Site of Action of the Local Anesthetic Tetracaine in a Phosphatidylcholine Bilayer with Incorporated Cardiolipin

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ABSTRACT Tetracaine (TTC) increases the permeability of phospholipid liposomal membranes to water, and this increase is reduced by the incorporation of cardiolipin into the membranes. We examined the molecular interaction of a phospholipid with the TTC cation in egg-yolk phosphatidylcholine (EyPC) liposomal membranes with incorporated bovine heart cardiolipin (BhCL) by IR spectroscopy and by determination of partitioning and the pK_a of membrane-bound TTC. The IR spectra indicated that TTC shifted the stretching band of the BhCL PO_2^- group, a potential site of hydration in the bilayer, to a lower frequency but did not shift that of EyPC. TTC intercalated into the BhCL bilayer shifted its aromatic C—N stretching band to a lower frequency. One molecule of TTC was found to bind approximately five molecules of EyPC, and the incorporation of negatively charged BhCL into EyPC membranes increased the degree of binding of TTC to the bilayer membranes. The pK_a values of TTC bound to membranes were determined as 7.7, 9.4, and 10.2 for EyPC membranes, EyPC membranes containing 50 mol % BhCL, and BhCL membranes, respectively, whereas that in an aqueous 10-mM NaCl solution was 8.5, as it was dependent on the manner of binding. The IR data together with the partitioning and the pK_a data suggested differences between the actions of the TTC cation on negatively charged BhCL and on neutrally charged EyPC polar groups in the region close to the aqueous interface of the lipid bilayer.

INTRODUCTION

Several studies have suggested that the intercalation of local anesthetics into model phospholipid membranes leads to reorientation of the charged head groups and disordering in the acyl chains (Neal et al., 1976; Boulanger et al., 1981; Seelig et al., 1988). Nuclear magnetic resonance studies have demonstrated that the local anesthetic tetracaine (TTC) is located closer to the head groups than to the center of the hydrocarbon region and that the charged form of TTC is effective in changing the head-group conformation (Boulanger et al., 1981; Seelig et al., 1988). The magnitudes of these effects depend on the nature and phase behavior of the phospholipid and on whether the anesthetic is charged (Auger et al., 1988; Smith et al., 1991). The structure and thermotropic properties of negatively charged phospholipid bilayers are sensitive to divalent cations such as Ca^{2+} and Mg^{2+} (Papahadjopoulos et al., 1977; Hauser et al., 1982), and the actions of local anesthetics on membranes are suggested to involve their displacement of Ca^{2+} bound to anionic phospholipids (Papahadjopoulos, 1970; Casal et al., 1987).

Recently we found that local anesthetics cause a unique uncoupling of oxidative phosphorylation in mitochondria, unlike that by weakly acidic uncouplers (Terada et al., 1990; Van Dam et al., 1990). This finding was not consistent with those from other laboratories (Sun and Garlid, 1992; Schonfeld et al., 1992). We suppose that the uncoupling by local anesthetics is essentially due to perturbation of the membrane structure of mitochondria. The anionic phospholipid cardiolipin with two negatively charged phosphate groups and four fatty acyl chains must be localized almost exclusively in the inner mitochondrial membrane (Hoch, 1992). As cardiolipin is reported to regulate the activity of coupling components involved in oxidative phosphorylation, such as the ADP/ATP carrier (Hoffmann et al., 1994), the effects of local anesthetics on oxidative phosphorylation are expected to be regulated by their interaction with cardiolipin. The precise role of cardiolipin in the coupling process of phosphorylation and electron transport is unknown, but possibly it functions as a proton-conducting chain along the polar surface of the mitochondrial membrane (Haines, 1983; Boggs, 1987), because cardiolipin in the bilayer membrane tends to form intramolecular hydrogen bonds between C—OH and P—OH groups that can retain protons (Hubner et al., 1991).

In this study we examined the molecular nature of the interaction of TTC with phospholipid molecules in liposomal membranes with incorporated cardiolipin by Fourier-transform IR spectroscopy and determined the partition of TTC to the membrane and the pK_a of membrane-bound TTC.

