength (without NaCl).

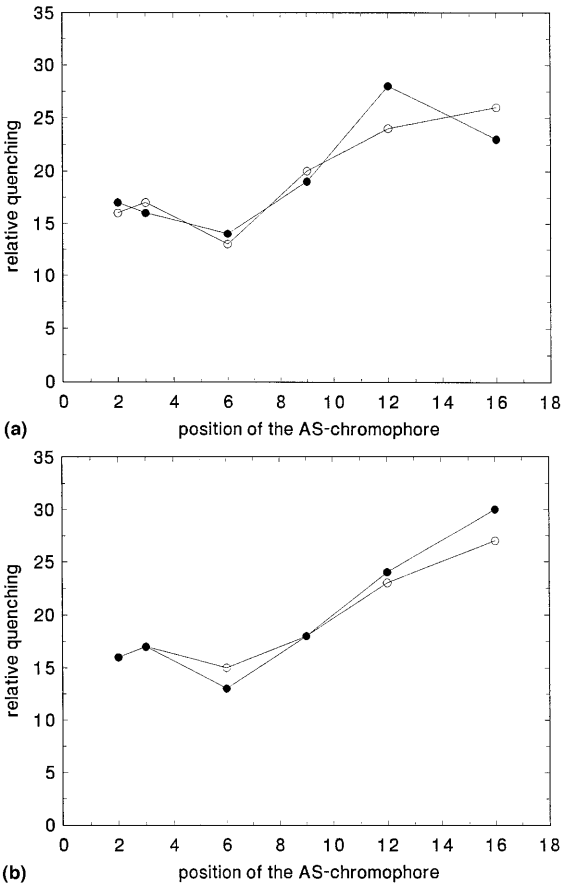

Fig. 7. Profiles of the quenching rates for tetracaine in DMPC vesicles in the absence (open circles) and presence (filled circles) of 0.1 M NaCl; (a) $T = 15$°C, (b) $T = 45$°C.

increasing amounts of free tetracaine. The temperature dependence of the fluorescence intensities of tetracaine in DMPC vesicles is unusual, as most fluorescent amphiphiles (i.e. Prodan, Patman) as well as hydrophobic probes like DPH or TMA-DPH show a continuous decrease of the fluorescence intensity with increasing temperature. This behaviour may be understood considering the larger partition coefficients for tetracaine in the liquid crystalline phase of bilayer membranes. Although we showed that the interaction of tetracaine with model membranes seems to follow saturable binding the published partition coefficients should give a reasonable estimate for the binding capacity of charged and neutral tetracaine. As binding occurs more easily to fluid phase membranes an increase in fluorescence intensity from 15 to 25°C may represent increased membrane binding (Kaminoh et al., 1988; Okahata and Ebato, 1991). Above T_c, binding might further increase, but this effect is outweighed by increased collisional quenching in fluid phase membranes, leading to the observed decrease in intensity. As the amount of bound tetracaine does not influence the decay times, only the effect of increased quenching is observed. Thus, the decay times do not show a maximum but a smooth decrease which is most pronounced near the phase transition temperature where the largest increase in quenching efficiency should occur.

The quenching of AS-dyes by tetracaine as a means to study the localization of the local anesthetic has been introduced by Sikaris and Sawyer (1982). However, this study did not take into account static quenching and was restricted to the gel phase and a single ionic strength ($I = 0.01$, pH 9.5). By using both steady-state and time-resolved measurements we were able to show the contribution of static quenching to the overall quenching efficiency. A higher efficiency was obtained for the liquid crystalline phase. This effect is predominantly due to increased collisional quenching, as

