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Dibucaine interaction with phospholipid vesicles

A resonance energy-transfer study

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Resonance energy transfer between a local anaesthetic, dibucaine (donor) and a set of functionalized probes [*n*-(9-anthroyloxy)stearic acids, $n = 2, 3, 6, 7, 9$ and 12 and 16-(9-anthroyloxy)palmitic acid] (acceptors) was found to be an efficient process with a critical radius of transfer $R_0 = 2.1$ nm, this interaction being used to locate the drug in a model membrane system, small unilamellar vesicles of dipalmitoylglycerophosphocholine, both above and below the temperature of the gel-to-the-liquid-crystal transition of the phospholipid.

From the sequence of relative quenching efficiencies of dibucaine fluorescence upon incorporation of the probes, it was concluded that the drug intercalates in the membrane near the glycerol backbone of the lipid. In addition, it was found that dibucaine location is not significantly affected upon crossing the phase-transition temperature of the phospholipid.

Dibucaine photophysics was also studied and the short lifetime of the neutral form of the anaesthetic with respect to that of the monoprotonated species was attributed to an intramolecular charge-transfer interaction.

From the study of its partition coefficient between the membrane and the aqueous phase, it was concluded that the only significant species present in the membrane is the charged one.

Two main groups of theories exist to explain the mechanism of action of local anaesthetics: (a) direct interaction with membrane proteins or (b) induced lipid structural alterations [1].The first theories are based on the hypothesis that proteins have non-specific hydrophobic binding centers for anaesthetics [2], a correlation between affinity for membrane proteins and potency being reported [3]. As for the interaction with lipids, the observations of an increase in the surface area [4] and fluidity [5], as well as an effect on lipid-phase transition of the membranes upon addition of the anaesthetics [6] supported the role of lipid-matrix structural alterations in their mode of action. The possibility of a specific interaction between these drugs and the acidic phospholipids, which may also interact competitively with divalent cations, has also been advanced [7]. In addition,the local anaesthetics may exert their action on the lipid-protein interface, the enzymatic properties of the proteins being affected indirectly in this way [8].

In the present study we were concerned with the lipid-anaesthetic interaction. Since there are only a few studies about this subject at the molecular level, our purpose was to determine the depth (transverse location) of a local anaesthetic, dibucaine, inside the phospholipid bilayer of a model membrane system, small unilamellar vesicles (SUV) of dipalmitoylglycerophosphocholine ($Pam_2GroPCho$), using resonance energy transfer, both below and above the phase-transition temperature (t_c) of $Pam_2GroPCho$. A set of donor-acceptor pairs exhibiting graded separations was used, dibucaine being the donor and the family of *n*-(9-anthroyloxy)stearic acids probes (*n*-SteOAnt) ($n = 2, 3, 6, 7, 9$ and 12) and 16-(9-anthroyloxy)palmitic acid (16-PamOAnt) the acceptors.

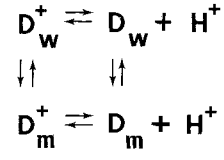

Fig. 1. *Multiple equilibria of dibucaine (monoprotonated, D^+, and neutral, D, forms), between water (w) and membrane (m)*

Dibucaine is a tertiary amine local anaesthetic existing both in neutral and cationic forms at physiological pH, the p*K* values of its aromatic N and of its tertiary amine being 1.77 and 8.85, respectively [9]. Both forms can interact with the membrane surface (Fig. 1), so a study of their partition coefficients between the aqueous phase and the membrane was carried out prior to the energy-transfer study.

MATERIALS AND METHODS

Materials

Dibucaine (free base) and $Pam_2GroPCho$ from Sigma (Chemical Co, St. Louis, MO) were used as received. The probes *n*-SteOAnt and 16-PamOAnt were obtained from Molecular Probes (Eugene, Oregon). The chemical structure of the anaesthetic is presented in Fig. 2. The solvents used were of spectroscopic grade.

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Abbreviations. SUV, small unilamellar vesicles; $Pam_2GroPCho$, dipalmitoylglycerophosphocholine; t_c, temperature of the gel-to-liquid-crystal transition; *n*-SteOAnt, *n*-(9-anthroyloxy)stearic acid; 16-PamOAnt, 16-(9-anthroyloxy)palmitic acid; MLV, multilamellar vesicles; cmc, critical micellar concentration.