MATERIALS AND METHODS

Bovine heart cardiolipin (BhCL) was isolated as its sodium salt from bovine heart (Faure and Morelec-Coulon, 1958). Egg-yolk phosphatidylcholine (EyPC) was purchased from Nichiyu Liposome Co. (Tokyo, Japan) and TTC hydrochloride from Sigma Chemical Co. (St. Louis, MO). A large unilamellar vesicle suspension of EyPC with incorporated BhCL was prepared by reverse phase evaporation (Szoka and Papahadjopoulos, 1978) in 10-mM *tris*-HCl buffer (pH 7.5) and filtered through 0.4- and 0.2-μm pore filters (Nuclepore Co., Pleasanton, CA). The mean diameter of the large unilamellar vesicle was 220 nm, as determined with a Nicomp (Santa Barbara, CA) model 370 particle sizer. Complete equilibration of the anesthetic in the lipid bilayer in the liquid crystalline state was achieved by incubating large unilamelar vesicle suspensions (60-mM EyPC) containing various amounts of TTC for 24 h at 30°C.

Infrared spectra were recorded in a Perkin-Elmer model 1720 Fourier-transform spectrometer equipped with a triglycine sulfate detector with KBr windows. For liquid samples (TTC aqueous solution and liposome

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suspension) a cylindrical attenuated total reflectance cell (circle cell; Spectratech, Stanford, CT) with a ZnSe crystal was employed. Unhydrated TTC was obtained by drying at 10^{-5} Torr for 24 h to ensure the complete removal of water. When required, samples were also examined in a KBr disk. Spectra were routinely accumulated and averaged with a standard resolution of 4 cm^{-1} and an encoding interval of 1 cm^{-1}.

Spectral analyses were performed as follows: peak positions were determined by Fourier self-deconvolution, and then the bandwidths, positions, intensities, and percent Lorentzian/Gaussian shape were varied to yield the best fit for the band shapes. The parameters of best fitting for the band shapes were varied until the residual spectrum was minimal (Singh et al., 1990; Shibata et al., 1994).

An equilibrium dialysis chamber (Bel-Art Products, Pequannock, NJ) was used for the partitioning studies. A volume of 5.0 mL of liposome suspension (60 mM) was added to one part and 5.0 mL of TTC solution (60 mM) to the other. The buffer used was 10-mM *tris*-HCl (pH 7.5). The dialysis chamber was then shaken in a water bath at 23°C for 6 h. The absorbances at 312 nm of aliquots taken from each side of the dialysis cells were determined. The moles of TTC bound per mole of phospholipid were evaluated (Eftink et al., 1985).

The pK_a value of TTC in the presence of membranes was determined by electrometric titration. For sample preparation, 40 mg of EyPC was dissolved in 3 mL of diethylether. The solution was dried first in an evaporator and then in vacuum overnight. The dry lipid film was hydrated in 5 mL of a 10-mM NaCl aqueous solution containing 7 mg of TTC hydrochloride, dispersed by vortex mixing, and then sonicated for 6 min. EyPC membranes with 50 mol% incorporated BhCL and BhCL membranes (10 mM) were also prepared. Titrations with a pH/ion meter (model 225, Iwaki Glass, Tokyo) were performed by adding aliquots of 0.25-M NaOH solution to TTC in the solution and suspensions of liposomes with incorporated TTC starting at pH 3 at 23°C under a nitrogen atmosphere. The pK_a was determined from plots of pH versus the volume of NaOH.

RESULTS

IR spectra of dry and hydrated TTC hydrochlorides

First we analyzed the effect of hydration on the spectrum of TTC hydrochloride. The blocking action of local anesthetics on nerve conduction is known to be more effective at pH values below the pK values of the anesthetics (Feinstein, 1964).

Figure 1 shows the IR spectra of dry (*a*) and fully hydrated (*b*) TTC hydrochloride in the frequency ranges of

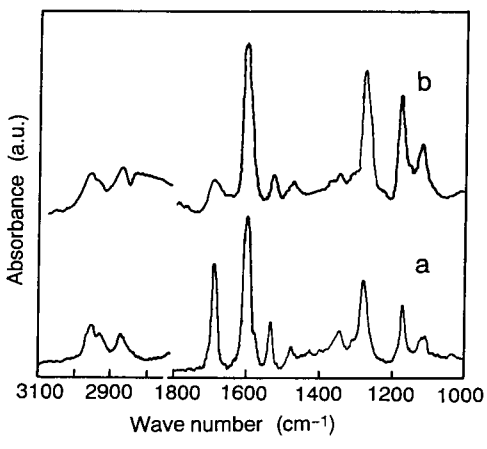

FIGURE 1 Infrared absorbance spectra of (*a*) dry and (*b*) fully hydrated TTC hydrochlorides.