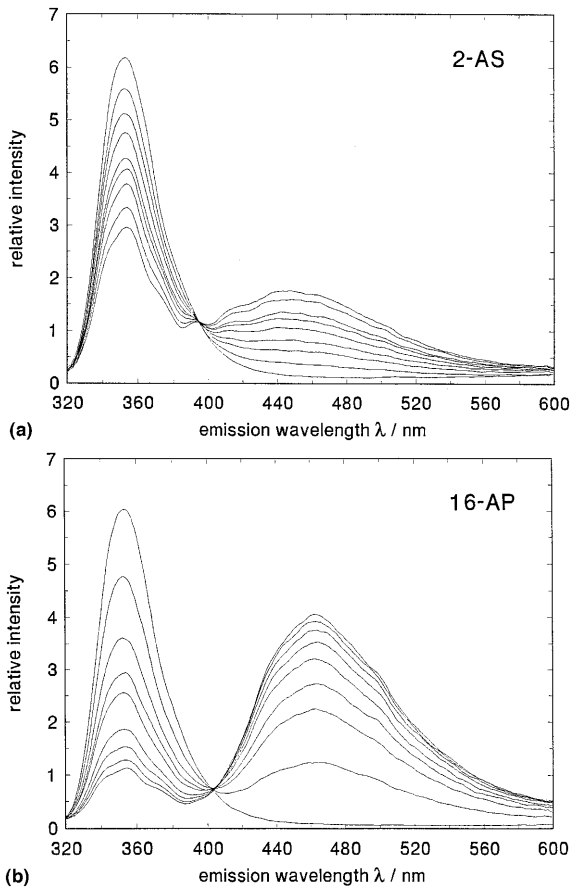

Fig. 8. Energy transfer from tetracaine to *n*-AS in DMPC vesicles (1 mM); pH = 9.0, 0.1 M NaCl, $\lambda_{ex} = 310$ nm, $T = 15$°C. The respective chromophore is given in the figure. The concentrations of 2-AS and 16-AP were (from top down at 350 nm): 0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0 μM.

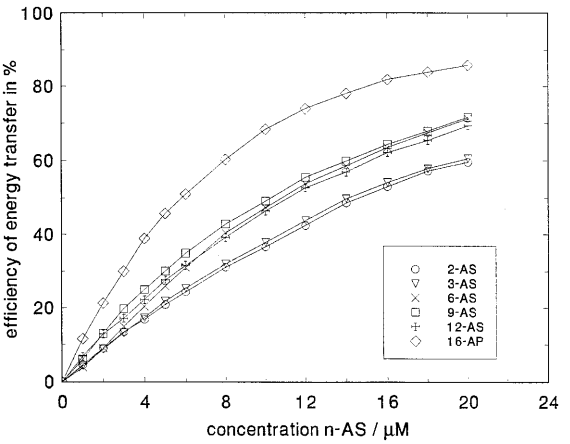

Fig. 9. Relative efficiencies of energy transfer, calculated according to Eq. (8), for the AS-dyes in DMPC vesicles (1 mM); pH = 9.0, 0.1 M NaCl, $\lambda_{ex} = 310$ nm, $T = 15$°C. The spectra were integrated from 336 to 373 nm.

revealed by the much larger decrease of the fluorescence lifetimes above T_c (Fig. 4). This result is reasonable because of the much faster diffusion in liquid crystalline compared to gel phase bilayers.

It is an old question whether the interaction of local anesthetics with phospholipid bilayers is better described by a saturable binding model or by a partitioning equilibrium (Barghouti et al., 1993). Negative deviations from linearity in Stern–Volmer plots are commonly interpreted in favor of a binding model while a linear behaviour is expected for partitioning (Stubbs and Williams, 1992). However, such negative deviations may be masked by contributions of static quenching, causing positive deviations from linearity in steady-state Stern–Volmer plots (Smoluchowski, 1917), if I_0/I-values are plotted versus the quencher concentration $[Q]$. Therefore, time-resolved measurements were performed in order to detect possible negative deviations in the presence of static quenching, as the lifetimes are unaffected by static quenching. In the gel phase of DMPC, large negative deviations from linear Stern–Volmer behavior were observed for all n-AS suggesting a saturable binding of tetracaine to gel phase bilayers. A similar behaviour was observed for the binding of tetracaine to erythrocyte ghosts (Sikaris and Sawyer, 1982). The transition to the liquid crystalline phase results in a considerable lateral expansion of the bilayer (Continho et al., 1990) and decreased lipid order in the region of the hydrophobic acyl chains. Thus, uptake of the amphiphilic tetracaine molecules should be facilitated. Much smaller deviations from linear behaviour have been obtained for plots of τ_0/τ versus $[Q]$ at 45°C. This shows that saturation of binding is much less a problem in the liquid crystalline state. Alternatively, a partitioning model may hold for the interaction of tetracaine with DMPC in the liquid phase. Both models have been compared for the binding of another local anesthetic, dibucaine (Barghouti et al., 1993) to DMPC bilayers at pH 5.0, using equilibrium dialysis. At 45°C the data could be nearly equally well fitted, assuming either a saturating (Langmuir) or a non-saturating (Henry's) model. Thus, while a clear decision between both models seems to be difficult in the liquid crystalline phase our data strongly suggest a saturable binding for the gel phase and also favour this model for the liquid crystalline phase.

