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LOCAL ANESTHETICS AND DIVALENT CATIONS HAVE THE SAME EFFECT ON THE HEADGROUPS OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE

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The effects of the local anesthetic dibucaine on the membrane headgroup conformations of phosphatidylcholine and phosphatidylethanolamine were determined using ^{2}H- and ^{31}P-NMR. The size of the deuterium quadrupole splittings of the two methylene segments of the choline and ethanolamine groups changed dramatically and the 31-phosphorus chemical shift anisotropy of the phosphatidylcholine headgroup decreased by about 7 ppm in the presence of local anesthetic. The quadrupole splittings of the 3-glycerol and choline methyl segments were relatively insensitive to the addition of dibucaine. The headgroup data for dibucaine addition paralleled similar data for the addition of various cations. These NMR results agree with the previous observation that these drugs displace calcium from phospholipids. The effects of this local anesthetic on these headgroups were distinctly different from the changes induced by cholesterol, heat and the general anesthetic chloroform.

Introduction

Despite almost a century of work, the mechanism of anesthesia still remains unclear [1–4]. One aspect of local anesthetic action which has been extensively studied is the ability of these drugs to displace membrane bound calcium in both natural and model membrane systems [3,5]. At this point, the phenomenon remains one of the more interesting effects of local anesthetics at clinical concentrations. In this report the effects of divalent cations and the local anesthetic dibucaine on the headgroup conformation of two zwitterionic phospholipids, phosphatidylcholine and phosphatidylethanolamine, will be compared. With the present technology, ^{2}H- and ^{31}P-NMR are the most straightforward methods for the study of the lipid headgroup conformation in fluid bilayer membranes [6–9]. Previously these methods were useful in the study of the nonperturbed DPPC [10] and DPPE [11] headgroups and in the analysis of the effects of various cations on the DPPC headgroup [12].

Methods

Dibucaine HCl was obtained from the Sigma Chemical Co.. Headgroup deuterated DPPC's were synthesized as described by Gally et al. [10] as well as deuterated DPPE's [11] with some alterations [13]. ^{2}H-NMR measurements were made at 61.4 MHz with a Bruker WH-400 spectrometer using a normal single pulse (90°) sequence. With this instrument, full, undistorted powder spectra gener-

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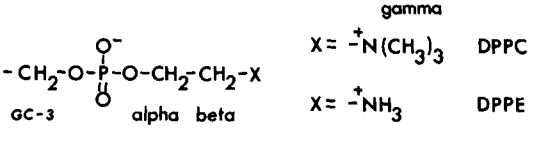
Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; Pipes, Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

ally are not obtained; however, this factor does not affect the measurement of the quadrupole splitting [14]. Proton decoupled ^{31}P-NMR spectra were measured at 36.4 MHz with a Bruker HX-90 spectrometer with 5–10 watts of broadband decoupling power being applied. Dispersed, multilamellar phospholipid samples were prepared in two ways each in 50 mM Pipes-Tris buffer (pH 7.0) with 0.1 M NaCl and 1.0 mM EDTA. In the first method, 30–50 mg of phospholipid were suspended in 0.3–0.5 ml of buffer by vortexing and heating to temperatures above the phase transition temperature. This sample was titrated with aliquots of an aqueous 0.5 M dibucaine HCl solution. At very high levels of drug, the pH shifted downwards pH 5.5. Titration back to pH 7.0 did not alter the quadrupole splitting nor the chemical shift anisotropies. At low pH values, a small (< 10%) isotropic signal could be observed in the phosphorus spectra which disappeared after titration to pH 7.0. After each aliquot addition the sample was mechanically mixed, heated to a temperature above the phase transition temperature of the pure lipid and remixed. In the second method, 30 mg of phospholipid was dispersed in 10 ml of buffer with the desired concentration of the drug. Following dispersal of the lipid, the mixture was centrifuged and the pellet measured. DPPE preparations with cations were prepared as described for DPPC [12]. Chloroform was added directly to the dispersed lipid preparations and extensively mixed.