TABLE 1 Assignments of prominent absorption band of TTC hydrochloride in the dry and fully hydrated states

Dry TTC Wave number (cm^{-1})	Hydrated TTC Wave number (cm^{-1})	Assignment
2955	2960	Asymmetric CH_3 stretching band of terminal CH_3
2930	2933	Antisymmetric CH_2 stretching band
2872	2872	Symmetric stretching band of terminal CH_3
2862	2862	Symmetric CH_2 stretching band
1689	1697	Ester C═O stretching band
1600	1604	Aromatic C═C stretching band
1284	1275	Aromatic amine C—N stretching band
1173	1176	Antisymmetric C—O—C stretching band
1118	1110	Symmetric C—O—C stretching band

2800 to 3100 and 1000 to 1800 cm^{-1}. The spectrum of the hydrated sample showed noticeable differences in band frequencies, intensities, and widths from those of the dry sample. The assignments of the bands and the vibrational frequencies of dry and hydrated TTCs are shown in Table 1. These results are consistent with those reported by Chatten et al. (1959). The vibrational spectrum of the hydrocarbon chains of dry TTC hydrochloride is characterized by two bands at 2955 and 2930 cm^{-1}, assigned to asymmetric CH_3 and antisymmetric CH_2 stretching vibrations, respectively, and the band at 1600 cm^{-1} has been assigned to the aromatic C═C stretching vibration (Chatten et al., 1959). These bands were shifted to higher frequencies (2960 and 2933 cm^{-1} for the C—H stretching vibrations and 1604 cm^{-1} for the C═C stretching vibration) on hydration, indicating the introduction of different conformers owing to "melting" of the hydrocarbon chains of TTC (Umemura et al., 1980; Casal and Mantsch, 1984). On the other hand, the polar bands in the dry state were seen as a C═O stretching band at 1689 cm^{-1}, as an aromatic amine C—N stretching band at 1284 cm^{-1}, and as antisymmetric and symmetric C—O—C stretching bands at 1173 and 1118 cm^{-1}. When large amounts of water were added to the TTC hydrochloride, the C═O stretching band was seen at 1697 cm^{-1}, indicating a shift to a higher frequency (Fig. 1 *b*). If a strong hydrogen bond were formed with the ester carbonyl oxygen, a frequency decrease would be observed for the C═O stretching mode. However, in addition to the increase in the frequency of the C═O stretching mode of hydrated TTC, the band profile of the C═O stretching mode showed a pronounced decrease in intensity associated with broadening compared with that of dry TTC. This hydration probably induces conformational rearrangement in the ester C═O group of TTC. The aromatic amine C—N stretching vibration at 1284 cm^{-1} in the dry state was seen at 1275 cm^{-1} and shifted to a lower frequency on hydration, indicating that hydrogen bonds were formed between amine moieties and water molecules (Hubner et al., 1991). A prominent vibration at 1118 cm^{-1} in the dry state, assigned to the symmetric C—O—C stretching mode, was broadened and

shifted to a lower frequency on addition of water. The broadening of the spectrum on hydration in the 1080–1140-cm^{-1} region probably represents the overlapping of several vibrational modes.

Effect of TTC on the spectrum in the region of the phosphate group

Details of the interaction of the TTC cation, as the active species of TTC, with phospholipid molecules in the liposomal membrane were next examined. In the absence of TTC molecules, the most remarkable change in the polar bands of phospholipids on hydration was observed for the antisymmetric PO_2^- stretching mode at approximately 1230 cm^{-1} (Fringeli and Gunthard, 1976; Ter-Minassian-Saraga et al., 1988; Choma and Wong, 1992). With hydration of the phospholipid, the PO_2^- stretching band was shifted to a lower frequency, whereas dehydration resulted in shift to a higher frequency.

