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A ^{2}H-NMR study on the interactions of the local anesthetic tetracaine with membranes containing phosphatidylserine

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The interaction of the local anesthetic tetracaine with phosphatidylserine-containing model membranes has been studied by ^{2}H-NMR. Charged tetracaine exhibited an unusually large partition coefficient into multilamellar dispersions of phosphatidylserine. The ^{2}H-NMR spectra consisted of a Pake doublet and a narrow line, with the former corresponding to tetracaine in the bilayer and the latter to tetracaine free in solution. A strong pH dependence of the quadrupole splittings indicated different membrane locations for charged and uncharged tetracaine. In equimolar mixtures of phosphatidylserine and phosphatidylcholine the partition coefficients and ^{2}H-NMR spectra were much more like those observed in neat phosphatidylcholine than in neat phosphatidylserine. Dilution studies at pH 5.5 indicated that in phosphatidylserine/phosphatidylcholine mixtures tetracaine experiences a three-site exchange similar to that found earlier for tetracaine in phosphatidylcholine. Tetracaine is in fast exchange between sites weakly bound to membrane and free in solution, and in slow exchange with a strongly bound site in the membrane.

Introduction

Local anesthetics are known to exert their pharmacological influence by blocking the sodium channels in the nerve axonal membrane [1]. Whether this blocking is the result of a specific anesthetic-channel interaction or a non-specific interaction with the lipid matrix is still unclear. However, the diverse range of chemical structures which induce anesthesia suggests the possibility of heterogeneous sites of anesthesia, of the type described by Trudell [2]. This could involve binding sites on the protein and/or in the lipid matrix.

The interaction of tetracaine (TTC) with model membranes of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) has been studied by a number of techniques, especially ^{2}H-NMR [3–7]. These studies indicated that, in model systems of PE, the TTC is in slow exchange between sites membrane-bound and free in solution [6]. However, in PC model systems TTC undergoes exchange between three sites – strongly bound in the membrane, weakly bound, likely at or near the surface, and free in solution [3,5]. Parallel experiments with TTC in specifically deuterated PE [7] and PC [4] indicated that the depth of anesthetic penetration into the bilayer is strongly dependent on the TTC charge in PC systems, but is charge-independent in PE systems.

We have now extended these studies to the interaction of specifically deuterated tetracaine with bilayers of phosphatidylserine (PS), as well as with mixtures of PS and PC. The results are compared to those for the PC and PE systems reported earlier [4–7].

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Materials and Methods

Tetracaine hydrochloride was purchased from Sigma Chemical Co. The synthesis of labelled anesthetics TTC-d_2, TTC-d_3 and TTC-d_6 has been described earlier [8] (see Fig. 1 for structures of the labelled tetracaines). Deuterium-depleted water, used for all samples, was obtained from Aldrich, Milwaukee, WI. Bovine brain phosphatidylserine (PS) was purchased from Lipid Products, South Nuffield, U.K. Phosphatidylcholine was isolated from fresh eggs by the method of Singleton [9].

The PS or 1:1 PS/PC mixtures (100 mg of total lipid), in a chloroform/methanol solution, were concentrated under a stream of nitrogen and then pumped dry on a vacuum line for at least 12 h. The labelled tetracaines and buffer were added and the sample was vortexed. In order to attain complete equilibration of the anesthetic between lipid and water, the samples were subjected to at least five freeze-thaw vortex cycles [5].

The borate-phosphate-citrate buffer (BPC) was made in deuterium-depleted water to minimize the H^2HO signal. The buffer consisted of citric acid (3.8 mM), boric acid (2.9 mM), sodium hydroxide (17.1 mM) and 85% H_3PO_4 (2.4 mM) in ^1H_2O. The pH was adjusted with concentrated HCl to 5.5 or 9.5.

The ^{2}H-NMR spectra were obtained on a Bruker CXP-300 spectrometer operating at 46.063 MHz. The spectra were acquired using the quadrupole echo sequence [10] with full phase cycling of the radiofrequency pulses. The $\pi/2$ pulse length was 5 μs, the pulse spacing was typically 50 μs, and the recycle time was always greater than $5 \times T_1$. Spectra were acquired on resonance, but folded in order to increase the signal-to-noise ratio. The sample temperature was 20°C unless otherwise indicated.

The partition coefficients were determined by the centrifugation method of Miller and Yu [11], with the TTC detected spectrophotometrically [6], under conditions comparable with those of the NMR experiment.