Results

For convenience the following nomenclature for the various headgroup positions will be employed:



Typical ^{2}H-NMR powder spectra of [α-C^2H_2]-DPPC in mixtures with dibucaine HCl are shown in Fig. 1 (drug was added in aliquots). Dibucaine leads to a decrease in the size of the α-position quadrupole splittings of both DPPC and DPPE, whereas an increase is observed at the β-position. Two signals were seen in the spectra at the α and β positions at dibucaine:phospholipid (molar) ratios of 0–0.4. One signal had a quadrupole splitting whose magnitude is roughly the same as that of the pure lipid, while the other changed in size as a function of the dibucaine concentration. The intensity of the signal associated with the unaltered lipid diminished with increasing amounts of dibucaine and above a molar ratio of 0.4, the signal disappeared. The appearance of two signals at low drug levels suggests two environments, i.e. relatively unaltered phospholipid and phospholipid

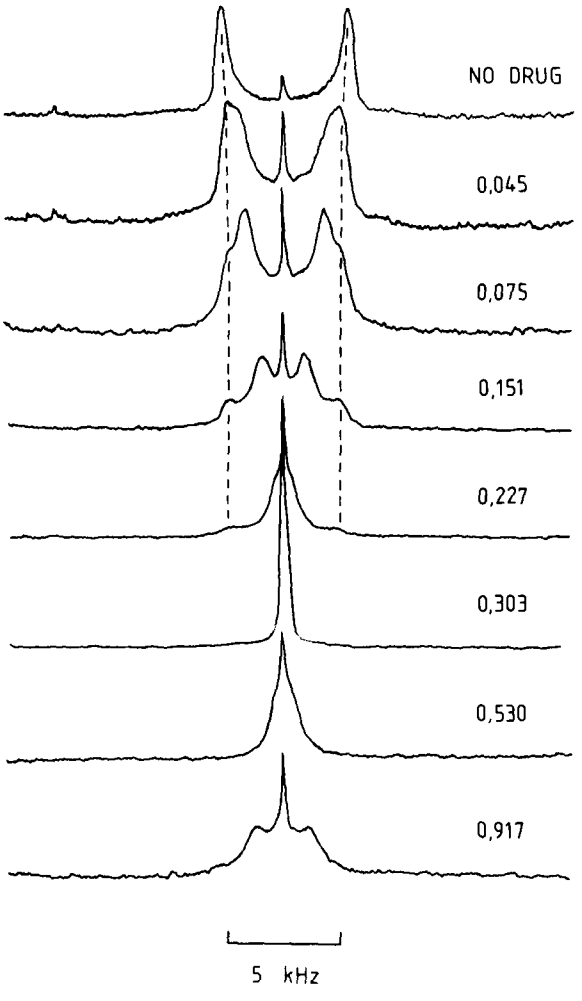


Fig. 1. ^{2}H-NMR spectra of (α-C^2H_2)-labeled DPPC in the presence of varying amounts of dibucaine HCl (molar ratios of drug:DPPC are given). Dotted lines indicate the quadrupole splittings which correspond to relatively unaltered lipid. The measuring temperature was 53°C.

with bound drug. Whether these two environments result from two types of drug binding sites as has been suggested previously [15] or simply from a slow exchange of the drug between the lipids is not clear.

The effects of dibucaine on the quadrupole splittings are quantitated in Fig. 2. The partition coefficient for dibucaine in phosphatidylcholine membranes lies between 40 and 2000 [15,16]. Therefore, under these conditions of 50 mg lipid in 0.5–0.6 ml buffer, most of the added drug will be dissolved in the membrane and the drug:phospholipid ratios given in Figs. 1 and 2 are close to the actual values. The [α-C^2H_2]DPPC quadrupole splitting is seen to approach zero and then to increase in size. This tendency reflects a change in the sign of the order parameter for this segment [12,17,18]. With this NMR technique, the absolute sign of the quadrupole splitting cannot be determined and the signs shown in Figs. 2 and 3 have been assigned arbitrarily. The pH of all the samples was between 5.5 and 7.0 and the quadrupole splittings did not change appreciably within this pH interval. A similar pH insensitivity of the phosphatidylcholine headgroup quadrupole splittings in mixtures with local anesthetics was mentioned [15]. The shape of the titration curves were