Figures 2 and 3 show the effects of TTC (30 mM) on the spectral features of the antisymmetric PO_2^- stretching band in the region 1150–1300 cm^{-1} of pure EyPC and pure BhCL in the bilayer membranes. The three intense bands observed in this frequency region are due to the phosphate band, to the TTC aromatic C–N, and to the TTC ester C—O—C band (Table 1). The phosphate band was seen at 1227 cm^{-1} for EyPC (Fig. 2 *a*) and at 1215 cm^{-1} for BhCL (Fig. 3 *a*). TTC aromatic C—N and the ester C—O—C bands were seen at 1275 and 1176 cm^{-1} in the EyPC–TTC system (Fig. 2 *a*) and at 1270 and 1175 cm^{-1} in the BhCL–TTC system (Fig. 3 *a*). The effects of TTC on the phosphate bands can be resolved by successive subtractions of the pure phospholipid spectrum (Fig. 2 *c* for EyPC or Fig. 3 *c* for BhCL) and the TTC spectrum (Fig. 2 *b* or 3 *b*) from the spectrum of the phospholipid–TTC system (Fig. 2 *a* for the EyPC–TTC system and Fig. 3 *a* for the BhCL–TTC system). The subtracted spectra in Fig. 2 *d* (=*a* − *b*) and *e* (*a* − *c*) show, respectively, the spectra of EyPC and of TTC in the EyPC–TTC system, and those in Fig. 3 *d* (=*a* − *b*) and *e* (=*a* − *c*) show the subtracted spectra of BhCL and of TTC in the BhCL–TTC system. For the EyPC–TTC system in Fig. 2 *d* and *e* the positions of the peaks for the EyPC phosphate group (1229 cm^{-1}), the TTC aromatic C—N group, (1275 cm^{-1}), and the ester C—O—C group (1176 cm^{-1}) are consistent with those in the absence of TTC.

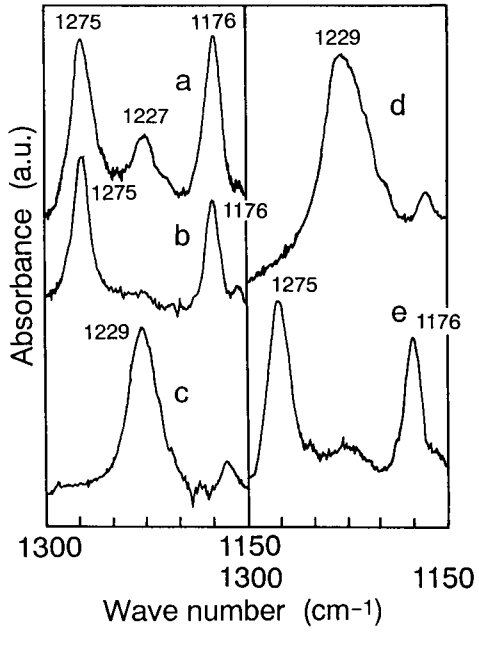

FIGURE 2 Effect of TTC on the spectrum of the antisymmetric PO_2^- stretching band in the 1150–1300-cm^{-1} region of EyPC in the bilayer. (*a*) EyPC in the presence of TTC, (*b*) TTC, (*c*) EyPC in the absence of TTC, (*d*) subtracted spectrum of EyPC (=*a* − *b*), (*e*) subtracted spectrum of TTC (=*a* − *c*). TTC was applied at 30 mM.

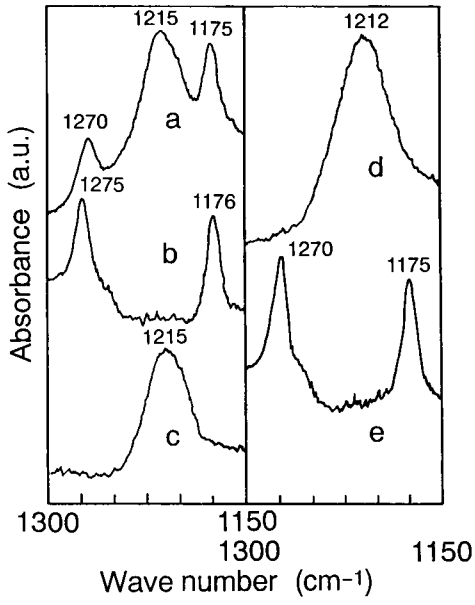

FIGURE 3 Effect of TTC on the spectrum of the antisymmetric PO_2^- stretching band in the 1150–1130-cm^{-1} region of BhCL in the bilayer. (*a*) BhCL in the presence of TTC, (*b*) TTC, (*c*) BhCL in the absence of TTC, (*d*) subtracted spectrum of BhCL (=*a* − *b*), (*e*) subtracted spectrum of TTC (=*a* − *c*). TTC was applied at 30 mM.