Results and Discussion

Partition coefficients

The partition coefficient, K_p, is the ratio of the anesthetic concentration in the lipid phase to that in the buffer (expressed in grams of anesthetic per gram of each phase). The partition coefficients for TTC into bilayers of PS, PC, and PS/PC mixtures are reported in Table I.

TABLE I

PARTITION COEFFICIENTS FOR TETRACAINE INTO BILAYERS OF PS, PS:PC (1:1) AND PC

Lipid	K_p	
	pH 5.5	pH 9.5
PS	700	425
PS:PC	80	550
PC [a]	22	600

[a] From Ref. 3.

At pH 9.5, when TTC is primarily uncharged ($pK_A = 7.5$–8.5 [3,22]) K_p values are very large, but there is not a large dependence of the partition coefficients on the nature of the lipid. However, at pH 5.5, when TTC is primarily charged, the K_p values depend strongly on the lipid type. In the neat PS system, at pH 5.5, K_p is 700, considerably greater than found in PC or PS/PC mixtures.

The large K_p found at pH 9.5 reflect the strong hydrophobic interactions between the primarily uncharged TTC and the hydrocarbon regions of the lipids. The sharp reduction in K_p for TTC in PC and PS/PC mixtures, when the pH is lowered to 5.5, reflects the diminishing importance of this hydrophobic interaction for charged TTC. However, for TTC in neat PS bilayers at pH 5.5 K_p is the largest recorded to date. This large value arises from a strong electrostatic interaction between the positively charged TTC and the negatively charged PS ($pK = 3.5$, [34]). Strong electrostatic interactions between the local anesthetics tetracaine and procaine and PS bilayers have also been suggested from ^{1}H-NMR [12,13] and fluorescence quenching data [14].

^{2}H-NMR of tetracaine in PS bilayers

The ^{2}H-NMR spectra of the specifically deuterated tetracaines in PS bilayers were recorded at pH 5.5 and pH 9.5. The spectra generally consisted of a Pake doublet, with quadrupole splitting $\Delta\nu_Q$, and a narrow central resonance. How-

TABLE II

QUADRUPOLE SPLITTINGS (kHz) FOR SPECIFICALLY DEUTERATED TETRACAINE IN PS, PC AND PS/PC MIXTURES AT pH 5.5 AND 9.5

	PS		PC [a]		PS/PC	
	pH 5.5	pH 9.5	pH 5.5	pH 9.5	pH 5.5	pH 9.5
TTC-d_6	1.8	1.2	1.8	[b]	1.8	[b]
TTC-d_2	12.8	16.1	14.4	15.2	14.6	12.6
TTC-d_3	0.65	[b]	[b]	[b]	[b]	[b]

[a] From Ref. 5.

[b] Only a narrow resonance was observed.

ever, for some TTC-d_3 samples only a narrow resonance was observed. The quadrupole splittings for the labelled tetracaines in PS bilayers are reported in Table II, along with those in PS/PC bilayers, and the earlier data for PC bilayers [3,5].

Fig. 2 shows the spectra of TTC-d_6 and TTC-d_3 in neat PS bilayers at pH 5.5 and 9.5. At pH 5.5 the spectra consist of a quadrupole pattern, with a large integrated intensity, and a weak narrow resonance in the center. The quadrupole pattern results from TTC in the bilayer, and the narrow line from TTC in solution. The very small overall intensity of this central line thus reflects the extremely high partition coefficients found for TTC in PS at low pH. On going to higher pH the quadrupole splitting for TTC-d_6 in PS is reduced and that for TTC-d_3 disappears into a single resonance. PS is the only lipid so far in which a detectable quadrupole splitting is seen for TTC-d_6 at high pH. In addition, the narrow component in the TTC-d_6 sample represents a larger portion of the total spectral intensity. This reflects the drop in K_p from 700 to 425 when the pH is raised from 5.5 to 9.5; under these conditions this results in twice as much anesthetic in the aqueous phase at pH 9.5.

TETRACAINE (TTC)

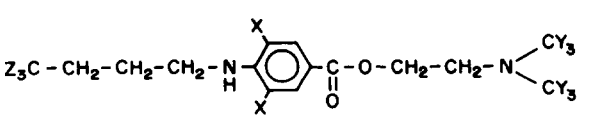

Fig. 1. Structures of the specifically deuterated tetracaines. TTC-d_2: X = ^{2}H, Y = H, Z = H; TTC-d_3: X = H, Y = H, Z = ^{2}H; TTC-d_6: X = H, Y = ^{2}H, Z = H.