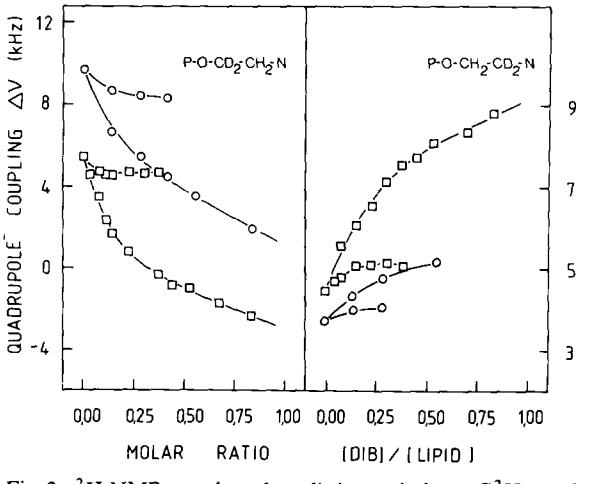


Fig. 2. ^{2}H-NMR quadrupole splittings of the α-C^2H_2 and β-C^2H_2 headgroup labels of dispersed multilamellar membranes of DPPC (□) and DPPE (○) as a function of the molar ratio of total dibucaine:phospholipid. The two curves shown for each position represent the two observed signals (c.f. Fig. 1). Temperatures were 53°C for DPPC and 68°C for DPPE.

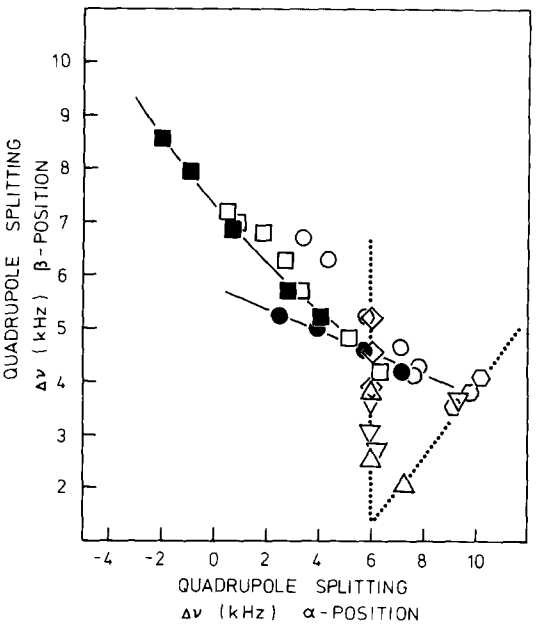


Fig. 3. Plot relating the headgroup α-C^2H_2 and β-C^2H_2 position quadrupole splittings for DPPC (□, ◇) and DPPE (○, ⬡) in the presence of varying amounts of dibucaine (■, ●), various organic and metallic cations (□, ○), cholesterol (△), chloroform (▽) and at various temperatures (◇, ⬡). Solid lines indicate the changes induced by dibucaine, while dotted lines show the effects of cholesterol, heat and chloroform. The α, β coordinates of the nonperturbed headgroups lie at the intersection of the solid and dotted lines. Data are shown for the following conditions. Molar ratios of dibucaine to phospholipid: DPPC 0.05, 0.1, 0.25, 0.50 and 0.75; DPPE 0.1, 0.25, 0.50 and 0.75. Cation additions are listed in order of increasing effect: DPPC 1.05 M $(CH_3)_4NBr$, 1.05 M NaCl, 0.35 M $CdCl_2$, 0.35 M $MgCl_2$, 0.35 M $CaCl_2$, 1.05 M $(phenyl)_4PCl$, 1.0 M $CaCl_2$; DPPE 0.35 M $CdCl_2$, 0.35 M $MgCl_2$, 0.35 M $CaCl_2$, 1.0 M $CaCl_2$, 0.35 M $LaCl_3$, 1.0 M $LaCl_3$. Molar ratios of cholesterol to phospholipid (data from Ref. 21): DPPC 0.5, 1.0; DPPE 1.0. Molar ratio of chloroform to phospholipid: DPPC 1.0, 1.5, 2.0; DPPE 0.7. Temperatures: DPPC 43, 53 and 63°C (data from Ref. 10); DPPE 63, 68 and 85°C (data from Ref. 11). All the data except the temperature data were obtained at 53°C (DPPC) and 68°C (DPPE). Most of the metallic cation and some of the anesthetic data were obtained by interpolation of the temperature dependence curves.