Nuclear magnetic resonance data have suggested that the head-group portion of phosphatidylcholine in a bilayer membrane undergoes a conformational change on interaction with the TTC molecule (Boulanger et al., 1981; Kelusky et al., 1986). In the IR spectra, this interaction should be reflected in a band-frequency shift of the PO_2^- group involving changes in the hydrated structure around the polar groups of the phospholipids; that is, in contrast to the shift to higher frequency of the PO_2^- band by dehydration, the binding of TTC to the phosphate group should result in its shift to a lower frequency. Therefore the absence of a frequency shift of the EyPC PO_2^- band in the presence of TTC was considered probably to be due to competitive

binding of TTC and water to the phosphate moiety of EyPC (Chiou et al., 1992). In Fig. 3 *d* and *e*, on the other hand, the intercalation of the TTC cation into the anionic BhCL bilayer shifted the phosphate band of the BhCL 3 cm^{-1} and the aromatic C—N stretching band 5 cm^{-1} to lower frequencies, compared with the frequencies in the absence of TTC (Shibata et al., 1994). These results indicate that the water molecules bound around the polar group of BhCL are replaced by TTC molecules. The directions of frequency shift of TTC aromatic C—N bands in EyPC and BhCL bilayers differ, suggesting a difference in the action mechanisms of the TTC cation inserted into these phospholipid membranes.

The interactions of TTC with the mixed bilayer membranes in conditions resembling those in biological membranes are, of course, of particular interest. In Fig. 4 the spectra show the antisymmetric PO_2^- stretching band (1220 and 1227 cm^{-1}) in the region of 1150 to 1300 cm^{-1} of the mixed BhCL (50 mol %)/EyPC (*a*) and BhCL (10 mol %)/EyPC (*b*) systems in the presence of TTC (30 mM). Subtraction of the spectrum of the TTC cation in an aqueous solution and the spectrum of the mixed BhCL(50 mol %)/EyPC liposomes from that of the mixed BhCL(50 mol %)/EyPC liposomes in the presence of TTC (30 mM) provides the spectrum of mixed phospholipid and TTC, both of which were modified by their mutual interactions (Fig. 4 *c* and *d*). The PO_2^- stretching vibration in the subtracted spectrum of the mixed BhCL(50 mol %)/EyPC liposomes was shifted 3 cm^{-1} to a lower frequency (Fig. 4 *c*). The aromatic amine C—N stretching band of TTC molecules inserted into the mixed BhCL(50 mol %)/EyPC bilayer membranes caused a shift of 5 cm^{-1} to a lower frequency (Fig. 4 *d*). The frequency shift of the C—N stretching band was less (2 cm^{-1}) with mixed BhCL(10 mol %)/EyPC membranes (data not shown). Taking into account the fact that there was no shift in frequency of the PO_2^- stretching vibration at 1229 cm^{-1} of pure EyPC in the presence of the TTC cation (Fig. 2), the present data indicate that TTC binds strongly to the phosphate moiety of BhCL molecules incorporated into BhCL/EyPC liposomal membranes.

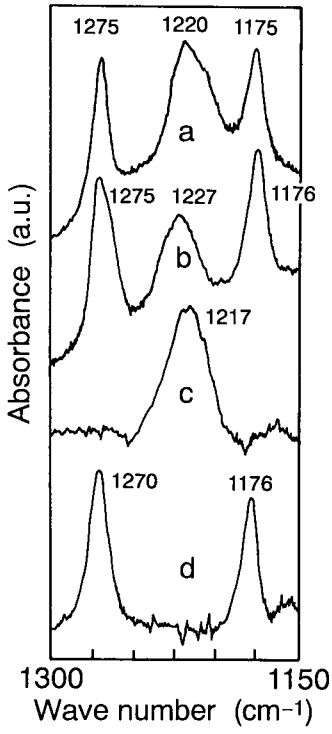

FIGURE 4 Infrared spectra of polar groups in the 1150–1300-cm^{-1} region of (*a*) mixed BhCL(50 mol %)/EyPC and (*b*) mixed BhCL(10 mol %)/EyPC bilayer membranes in the presence of 30-mM TTC. (*c*) Spectrum after subtraction of the spectrum of 30-mM TTC in aqueous solution from spectrum (*a*); (*d*) spectrum after subtraction of the spectrum of the mixed BhCL (50 mol %)/EyPC liposome suspension from spectrum (*a*).