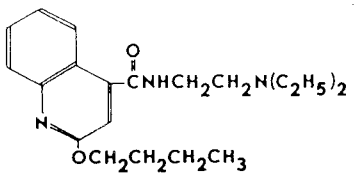

Fig. 2. *Chemical structure of dibucaine*

Preparation of phospholipid vesicles containing donors and acceptors

A film of lipid obtained from evaporation of a chloroform solution was kept under vacuum for more than 5 h and then solubilised in 70 mM K_2HPO_4/KH_2PO_4, pH 7.4, by vortexing and warming the solution above t_c. These multilamellar vesicles (MLV) were disrupted to SUV by sonication (Branson, 40 W). Usually six 30-s cycles were performed until no significant decrease in the scatter intensity of the suspension was obtained. The vesicles were annealed for 10 min at 55°C to decrease structural defects in the bilayer which would induce its fusion [10] and were then submitted (2 min) to low-speed centrifugation to separate titanium particles arising from the sonication.

To this lipid solution (1 mM), donor was added from a stock solution in ethanol and after an incubation time of 30 min, the solution was divided into aliquots to which different concentrations of acceptor were added, also from an ethanolic solution. The final concentration of donor in each sample was 50 μM (donor/phospholipid ratio, 1:20) and the maximum concentration of acceptor used was 56 μM. For the highest concentration of ethanol used, 2% by vol., no alteration in the bilayer structure is reported [11] and no change in the intensity of light scattered by the vesicles was found. The incubation time in the dark for incorporation of the acceptor probes was 1.5 h, a steady intensity of fluorescence being observed in all cases after uptake of the probes into the vesicles. The energy-transfer study was usually carried out during the subsequent 3 h to prevent significant translocation of the probe to the inner half of the bilayer membrane.

Determination of partition coefficients of dibucaine

Partition coefficients were measured using MLV prepared as reported above. A different amount of dibucaine was added to each sample from a stock solution in ethanol, the lipid concentration being kept constant. The following buffer systems were used: (a) 50 mM K_2HPO_4/KH_2PO_4 containing 100 mM NaCl, pH 5.5; (b) 70 mM K_2HPO_4/KH_2PO_4, pH 7.4; (c) 50 mM $NaHCO_3/Na_2CO_3$ containing 100 mM NaCl, pH 11. In order to promote the attainment of equilibrium of the drug between the buffer solution and the MLV, several cycles of freeze-thawing and vortexing at room temperature were performed [12]. The samples were then centrifuged at 25°C and $30000 \times g$ for 60 min in a Beckman J2−21 M/E centrifuge.

The dibucaine concentration in the supernatant was determined from its absorbance, a molar absorption coefficient of 4400 $M^{-1}cm^{-1}$ at 326 nm being used [9]. The presence of lipid remaining in the supernatant was monitored by the light-scattering intensity ($\lambda = 450$ nm) of the solution, the concentration of the lipid determined in this way always being less than 5% of the total lipid concentration.

For the study of the effect of *n*-SteOAnt on dibucaine incorporation into the membrane, this probe was co-solubilised with the lipid in chloroform, 9-SteOAnt being chosen as the model compound. Its concentration (56 μM) was the same as the highest one used in the energy-transfer study.

Absorption spectra were recorded on a Perkin-Elmer Lambda 9 spectrophotometer.

Fluorescence measurements

Steady-state excitation and emission spectra were obtained at 25°C and 49°C, using 5 mm × 5 mm cuvettes in a Spex F112A Fluorog spectrofluorimeter equipped with a double emission monochromator and a thermostatting unit. The excitation and emission bandwiths used were 4.5 nm, and 2.25 nm, respectively. Correction of excitation and emission spectra were performed using a rhodamine-B quantum counter solution [13] and a standard lamp [14], respectively.

The energy-transfer efficiency was experimentally evaluated from the excitation spectra ($\lambda_{ex} = 326$ nm, $\lambda_{em} = 370$ nm) of dibucaine. When necessary, background and scatter intensities were subtracted from the spectra.