similar for both the α and β positions as expected and also between the two headgroups, DPPC and DPPE. These data are qualitatively very similar to the results obtained from mixing DPPC with various metallic cations [12]. At high concentrations, the effects of La^{3+} and Ca^{2+} on the DPPC

headgroup were saturating. With local anesthetic addition, the extent of the changes in the headgroup quadrupole splittings was larger than that achievable with cation addition and complete saturation was not observed even at drug:phospholipid levels approaching 1.0.

When lipids were dispersed in a large quantity of buffer with dibucaine, the same trends were observed. For example, with DPPC at drug concentrations of 0.5, 2, 5 and 10 mM the headgroup quadrupole splittings were the same as those obtained with molar ratios of 0.02, 0.1, 0.24 and 0.33 (drug:phospholipid), respectively. The temperature dependencies of the quadrupole splittings at all positions were essentially identical to those observed with cation addition [12].

The terminal methyl (γ) segment of the DPPC headgroup and the glycerol backbone (GC-3 segment) were also examined. The effects of dibucaine on the γ segment were very small (a slight increase in the quadrupole splitting) in agreement with previous reports [15,19]. No appreciable effect of cations on this segment was reported [12]. The glycerol backbone quadrupole splitting (GC-3) decreased from about 28 kHz [11] in the absence of drug to 24.5 kHz at a drug:phospholipid molar ratio of 0.75. Likewise, a small decrease of 0.5 kHz in this quadrupole splitting was reported in the presence of 0.35 M Ca^{2+} [12]. Using the dependence of the quadrupole splittings on the drug concentration given in Figs. 2 and 3, one can see that dibucaine at a drug:phospholipid ratio of 0.15 induces quantitatively the same changes in the choline headgroup quadrupole splittings as 0.35 M $CaCl_2$. With a 0.15 dibucaine:phospholipid ratio, the GC-3 position quadrupole splitting was decreased by about 1.0–1.5 kHz (average of the two deuteron signals). Thus the local anesthetic affect at this position is larger than that induced by metallic cations, although both agents have relatively small effects on this segment. The apparently larger decrease in the quadrupole splitting is reasonable considering the large perturbation introduced by a local anesthetic at this level. The GC-3 position of DPPE was not examined.

Structural changes in the headgroup can also be studied by means of ^{31}P-NMR [8]. Here the characteristic parameter is the separation of the edges of the powder spectrum which is the chemical shift anisotropy. A roughly linear decrease in the chemical shift anisotropy from about -46 ppm (no drug) to -55 ppm (at a molar ratio drug:phospholipid of 1.0) was observed with DPPC. The chemical shift anisotropy of DPPE varied only slightly from -43 ppm to -44 ppm over the same concentration range. The same result was reported for the addition of the tranquilizers diethazine or chlorpromazine to phosphatidylcholine where the chemical shift anisotropy decreased from -46 ppm to -62 ppm (fluid phase data, Ref. 20). These two drugs have chemical structures similar to dibucaine.

The effects of an inhalation anesthetic, chloroform, on the headgroups were examined using these techniques. With DPPC, in contrast to the effects of local anesthetics or cations, the addition of chloroform decreased the β-quadrupole splitting while the α-splitting remained unchanged. This trend is identical to that obtained by heating the sample [10] or adding cholesterol [21]. With chloroform:DPPC molar ratios greater than 1.0, an isotropic signal appeared in both the ^{31}P and ^{2}H spectra. With DPPE, the quadrupole splitting data remained unaltered up to molar ratios of 0.7. Above this range, two signals were observed, one with the original quadrupole splitting and a second with a quadrupole splitting one-half that of the pure lipid. Above a molar ratio of 1.5 only the signal with the smaller quadrupole splitting and an isotropic signal were observed. Such behavior is typical of a lamellar to hexagonal phase transition [22]. ^{31}P-NMR also indicated the appearance of a new phase in addition to the original lamellar and an isotopic phases as has been reported previously by Cullis et al. [23].