Effects of TTC on the spectrum of the ester carbonyl group region

The region of the ester C=O stretching vibration of the interfacial region of the lipid molecule is useful for determination of the structural phase of membrane lipids. A complication in analysis of the C=O vibrational bands of phospholipids is, however, that the bands are broad and no are longer symmetric because of at least two overlapping components of different frequencies, intensities, and bandwidths (Wong and Mantsch, 1988). We found by superposition that the two bands of the C=O vibration are located at 1737 and 1726 cm^{-1} for EyPC and at 1741 and 1725 cm^{-1} for BhCL (Shibata et al., 1994). The splitting of the ester C=O vibrational bands of phospholipids was caused by hydrogen bonding of water molecules to the C=O groups, and the lower-frequency band was attributed to the hydrogen-bonded C=O band (Blume et al., 1988). Because for mixed lipid membranes the C=O vibrations are displayed by superpositions of at least four bands, it is difficult to analyze their overlapping C=O bands quantitatively. Therefore further studies were carried out on the interaction of TTC with only pure phospholipid (EyPC or BhCL) bilayer membranes.

Figure 5 shows the effect of TTC (30 mM) on the spectra of the C=O groups of pure EyPC and pure BhCL bilayer membranes. In the region between 1650 and 1785 cm^{-1} the relatively intense band at approximately 1730 cm^{-1} is due to the ester C=O stretching vibration of the phospholipid, and the shoulder on the lower-frequency side of the lipid C=O band to the ester C=O band of TTC (Fig. 5 *a* for EyPC and *b* for BhCL). For the overlapping of the different components of the C=O stretching bands for the phospholipid and TTC we determined the frequencies of the two C=O bands by curve-fit analysis after subtraction of the spectrum of the C=O band for TTC from that of the C=O band for the mixed EyPC(or BhCL)–TTC system. The results are shown in Fig. 5 *c* together with the calculated spectrum for the mixed EyPC–TTC system. The TTC cation shifted the hydrogen-bonded C=O stretching band of EyPC 6 cm^{-1} to a lower frequency but did not change the fre-

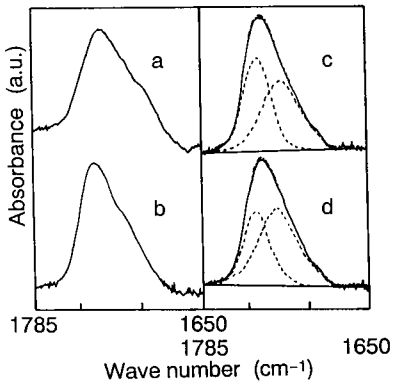

FIGURE 5 Effects of TTC on the spectra of the C=O stretching band in the 1785–1650-cm^{-1} region of pure EyPC and pure BhCL in the bilayers. (*a*) pure EyPC, (*b*) pure BhCL, (*c*) calculated spectrum of pure EyPC, (*d*) calculated spectrum of pure BhCL. Solid curves, observed spectra; dotted curves, calculated spectra; dashed curves, sum of individual calculated spectra. TTC was applied at 30 mM.

quency of the higher-frequency band (1737 cm^{-1}). For the BhCL bilayer membrane, subtraction of the spectrum of TTC in an aqueous solution from that in the BhCL–TTC membrane system showed no frequency shift of either the nonhydrogen or the hydrogen-bonded C=O group of BhCL (Fig. 5 *d*). However, the TTC cation had an influence on the intensities of the two C=O absorption bands of both EyPC and BhCL molecules (Fig. 5 *c* and *d*). Although the aromatic amine C—N bands of TTC in EyPC–TTC did not shift, those in the BhCL–TTC systems shifted to lower frequencies (Figs. 2 and 3). The present IR data suggest that the aromatic amine group of the TTC molecule interacts with the C=O groups of BhCL and EyPC molecule in different ways in competition with water molecules.

To obtain further information on the interaction of TTC with the ester C=O group, we examined the effect of TTC on the relative changes in the integral intensities of the two C=O bands of EyPC and BhCL (Fig. 5). The ratios of R ($=I_L/I_H$) of the intensity of the lower-frequency band (I_L) to the higher-frequency C=O band (I_H) were, respectively, 0.93 and 1.13 for EyPC membranes with and without TTC and 1.21 and 1.33 for BhCL membranes with and without TTC. Thus TTC decreased the R values of both EyPC and BhCL membranes. An increase in the R value indicates increased hydration of C=O groups of phospholipid (Blume et al., 1988), and TTC had the reverse effect on the R values for EyPC and BhCL membranes. These results are attributed primarily to a difference in the proportion of the hydrogen-bonded ester carbonyl groups to water in the presence and absence of membrane-bound TTC. However, inasmuch as TTC molecules interact with the C=O groups of phospholipid molecules in competition with water molecules (Fig. 5) and destabilize the phospholipid membrane structure (Shimooka et al., 1992), the interaction is expected to result in the conformational change of the phospholipid polar groups in their membranes. Therefore binding of TTC to the membrane causes a change in the intensity of the ester C=O bands. To understand details of the interaction of liposomal membranes with TTC, we next determined the partitioning of TTC and the pK_a of membrane-bound TTC.