Values of relative fluorescence intensity in the presence and absence of acceptor (ϕ/ϕ_0) were obtained from these measurements with a maximum error estimated at 5%. In replicate experiments, the maximum deviation observed was not greater than 2%.

All the fluorescence measurements were carried out in a right-angle geometry and the possible errors arising in a steady-state experimental approach to energy transfer in this geometry were taken into account. (a) Significant variation of absorbance (geometry factor) at the excitation wavelength; under the experimental conditions described no correction is needed [15]. (b) Absorption by the acceptor at the excitation wavelength; the consideration of this factor for the highest concentration of acceptor implied a correction of 8% in the ϕ/ϕ_0 intensity. In the lower concentration range, its importance is negligible. (c) Absorption of fluorescent light (trivial effect); for the highest concentration of acceptor it amounts to a correction of 6%. Effects were minimized by the use of 5 mm × 5 mm fluorescence cells.

As the energy-transfer efficiencies were studied over a relatively small range of donor quenching (minimum ϕ/ϕ_0 about 0.5), a marked variation in the mean lifetime of the donor does not occur, and the polarization of donor fluorescence is not expected to change significantly, unless the rotational correlation time (ϕ) is very short. It was therefore deemed unnecessary to use a magic angle arrangement of polarizers in the excitation and emission paths.

Quenching of *n*-SteOAnt probes by dibucaine in homogeneous media [ethanol (95% by vol.) and dioxane] was carried out in aerated solutions using excitation and emission wavelengths of 380 nm and 475 nm, respectively.

Relative quantum yields of neutral and monoprotonated dibucaine were determined in aerated buffer solutions (at pH 11 and 7.4, respectively) using an excitation wavelength of 320 nm. The quantum yield of the neutral species in the membrane was determined by comparison with that observed in buffer solution ($\phi_D = 0.033$ [9]).

Fluorescence decays were measured using the time-correlated single-photon−counting technique. The excitation source was a nitrogen-filled flash lamp (Edinburgh Instruments, 119 F). Collection of pulse and sample profiles, detected with a Phillips XP2020Q photomultiplier, was alternated with cycle times of 3 min and 7 min, respectively. The decay curves were deconvoluted on a Digital Equipment Corpor-

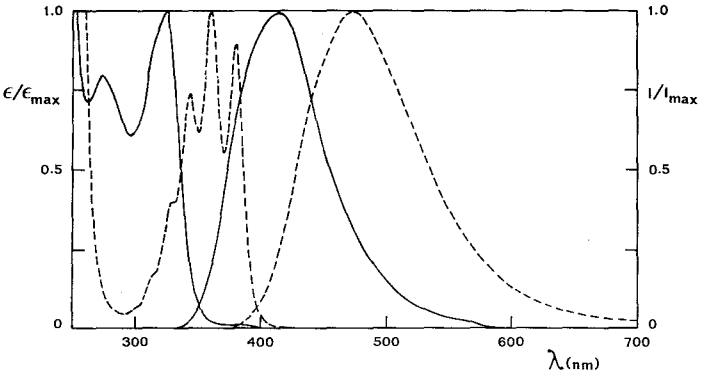

Fig. 3. *Corrected excitation and emission spectra.* Donor (——) dibucaine, λ_{ex} = 326 nm, λ_{em} = 410 nm and acceptor (– – –) 6-SteOAnt, λ_{ex} = 365 nm, λ_{em} = 490 nm in 1 mM $Pam_2GroPCho$ small unilamellar vesicles at 25 °C