The observed quenching of n-AS by tetracaine has been attributed to an intermolecular charge-transfer mechanism with the aromatic amine as the donor due to its lower ionization potential (Sikaris and Sawyer, 1982). Different relative quenching efficiencies were obtained for the gel- and the liquid phase. While in the gel phase 12-AS was quenched most strongly, the relative order in the fluid phase was 16-AP $\gg$ 12-AS $\gg$ 9-AS. This observation suggests that tetracaine is incorporated more deeply into the fluid phase membrane. However, we have also shown that the relative quenching efficiencies depend on ionic strength. Low ionic strengths lead to smaller differences in the relative quenching efficiencies, with most efficient quenching of 16-AP also in the gel phase. Above the phase transition the relative quenching order is the same both at low and high ionic strength.

The exact reason for the influence of the ionic strength is not yet known but the results demonstrate that the effects of ionic strength have to be born in mind when the localization of a local anesthetic or another amphiphile in a bilayer is determined.

The favourable overlap of the tetracaine emission and the n-AS absorption spectra suggest the n-AS dyes as good candidates for locating a donor molecule in the membrane by resonance energy transfer measurements. Because the absorption spectra of all n-AS dyes are identical, the overlap with tetracaine emission is also identical, i.e. R_0 is the same for all pairs (Continho et al., 1990). Thus, no errors in determining different R_0-values are introduced. Although a minor portion of tetracaine will reside free in solution, energy transfer will only be significant between donor and acceptor molecules incorporated into the vesicles. A qualitative comparison of distances is therefore possible. The Φ_0/Φ-values for the same concentrations of acceptor in the series of probes reflect the relative distance between donor and acceptor (Frank and Wawilow, 1931), yielding information on the localization of the local anesthetic.

We have used this method for the *n*-AS in DMPC at pH 9.0 in the presence of 0.1 M NaCl at 15°C. The much higher transfer efficiency to 16-AP can be rationalized as follows. The neutral tetracaine should be present in both leaflets of the membrane. The *n*-AS are deprotonated at pH 9.0; thus, they will be incorporated mainly in the outer leaflet when added to the vesicle suspension. 16-AP is localized close to the middle of the bilayer, yielding energy transfer from tetracaine in both the outer and inner leaflet. For the other *n*-AS, energy transfer will be predominantly from tetracaine in the outer monolayer. The transfer efficiencies were found to be very similar for 12-AS, 9-AS and 6-AS and considerably smaller for 2- and 3-AS. This suggests a localization of the aromatic ring of tetracaine in the region between 6- and 12-AS. These results may be compared with the quenching efficiencies.

Quenching has been shown to be highest for 12-AS, followed by 16-AP and 9-AS. The amino group bound to the aromatic ring is the molecular domain of tetracaine assumed to be responsible for the fluorescence quenching. This part of the molecule is located somewhat deeper in the membrane than the aromatic ring responsible for energy transfer. Thus, the maximum of quenching efficiency of 12-AS compared to the order of transfer efficiency (maximum for 9-AS, with exclusion of 16-AP) is reasonable.

While 2- and 3-AS show the lowest transfer efficiency they are more strongly quenched than 6-AS. This fact suggests the contribution of secondary effects to the high quenching of 2- and 3-AS. The intercalation of local anesthetics in the bilayer leads to an expansion of the headgroup region, as shown by Langmuir film balance studies (Seelig, 1987). ^{1}H NMR studies (Eriksson, 1986) also suggest a perturbation of the conformation of the phospholipid headgroups. Such perturbations should lead to increased penetration of water molecules into the headgroup region contributing to the quenching of 2- and 3-AS. *n*-AS dyes are effectively quenched by water, as shown by their low quantum yields in this solvent. The shorter lifetimes of 2- and 3-AS compared to 6-, 9-, 12-AS and 16-AP as well as their much larger time-dependent Stokes shift and faster solvent relaxation (Hutterer et al., 1997) also show the important role of bilayer water for the fluorescence behaviour of 2- and 3-AS.

5. Conclusion

The fluorescence properties of tetracaine in DMPC vesicles reflect the previously determined partition coefficients which are sensitive to pH and temperature. The measurement of fluorescence decays of the series of *n*-AS as a function of tetracaine concentration in comparison with steady-state data reveals a large contribution of static quenching and suggests a binding rather than a partitioning mechanism because of pronounced negative deviations of plots of τ_0/τ versus quencher concentration from linear Stern–Volmer behaviour. As shown by the maximal relative quenching efficiencies for 12-AS and 16-AP in the gel- and fluid phase, respectively, tetracaine is incorporated deep in the membrane. Measurements of relative resonance energy transfer efficiencies give additional support for a localization of tetracaine in the region of the chromophores of 9- and 12-AS in DMPC vesicles.

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