For TTC-d_2, where the aromatic ring is deuterated, the quadrupole splittings are substantially larger. This indicates that the aromatic moiety is in an area of high order, probably near the

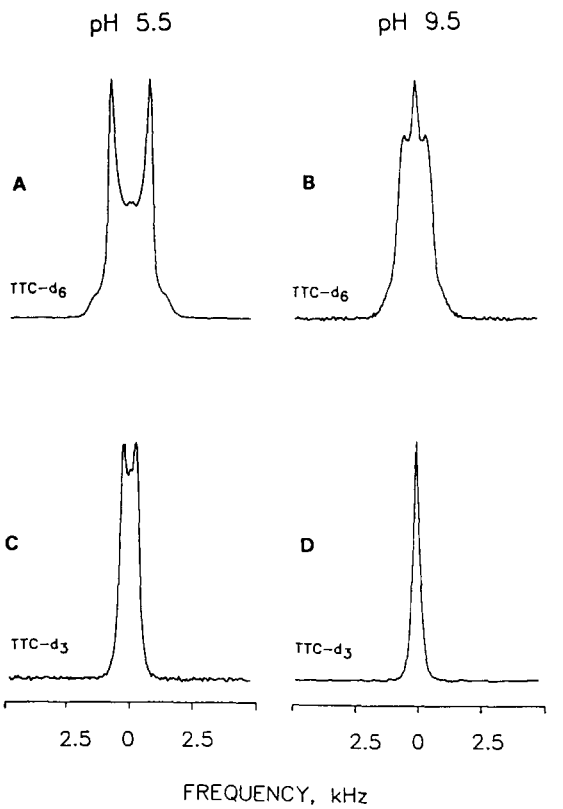

Fig. 2. ^{2}H-NMR spectra of labelled tetracaines in PS model membranes. (A) TTC-d_6 (33 mM) and PS (140 mM) in borate-phosphate-citrate (BPC) buffer at pH 5.5. (B) TTC-d_6 (33 mM) and PS (140 mM) in BPC buffer at pH 9.5. (C) TTC-d_3 (33 mM) and PS (140 mM) in BPC buffer at pH 9.5. (D) TTC-d_3 (33 mM) and PS (140 mM) in BPC buffer at pH 5.5.

glycerol backbone. The quadrupole splitting is even larger at higher pH, suggesting that the anesthetic changes location within the bilayer on deprotonation. Similar behaviour has also been noted for TTC-d_2 in PC bilayers [3,5].

The quadrupole splittings observed for the TTC-d_2 species are indicative of extremely high order for the aromatic moiety of TTC. Since the aromatic moiety is rigid, and assuming that the axis of motional averaging passes through the 1,4 positions of the ring [4,6,15], a molecular order parameter, S_{mol}, can be defined which describes the anisotropic fluctuations of the aromatic ring about its equilibrium position;

$$S_{mol} = 0.061\ \Delta\nu_Q \quad (1)$$

where $\Delta\nu_Q$ is the observed quadrupole splitting [6].

For TTC-d_2 in PS at pH 9.5 the quadrupole splitting of 16.1 kHz is indicative of an S_{mol} of 0.98. This implies that for TTC in PS at pH 9.5 the aromatic ring undergoes almost no fluctuations from its equilibrium position. This S_{mol} value is higher than has been observed in other lipids or lipid mixtures. It is also higher than that observed for cholesterol, which ranges from 0.87 in egg PC [16] to 0.76 in the membrane of human erythrocytes [17]. It is also considerably larger than the 0.45 observed for the plateau region of dipalmitoylphosphatidylserine [18]. This suggests that for uncharged TTC in PS bilayers the aromatic moiety is in a highly ordered environment. Note that the very high order parameter for the aromatic ring is due to the rigidity of the ring, and does not imply that the acyl chains in its vicinity have equally high order parameters [23].

^{2}H-NMR of tetracaine in PS/PC bilayers

The ^{2}H-NMR spectra of the labelled tetracaine species in equimolar PS/PC mixtures were markedly different from those in neat PS. After extensive freeze-thawing to reach an equilibrium, quadrupole splittings were only observed for TTC-d_2 and for TTC-d_6 at pH 5.5 (Table II). In addition, the spectra of all deuterated species contained an intense narrow central resonance.