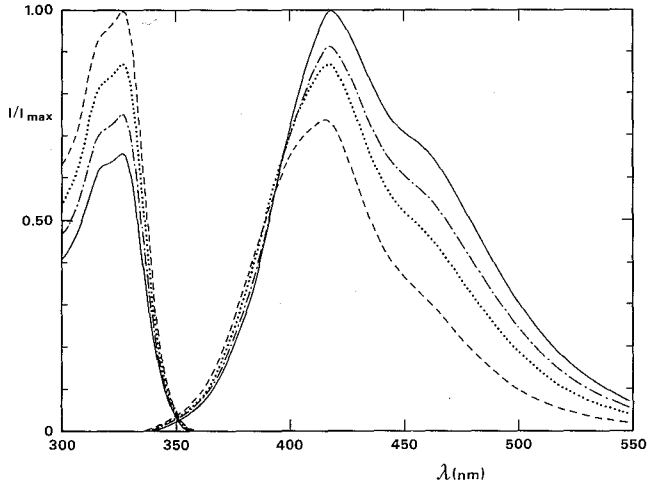

Fig. 4. *Excitation and emission spectra for dibucaine- and 7-SteOAnt-labeled 1 mM $Pam_2GroPCho$ vesicles, at 25 °C.* The concentrations of 7-SteOAnt used were 0 (– – –); 19 μM (····); 31 μM (· – · – ·) and 56 μM (——); λ_{em} = 370 nm; λ_{ex} = 290 nm; donor/phospholipid ratio, 1:20

liquid-crystal transition of $Pam_2GroPCho$. From these plots it is apparent that the quenching efficiency at 25 °C varies in the sequence 3-SteOAnt > 9-SteOAnt > 2-SteOAnt > 16-PamOAnt ≈ 12-SteOAnt > 7-SteOAnt ≈ 6-SteOAnt. Above t_c, larger efficiencies of energy transfer were observed for all the anthroyloxy probes with the exception of 2-SteOAnt which showed no significant variation. An alteration is apparent in the relative quenching efficiencies of the different probes, the sequence now being 3-SteOAnt ≈ 9-SteOAnt > 16-PamOAnt > 7-SteOAnt > 12-SteOAnt > 6-SteOAnt > 2-SteOAnt.

DISCUSSION

Dibucaine photophysics

Due to the pK_a of its aliphatic amino group there is a multiple equilibrium of monoprotonated and neutral forms of dibucaine between the buffer solution and the membrane, as previously mentioned. These two forms of the anaesthetic have distinct photophysical parameters, the quantum yield and the liefetime of the cationic and the neutral species being $\phi_D^+ = 0.25$: $\tau_D^+ = 3.33$ ns and $\phi_D = 0.033$; $\tau_D = 0.77$ ns, respectively [9]. We obtained $\tau = 3.2 \pm 0.1$ ns in phosphate buffer, pH 7.4, this value being also close to that reported by Papahadjopoulos [5]. The pH dependence of quantum yield and lifetime is due to an intramolecular charge-transfer quenching [28] operative in neutral dibucaine, the terminal aliphatic amine acting as the electron donor and the quinoline moiety as the acceptor. Upon protonation of the amine group its ionization potential increases, rendering electron donation unfavourable. In agreement with the small distance between the electron donor and acceptor moieties (two methylene groups), the ratio of quenching efficiencies evaluated from steady state (ϕ_D^+/ϕ_D) is larger than that observed from the lifetime ratio (τ_D^+/τ_D), evidence for the existence of a static contribution in the quenching mechanism. This observation is also supported by the spectral red shift observed in the emission spectra of the charged species relative to the neutral one [9]. In this last case, the aliphatic amine is in the vicinity of the aromatic moiety, the solvation by water molecules being less efficient.

Sikaris et al. [29] located a family of related local anaesthetics, e.g. butesin and tetracaine, in a model system of membranes by studying the dynamic quenching of the *n*-SteOAnt probes by these drugs. The quenching operates through an intermolecular charge-transfer mechanism, the aromatic amine (aniline) being the donor moiety because it has a lower ionization potential (I_p = 7.30 eV [30]) than the terminal aliphatic amine group of these molecules (I_p = 7.82 eV [30]).

We observed that dibucaine is not an effective collisional quencher of the excited 9-SteOAnt probe in ethanol (95% by vol.). In this solvent, most of the aliphatic amine groups are protonated and cannot act as electron donors to the *n*-SteOAnt. On the other hand, the large I_p of the quinoline group prevents its involvement in a charge-transfer process. In dioxane, linear Stern-Volmer relationships for the quenching of 9-SteOAnt by dibucaine were obtained both in the steady- and transient-state measurements, the quenching constant $k_q = (2.2 \pm 0.1) \times 10^9$ $M^{-1}s^{-1}$ being close to the diffusion-controlled limit as expected.