Fig. 3 summarizes in the form of an α- vs. β-quadrupole splitting plot the effects of dibucaine, cations, cholesterol, heating and chloroform on the DPPC and DPPE headgroup ^{2}H-NMR data. This figure dramatically illustrates the similarity of the cation and local anesthetic data. Cations and dibucaine induce essentially identical changes in the phosphatidylcholine headgroup. With the phosphatidylethanolamine headgroup, these two agents have similar, but not identical effects. Despite these relatively small differences, the effects can be grouped into two clear classes, one group containing local anesthetics and cations

and the other cholesterol, heat and chloroform.

The size of the quadrupole splitting is determined by both the average conformation of the headgroup and the amplitude of the fluctuations about this conformation. Thus a shift in the 'α, β' coordinates shown in Fig. 3 can be attributed to a change in the conformation and possibly the dynamics of the headgroup. In the case of the cation and local anesthetic effects, the data cannot be explained purely on the basis of altered dynamic properties, but rather a conformational change must occur. For the cholesterol, heat and chloroform alterations changes in only the dynamics properties could explain the results, but here again some conformational change is expected. The reader is referred to more comprehensive treatments of these effects [12,18]. The cholesterol effects have been previously reported although at that time it was not noticed that the addition of cholesterol results in almost identical physical states (identical 'α, β'coordinates) for both the DPPC and DPPE headgroups [21]. This observation lends support for the placement of both DPPC and DPPE in the same quadrant in this α-vs. β-quadrupole splitting plot, despite the lack of knowledge of the relative signs of the various quadrupole splittings.

Similar trends are found with the ^{31}P chemical shift anisotropies. The DPPC chemical shift anisotropy is known to increase by several ppm in the presence of cholesterol [21,24], an effect opposite to that of cations or local anesthetics. Such dramatic effects are not observed with DPPE indicating further that there are some differences between these two lipids. The glycerol backbone (GC-3) position quadrupole splitting increases slightly in the presence of cholesterol (Ghosh, R., unpublished data) and this is again an effect opposite to that induced by local anesthetics and cations.

Discussion

These data show that both local anesthetics and metallic cations induce similar changes in the physical state of the phosphatidylcholine and phosphatidylethanolamine headgroups. In general the effects on these physical states could be grouped into two classes. One type perturbation is associated with local anesthetics and cations and another with cholesterol, heat and chloroform. What are the common factors in these sets of rather diverse agents?

The positive charge is the only common element in the cation binding class. Previous studies on ion binding to phospholipids have shown that the phosphate moiety is the site of cation binding (for review, see Ref. 25). Dibucaine as well as many other local anesthetics consist of a hydrophobic portion connected to a tertiary amine. The hydrophobic portion positions the molecule in the membrane most likely with the amine oriented in the lipid:water interface region near the positively-charged amines of neighboring phospholipid molecules. This interaction between the phosphate and the drug could interfere with potential interactions between the phosphate and the amines of neighboring headgroups. Metallic cations via binding to the phosphate (or possibly simple charge screening) could also interfere with these interactions between neighboring headgroups. Thus the positive charge is viewed as disrupting weakly bound ligands from the phosphates. These ligands could be the amines of neighboring lipids or the solvation shell of the phosphate. Bound water is known to be displaced from the phosphatidylserine headgroup with calcium binding [26].