Binding of TTC to bilayer membranes and pK_a values of membrane-bound TTC

The degree of binding in the interaction of a TTC cation with phospholipid membranes at pH 7.5 was evaluated by an equilibrium dialysis procedure. The moles of TTC bound per mole of phospholipid, n, are given in Table 2. The value of n of 0.213 with EyPC indicates that one molecule of TTC binds to approximately five molecules of EyPC. The degree of binding of TTC to the liposomal membranes increased with the content of negatively charged BhCL incorporated into the EyPC membranes: the incorporation of 50 mol % of negatively charged BhCL into EyPC membranes and pure BhCL membranes resulted in 1.6- and 2.3-fold increases, respectively, in the binding of TTC molecule.

The electrometric titration of TTC at 0.8 mM in the absence of membranes yielded a value of pK_a = 8.5 for aliphatic amine groups, which is in good agreement with reported values (Kamaya et al., 1983; Schreier et al., 1984). The apparent values of pK_a were also determined in the presence of EyPC membranes with and without BhCL (Table 2) under conditions in which the amount of unbound TTC was negligible. The pK_a of EyPC membrane-bound TTC showed a downward shift, whereas that of TTC associated with EyPC membranes with incorporated negatively charged BhCL showed an upward shift compared with that of TTC in the absence of the membranes. The downward shift of pK_a of TTC is associated with a lowering of the medium dielectric constant through stabilization of the uncharged base (Garcia-Soto and Fernandez, 1983; Schreier et al., 1984; Miyazaki et al., 1992). From the pK_a value of bound TTC, EyPC-bound TTC was calculated to be 39% uncharged and TTC associated with mixed BhCL(50 mol %)/EyPC membranes to be almost 100% charged. Considering the different types of binding of charged and uncharged forms of local anesthetics to membranes (Ohki, 1984), charged TTC would bind to the membrane surface only, and its amount bound would depend on the surface electric potential of the membranes and its partitioning,

TABLE 2 Values of pK_a of TTC in the absence and presence of phospholipid membranes and moles of TTC bound per mole of phospholipid (n)

Lipid	pK_a	n
Without lipid (aqueous solution)	8.5	–
EyPC	7.7	0.213
BhCL (10 mol %)/EyPC	8.8	0.284
BhCL (50 mol %)/EyPC	9.4	0.342
BhCL	10.2	0.493

whereas uncharged TTC would bind not only to the membrane surface but also in the bulk membrane phase.

TTC molecules interact with the ester C=O groups of phospholipid molecules in competition with water molecules (Fig. 5). The conformational change of the phospholipid polar groups at the membrane surface by the binding of TTC (Shimooka et al., 1992) is closely linked to lowering of the absorption coefficient of the IR absorption band (Blume et al., 1988). Although the decrease in the *R* values in the presence of membrane-bound TTC can be attributed primarily to dehydration by TTC as described above, this also involves a difference between the absorption coefficients of the ester C=O groups of phospholipids in the presence and in the absence of membrane-bound TTC.

DISCUSSION

In a previous study we examined the effect of the TTC cation on the water permeability of EyPC liposomal membranes with and without BhCL by monitoring their osmotic shrinkage in hypertonic glucose solution (Shimooka et al., 1992). At a certain concentration TTC induced a sharp increase in the permeability of pure EyPC liposomal membranes to water. The incorporation of small amounts of BhCL into the EyPC bilayer was effective in inhibiting the increased permeability induced by TTC. The incorporation of BhCL into the EyPC membranes may stabilize the intermolecular hydrogen-bonded network formed among BhCL, EyPC, and water molecules in the hydration layers at the membrane surface, causing a significant decrease in their water permeability associated with stabilization of the membrane structure.

In the present study IR spectroscopy was used to prove, at a molecular level, that the TTC cation interacts with the phospholipid polar groups in competition with water. The analysis was performed in terms of the PO_2^- moiety in the head group and the ester C=O group in the interfacial region of phospholipids and the aromatic amine C—N group of TTC, as potential sites during hydration.