In this way, the energy-transfer approach used to locate the anaesthetic inside a model membrane system is the only feasible one using photophysical methods for this pair of molecules, at physiological pH.

Dibucaine/n-SteOAnt energy transfer

The assumption used in this energy-transfer study, namely that the system is static except for the rotational motion of the two chromophores, should be analysed considering the

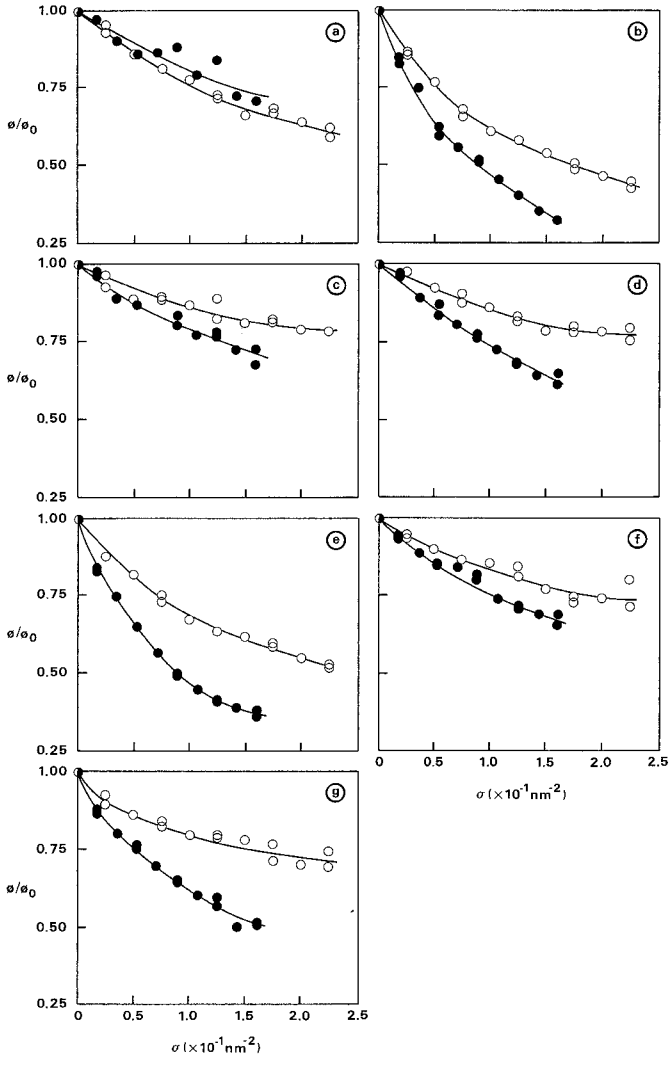

Fig. 5. *Relative quantum yields of fluorescence, ϕ/ϕ_0, for various probes as a function of surface concentration of acceptor, σ in 1 mM $Pam_2GroPCho$ vesicles, at 25°C (○) and 49°C (●).* (a) 2-SteOAnt; (b) 3-SteOAnt; (c) 6-SteOAnt; (d) 7-SteOAnt; (e) 9-SteOAnt; (f) 12-SteOAnt and (g) 16-PamOAnt. The areas/phospholipid head-group assumed are 0.50 ± 0.02 nm^2 below t_c [23] and 0.70 ± 0.04 nm^2 above t_c [24]. Donor/lipid ratio, 1:20

specific photophysical parameters of the donor/acceptor system used.This assumption is valid once R_0 is greater than the mean diffusion path length, $r = \sqrt{2D\tau_0}$ [31], where D is the sum of the diffusion coefficients of both species. Considering the lifetime of dibucaine $\tau_0 = 3.2 \pm 0.1$ ns, an upper boundary for D of $(7.6 \pm 0.1) \times 10^{-7}$ cm^2s^{-1} is obtained. A protonated drug related to dibucaine (tetracaine) was reported to diffuse slowly in the ^{2}H-NMR time scale when incorporated in a phosphatidylglyceroethanolamine matrix [32], so a significant diffusion of dibucaine in the nanosecond time scale (fluorescence) should be negligible. The other partner of the Förster pair would eventually have a higher diffusion coefficient than the ones reported for lipids ($10^{-8}-10^{-9}$ cm^2s^{-1} [33]) but, in any case, lower than the limit value obtained for the upper boundary.