The second type of headgroup alteration induced by cholesterol, heat and chloroform could be attributed to membrane expansion. Since the 3-OH group of cholesterol in a membrane is situated at the level of the glycerol backbone [27], the surface area available to a phospholipid headgroup (e.g. the phosphocholine portion of DPPC) is effectively increased in mixtures with cholesterol. Monolayer studies show that the available area per headgroup increases from about 60 $Å^2$ to 90 $Å^2$ (fluid state) in a 1:1 molar mixture (for review, see Ref. 28). Chloroform can be considered a typical member of the class of inhalation anesthetics which are known to laterally expand membranes (for review, see Ref. 4). These drugs are believed to partition preferentially into the hydrocarbon interior and their action can be compared roughly to that of cholesterol. Likewise, heating may also have a similar effect. A temperature increase of 30°C expands the surface area per phospholipid by 5–7 $Å^2$ as judged by monolayer

data (dimyristoyl- or dipalmitoylphosphatidylcholine in the fluid phase at a surface pressure of 30–35 dynes/cm [29,30]), although calculations using thermal volume [31] and transbilayer expansion [32] coefficients yield smaller values. Lateral expansion would create more space for the relatively bulky phosphatidylcholine headgroup and result in a reorientation. The accepted headgroup structure of DPPC has the C_α-C_β bond approximately parallel to the membrane surface and C_β-N bond directed upwards [6,7,25,33]. If such a reorientation involves a rotation about the C_α-C_β bond, it would affect primarily the β-C^2H_2 but not the α-C^2H_2 quadrupole splittings as was observed here.

On this basis, local and general anesthetics differ fundamentally in their effects on these two lipid headgroups. The general anesthetic effect appears to be related to membrane expansion, while the local anesthetic effect can be attributed to the approach of a positive charge to the phosphate. The insertion of a local anesthetic into a membrane also results in membrane expansion and a large perturbation in the region of the glycerol backbone. Since the same headgroup physical state is achieved in the expanded state (with local anesthetics) and in a nonexpanded or possibly even slightly contracted state [34] (with divalent cations), the inter-headgroup spacing is not the fundamental determinant of drug effects on the physical state of the headgroup. Rather, these packing changes manifest themselves in small shifts in the phase transition temperatures. The slight (if any) change in the packing induced by polyvalent metallic cations does not appreciably affect the phase transition temperatures of the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine [35–37]. On the other hand, the local or general anesthetic induced expansion slightly lowers the phase transition temperature [2,16,37].

These results are in good agreement with the known ability of local anesthetics to displace cations from phospholipids. Only negatively-charged lipids can bind calcium appreciably in the millimolar concentration range [16,25,38] and in this sense a study on phosphatidylcholine or phosphatidylethanolamine is not particularly relevant. The weak nature of the cation interaction with phosphatidylcholine and phosphatidylethanolamine is reflected in the high cation concentrations necessary to induce a change in the headgroup conformations. However, these studies are useful in that they indicate the types of phenomena to be expected with the negatively-charged phospholipids. Most likely the efficacy with which local anesthetics displace cations from phospholipids results from their ability to place a positive charge in the proper position at the membrane surface. By virtue of their amphiphatic nature, the amine is always positioned in the interface region [39].

Important from the viewpoint of anesthesia is the relationship between the drug levels necessary for the physiological effect and those required for the present observations. Whether the dibucaine concentrations employed in this study are in clinical range is a difficult question. Present estimates of the amount of anesthetic in a membrane needed for anesthesia are about 0.04 mol per kg dry erythrocyte membrane [4] corresponding to a drug:phospholipid ratio of 1:10 (assuming 50% protein and 1:1 molar ratio of cholesterol:phospholipid). However, this ratio could be much higher in certain regions. This may result because local anesthetics appear to partition preferentially into the inner erythrocyte membrane face [40–43] due to the transbilayer lipid asymmetry and a higher partition coefficient with negatively-charged phospholipids [16,44]. Nevertheless, the amounts of anesthetic required to alter appreciably the headgroup conformation appear to be considerably larger than the levels required for anesthesia. The same conclusion is reached when one considers the relatively small effect induced by 10 mM dibucaine. This concentration is already two orders of magnitude higher than a normal anesthetic concentration [3,4]. Likewise, the calcium concentrations necessary to significantly perturb these headgroups are certainly not physiological. These results suggest that the effect of local anesthetics on model phosphatidylcholine or phosphatidylethanolamine membranes appear to be too small to be important at physiological anesthetic concentrations. Rather, with these amine-type anesthetics, one should examine better the role of the negatively-charged lipids.

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