Fig. 3 shows the ^{2}H-NMR spectra of TTC-d_3 and TTC-d_6 in PS/PC bilayers at pH 5.5. For the spectrum of TTC-d_6, the integrated intensity of the central line is clearly much greater than would be predicted from the partition coefficients. The spectrum of TTC-d_6 exhibits a markedly different lineshape than that observed in neat PS alone (Fig. 2A) although the quadrupole splitting appears the same. Instead, the lineshape is similar to that observed for TTC-d_6 in neat PC [3,5].

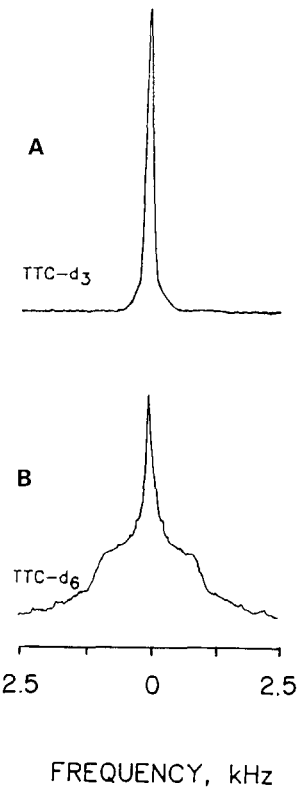

Fig. 3. ^{2}H-NMR spectra of (A) TTC-d_3 (30 mM) and (B) TTC-d_6 (30 mM) in PS/PC (150 mM) model membranes at pH 5.5.

Dilution studies on TTC-d_6 in PS/PC indicate that the central resonance narrows appreciably as buffer is added, but that the quadrupole splitting remains unaffected. This behaviour is identical to that observed for TTC in PC membranes and has been modelled in terms of a three-site anesthetic exchange. In this model TTC is in fast exchange between a weakly bound site and anesthetic free in solution, giving rise to the narrow line, but in slow exchange between a membrane bound site and either or both of the free and weakly bound sites. It is the TTC in the PS/PC bilayer which gives rise to the quadrupole pattern. From the general

similarity of the lineshapes noted for TTC-d_6 in PS/PC and PC alone, it would appear that the slow exchange rate in PS/PC must be similar to the $1.5 \cdot 10^3$ s^{-1} estimated for TTC in egg PC bilayers [5].

At pH 9.5, when TTC is primarily uncharged, a quadrupole splitting is observed for only the aromatic deuterons. This behaviour is again qualitatively similar to that observed for TTC in neat PC.

The influence of unlabelled TTC on the ^{2}H-NMR spectra of specifically deuterated PC, in PS/PC mixtures, has also been observed to be qualitatively similar to the effect of TTC on PC alone [4]. Tetracaine was observed to cause a general disordering of the hydrocarbon region and to induce corresponding changes in the orientation of the choline headgroup in the neat PC and PS/PC mixtures.

Conclusions

The charged form of TTC experiences a strong electrostatic interaction with neat PS, leading to an unusually large K_p. The ^{2}H-NMR quadrupole splittings indicate that charged and uncharged TTC occupy different sites in the PS bilayer. The ^{2}H lineshapes observed indicate that the TTC is exchanging very slowly between states free in solution and membrane-bound. A slow exchange of this type has also been observed for TTC in PE bilayers [6].

In equimolar mixtures of PS and PC the behaviour of TTC parallels that observed in neat PC bilayers. The ^{2}H-NMR results suggest that TTC experiences exchange between three sites. There is fast exchange of TTC between states weakly bound to membrane and free in solution, and slow exchange of the strongly bound species.

The strong electrostatic interaction noted for charged anesthetics in PS bilayers is noteworthy and has been speculated upon as relevant to the molecular mechanism of anesthesia [19–21]. High PS levels have been found in several preparations of excitable membranes [24–28], and data obtained from different tissues suggest that PS is present in greater proportion in excitable tissues [29] than in nonexcitable ones [30]. In addition, it has been seen that the stability of a reconstituted sodium channel preparation can be highly improved by adding PS [31]. Finally, it has been suggested that the sodium channel is surrounded by PS [32,33]. Thus, the present results may be very relevant to the interaction between local anesthetics and nerve membranes in vivo.

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