The intensities of dibucaine fluorescene in the energy-transfer experiments have a contribution from the fraction of the anaesthetic present in the aqueous phase. In fact, when the lipid concentration used in the preparation of the vesicles was increase from 1 mM to 5 mM, a larger efficiency of energy transfer between the dibucaine and the 9-SteOAnt probe was obtained (results not shown) as a larger fraction of dibucaine was incorporated into the membrane.

The uncertainty involved in the determination of the partition coefficients did not allow the correction of relative quantum yields for the presence of dibucaine in solution, preventing the estimation of absolute distances, e.g. from fitting the experimental results to Eqn A2 in [20].

Nevertheless, some relative information on dibucaine location is possible because energy transfer is only significant between donors and acceptors incorporated into the vesicles. Two arguments support this conclusion. (a) The possibility that the molecules present in solution or adsorbed at the interface were efficient in the process of electronic energy transfer can be ruled out. In the most extreme situation all *n*-SteOAnt probes would be solubilised in the buffer, in spite of their large partition coefficients, and the dynamic limit for energy transfer would be attained [34]. For the largest concentration of acceptors used, the calculated relative quantum yields for energy transfer alone is 0.91, which is higher than the experimental values obtained with any of the probes used. (b) The existence of mixed micelles of donors and acceptors in solution is also contradicted by several lines of evidence. In all experiments a law concentration of donor was used (50 μM) which must be below its critical micellar concentration (cmc), since tetracaine, a similar anaesthetic, has a cmc of 70 mM [35]. On the other hand, the large partition coefficients of the *n*-SteOAnt probes, together with the observation of an increase in the energy-transfer efficiency with the increase of lipid concentration used in the preparation of SUV, support the occurrence of energy transfer only, or at least very predominantly, between molecules incorporated into the membranes.

Two advantages arise from the use of the anthroyloxy family of probes in a systematic study of locations inside a membrane. First, these probes are known to be located at a graded series of depths [36], so a precise mapping can be obtained. Second, as the spectral overlap between dibucaine emission and the absorption of the anthroyloxy chromophore is identical for all the acceptor probes, the same R_0 holds for all the donor/acceptor pairs, at least with the assumptions for the orientation factor made here. This implies that: (a) no error in determining different R_0 values is introduced in the analysis; (b) an immediate qualitative comparison of distances is possible, i.e. the ϕ/ϕ_0 values for the same concentration of acceptor in the series of probes immediately reflect the relative order of separation between donors and acceptors.

As stressed in Results, the study of the partition coefficients allowed us to conclude that at pH 7.4 the monoprotonated form of dibucaine is largely dominant in the membrane and buffer, being the only important species involved in the electronic energy-transfer experiments.

Direct inspection of Fig. 5 allows one to locate dibucaine away from the center of the bilayer where 16-PamOAnt and 12-SteOAnt are lying, at 25°C. Larger efficiencies of transfer were observed for 2-SteOAnt and 9-SteOAnt, the 3-SteOAnt probe being that for which maximum donor quenching was obtained. From the study of this set of probes it can be concluded that dibucaine is located near the interface of the membrane inbetween 3-SteOAnt and 9-SteOAnt, in agreement with the polar nature of the chromophore. It should be expected then that the largest quenching efficiencies would be obtained for the 6-SteOAnt and 7-SteOAnt probes. However, these two probes do not fit with the previous trend, as the

observed relative quantum yields locates them in the bilayer interior, this conclusion being physically unacceptable. Considering the reasonable assumption that dibucaine and these probes are nearly on the same plane, two explanations can be put forward. First, due to steric reasons a non-random distribution happens; in this case the chromophore group of the probes excludes the dibucaine molecules from its vicinity. Second, the relevance of an unfavourable orientational factor would be larger in the case where donors and acceptors are on the same plane. As the dynamic isotropic regime was assumed for all cases in the calculation of the critical distance, R_0, this would lead to an overestimation of the quenching efficiency of these two probes.