When TTC was added to the liposome suspensions, both the PO_2^- band of negatively charged BhCL and the C—N band of TTC shifted to lower frequency. In the neutrally charged EyPC–TTC system, however, no frequency shift of the PO_2^- or the C—N band was observed (Figs. 2 and 3). This reflects a difference in the affinities of TTC to the phosphate groups of BhCL and EyPC molecules composing bilayer membranes. The consensus is that charged TTC inserted into the bilayer is located at the phospholipid headgroup level by interaction with the PO_2^- moieties of phospholipid molecules (Boulanger et al., 1981; Seelig et al., 1988). We found from the partitioning and the pK_a of membrane-bound TTC that the amount of TTC bound depends significantly on the membrane's surface properties (Table 2). It follows that alterations of the membrane's surface charge density by charged TTC cause a conformational change in the phospholipid head group and, at the same time, exert a significant influence on hydration of the lipid bilayer. Therefore the absence of a frequency shift in the EyPC PO_2^- band implies not no interaction with the TTC cation but involvement of dehydration from the phosphate surface by competitive binding (Chiou et al., 1992).

TTC also had a characteristic effect on the C=O frequency in the carbonyl group region, another potential site for hydration by phospholipid molecules (Fig. 5). TTC shifted the hydrogen-bonded C=O stretching band of EyPC to lower frequency but caused no apparent shift of the band of BhCL, indicating that it interacts with the lower-frequency C=O group of EyPC close to the polar head region in the bilayer membrane. On the other hand, the band of the aromatic amine of TTC in bilayers shifted to a lower frequency in BhCL but not in EyPC membranes (Figs. 2 and 3). Possibly this shift to lower frequency was due to hydrogen bonding between the phospholipid C=O group and the TTC aromatic amine.

The binding data indicated that one molecule of TTC bound to approximately molecules of EyPC. The degree of binding of TTC to the liposomal membranes increased with the content of negatively charged BhCL incorporated into the EyPC membranes (Table 2). From the pK_a value of bound TTC, EyPC-bound TTC was 39% uncharged, and TTC associated with mixed BhCL (50 mol %)/EyPC membranes was almost 100% charged. The decreases of the *R* values by binding of TTC to EyPC or BhCL membrane could be attributed mainly to a difference in the proportion of the ester C=O groups hydrogen bonded to water in the presence and absence of membrane-bound TTC.

The overall effect of the TTC cation can be explained by the following reactions: (1) For the BhCL molecule in the bilayer, the TTC cation binds strongly to the phosphate moiety and weakly to the carbonyl group in competition with water, which is associated with a significant change in hydration of the TTC molecule itself. (2) For the EyPC molecule in the bilayer, TTC binds weakly to the phosphate moiety and effectively establishes formation of hydrogen bonds with the carbonyl moiety.

The actions of local anesthetics are thought to be due primarily to their actions on membrane phospholipids, although their interactions with some receptor proteins are proposed to be decisive. Information on the action mechanisms of local anesthetics in phospholipid membranes is very important in connection with their effects on membranes such as induction of uncoupling of oxidative phosphorylation in mitochondria. The present results show that the cationic form of the local anesthetic TTC may interact with the phosphate moieties of negatively charged phospholipids such as cardiolipin and the ester carbonyl moieties of neutral phospholipids such as phosphatidylcholine in the membrane surface. As the interaction of TTC with the phosphate moiety of cardiolipin is much stronger than that with the carbonyl moiety of phosphatidylcholine, and, as insertion of a small amount of cardiolipin greatly stabilizes the membrane integrity of the bilayer structure of phosphatidylcholine liposomes (Shibata et al., 1994), the interaction

of the TTC cation with cardiolipin should be decisively important for its action. The interaction of the TTC cation will generate rearrangements of the intermolecular hydrogen-bonded network among phospholipid molecules associated with liberation of hydrated water molecules on the surface of the membranes and change the orientation of the P-N dipole of phospholipid molecules (Scherer and Seelig, 1989). These changes should cause disordering of the bilayer structure, and thus they could affect the transport of Na^+ and K^+ in nerve membranes, leading to the anesthetic action. They could also cause changes in the proton permeability in energy transducing membrane systems, such as mitochondria, resulting in a unique uncoupling action. The mechanism of the uncoupling action of TTC in mitochondria will be reported elsewhere based on the present results.

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