Above the phase-transition temperature larger efficiencies of energy transfer were obtained for all the *n*-SteOAnt probes with the exception of 2-SteOAnt, which was almost insensitive to the membrane state. These results can be explained by an increase in the partition coefficient of dibucaine with temperature, as varified with tetracaine [37].

The most pronounced increase in quenching efficiency with temperature is found for 16-PamOAnt. This probe has a peculiar structure in the anthroyoxy family, as the polar chromophore is not attached to a position along the alkyl chain but occupies a terminal position. This fact, as well as its location in the more fluid area of the bilayer, makes its inclusion in a palisade-type structure much less stringent and the probe probably can make loops, approaching the anthroyloxy moiety to a more favourable environment near the interface. This effect must have some importance in the gel state as the 12-SteOAnt and 16-PamOAnt showed similar efficiencies of quenching of the emission of dibucaine at 25°C.

It can be inferred from the results discussed above that the phase transition of $Pam_2GroPCho$ does not induce an alteration in the relative location of the drug in the bilayer.

The information on dibucaine location obtained in the present study by a fluorescence method should be compared with studies using other spectroscopic techniques. While references to dibucaine are scarce [27], studies on the related anaesthetics tetracaine and procaine have been reported by NMR [32, 38, 39] and X-ray diffraction [40]. In all of them evidence is presented locating the monoprotonated compounds near the head-group of the lipids.

Coster et al. [40] using X-ray diffraction observed an increase in electron density in lecithin-cholesterol multilayer membranes upon procaine incorporation, locating the aromatic moiety 1.0 nm away from the center of the multilayer and the protonated amine terminal group near the interface. The NMR studies reported used deuterated lipids [38, 39] or deuterated anaesthetics [32], ^{31}P resonance of the phospholipids was also presented [27, 38]. Boulanger et al. [38] concluded that the protonated form of tetracaine is located in the vicinity of the glycerol-backbone region, spectral alterations being observed both in the head groups and in the acyl-chain region. Interestingly, the addition of negatively charged phosphatidylserine did not affect the location of the anaesthetic in the membrane. For this reason, complicating effects in the present work due to alterations in the depth of dibucaine transverse in the membrane upon increasing concentration of the *n*-SteOAnt probes are very unlikely.

Recently, dibucaine penetration was assessed from the ^1H-^1H nuclear Overhauser effect [27] between the anaesthetic and the lipid (sonicated egg-yolk glycerophosphocholine). It was inferred that the quinoline ring penetrates no further than the rigid glycerol moiety and a concomitant high-field shift of the ^{31}P resonance was observed, eventually due to the strong interaction of the charged diethylammonium moiety of the drug with the phosphate group.

The proposed dibucaine location is in agreement with the previously described studies [27, 32, 38–40]. The photophysical technique used in the present study allows localization of the optical electron, i.e. in this case, the quinoline moiety of the molecule. It is apparent from the results that this aromatic group is located somewhere in the vicinity of the glycerol moiety of the lipid, near the 3-SteOAnt probe.

It should be stressed that the NMR studies reported so far were concerned with lipids in the liquid-crystal state, high-resolution spectra being obtained in that case. However, fluorescence measurements are not impaired by this requirement, so that results below t_c could be obtained and, from these, it could be inferred that there was no change in the location of the drug with temperature.

CONCLUSIONS

Electronic energy transfer between dibucaine (donor) and the *n*-SteOAnt ($n = 2, 3, 6, 7, 9, 12$) and 16-PamOAnt probes (acceptors) was studied, in order to determine the location of the anaesthetic in a model membranes system (SUV of $Pam_2GroPCho$) both above and below the t_c of the phospholipid.

In addition, dibucaine photophysics was reviewed and the short lifetime of the neutral form of the anaesthetic with respect to that of the monoprotonated species was attributed to an intramolecular charge-transfer interaction.

The drug is known to exist in these two forms at pH 7.4 in aqueous solution. A study of their partition coefficients was carried out, it being concluded that the only significant species in the membrane is the charged one.

From the sequence of relative quenching efficiencies it was concluded that the dibucaine intercalates into the vesicles near the glycerol backbone of the lipid, in agreement with information obtained by NMR spectroscopy [27]. Upon increasing the temperature above t_c no significant alteration of the drug location is observed.

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