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Interaction of local anesthetic heptacaine homologs with phosphatidylcholine bilayers: spin label ESR study

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Abstract

Local anesthetic monohydrochlorides of [2-(alkoxy)phenyl]-2-(1-piperidinyl)ethyl esters of carbamic acid (CnA, $n = 2$, 3, 4, 6, 8, 10, 12 is the number of carbon atoms in the alkyloxy substituent) increase the probability of formation of *gauche* isomers p_g and decrease the effective energy difference between *gauche* and *trans* conformation E_g in egg yolk phosphatidylcholine (EYPC) acyl chains, as determined by electron spin resonance spectroscopy using dipalmitoylphosphatidylcholines labeled with the paramagnetic dimethyloxazolidinyl group on the 12-th or 16-th carbon atoms of their *sn*-2 acyl chain, and oriented EYPC bilayers hydrated at 81% relative water vapour pressure. CnAs also increase the hydration of EYPC in non-oriented bilayers at the same relative water vapour pressure. At the molar ratio of CnA:EYPC = 0.4:1, the maximum effect on p_g, E_g and hydration has been observed for intermediate alkyloxy chain lengths $n \approx 4 \div 6$.

Keywords: Lipid bilayer; Phosphatidylcholine; Local anesthetic; Spin label; ESR; Hydration

1. Introduction

Most of numerous experimental studies of interactions of model and biological membranes with tertiary amine local anesthetics have been performed with procaine, lidocaine, tetracaine and dibucaine (cinchocaine). It is well known that the local anesthetic potency and the ability to reduce the action potential on the isolated nerve increase in the order procaine < lidocaine < tetracaine < dibucaine [1–4]. The same order of potency has been observed in the ability of these drugs to affect various reactions catalyzed by the transmembrane (Ca-Mg)ATPases of sarcoplasmic reticulum and of brain synaptosomes [5–7]. Since all these effects correlate well with the effects of these drugs on the fluidity of synaptosomal and model membranes [3,8] and on the gel-liquid crystal phase transition temperature of model membranes prepared from synthetic phospholipids [9–13], it seems that the local anesthesia and the effects on ATPases might be primarily caused by interactions of local anesthetics with the lipid part of the target membrane or/and with the lipid–protein interface in this membrane. However, the partition coefficients of these drugs between aqueous phase and *n*-octanol increase in the same order as above [12,14], and thus all these correlations may simply indicate an increased affinity in the series of these drugs for the hydrophobic binding sites in the studied systems.

The local anesthetic heptacaine, [2-

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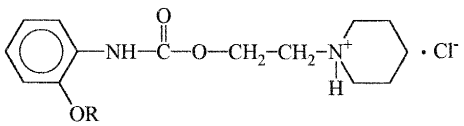
(heptyloxy)phenyl]-2-(1-piperidinyl) ethyl ester of carbamic acid [15], is more potent than the other anesthetics mentioned above in local anesthesia [15,16], in blocking the action potential on the nerve [4] and in inhibiting the sarcoplasmic reticulum (Ca-Mg)ATPase activity [17]. Heptacaine is also more potent in perturbing the fluidity of synaptosomal and model membranes [3] and in decreasing the gel-liquid crystal phase transition temperature of dipalmitoylphosphatidylcholine (DPPC) model membranes [11,12]. Since its aqueous phase/*n*-octanol partition coefficient is higher than that of the other anesthetics above [12,18], these findings should not be surprising. However, in the series of its alkyloxy homologs



where $R = C_nH_{2n+1}$, the local anesthetic potency [15,16] and the inhibiting effect on the sarcoplasmic reticulum (Ca-Mg)ATPase activity [17] increase with the number of carbon atoms in the alkyloxy substituent only up to heptyloxy and hexyloxy substituent, respectively, and then decrease, though the aqueous phase/*n*-octanol and aqueous phase/model phosphatidylcholine membrane partition coefficients increase exponentially [18,19]. Not only anesthetic but also other biological potencies of long chain amphiphilic substances show such a biphasic dependence on the chain length in homologous series [20]. Because of the decrease of activity for more lipophilic substances within the homologous series, it is often called cut-off effect. In the series of heptacaine homologs a typical cut-off effect has been observed not only in their potency in local anesthesia and in inhibition of the ATPase activity but also in their antimicrobial activity [21].

The aim of the present paper is to study the lipid bilayer perturbation effects of heptacaine homologs. Their interaction with oriented multilayers of egg yolk phosphatidylcholine (EYPC) is studied by spin label ESR spectroscopy. To avoid effects of anesthetic partition equilibria between the aqueous and lipid phases, oriented bilayers with low level of hydration are used.

2. Materials and methods

Phosphatidylcholine from hen egg yolks (EYPC) was prepared and purified according to Singleton et al. [22]. Its purity was checked by a thin-layer chromatography. The content of peroxides determined spectrophotometrically according to Klein [23] was less than 1%. Monohydrochlorides of [2-(alkoxy)phenyl]-2-(1-piperidinyl)ethyl esters of carbamic acid (abbreviation CnA, $n = 2, 3, 4, 6, 8, 10, 12$ is the number of carbon atoms in the alkyloxy substituent) synthesised according to Ref. [24] were a kind gift from Prof. J. Čižmárik. Dipalmitoylphosphatidylcholine labeled with the paramagnetic dimethyloxazolidinyl group on the m-th carbon atom of the *sn*-2 acyl chain (m-DPPC, $m = 12, 16$) was kindly provided by Dr. K. Ondriaš. The other chemicals of analytical grade were purchased from Lachema (Brno, Czech Republic). The solvents were redistilled before use. The glass and quartz plates used for deposition of oriented EYPC bilayers were cut from broken spectrophotometric cells. To make them free of defects, cracks, and scratches, their surfaces were polished to the optical quality.

Oriented samples for ESR spectroscopy were prepared by the spattering method introduced by Kawano et al. [25] and modified as described in detail by Gallová et al. [26]. The glass or quartz plates for oriented samples had to be scrupulously clean. Their surfaces were made hydrophobic according to the procedure suggested by Kuo and Wade [27]. EYPC, m-DPPC and CnA were dissolved in ethanol or methanol at the concentration of 125 mg EYPC/ml and molar ratios of m-DPPC:EYPC $\leq$ 1:100 and CnA:EYPC = 0.4. The Hamilton microsyringe was filled with 40 μl of the EYPC + m-DPPC + CnA solution. The end of the microsyringe needle was placed into a stream of nitrogen gas expired from a Pasteur pipette which enables atomizing of the solution by the stream of gas. The angle between the needle and the Pasteur pipette was about 45°. The glass/quartz plate was placed into a holder consisting of two copper plates. First the beam of atomized solution passed through a 3 mm $\times$ 6 mm rectangular orifice in the upper copper plate. The atomized particles were deposited as a spot at the center of the glass/quartz plate laying perpendicularly to the beam of atomized particles on the lower copper plate. The

schema of the atomizer apparatus was depicted and described in detail earlier [25,26]. The glass or quartz plate with the deposited lipid film was inserted into a cylindrical glass tube and was then evacuated at about 10^{-3} Pa in the presence of P_2O_5 for several hours. The samples were hydrated for at least 30 h in the nitrogen atmosphere with relative water vapour pressure of 81% produced by saturated aqueous solution of $(NH_4)_2SO_4$ at 20°C. This method of sample preparation produces well oriented multilayers of EYPC parallel to the plate surface [25,26].

Samples for gravimetric experiments were not oriented. EYPC and CnA were mixed by dissolving in chloroform at molar ratio CnA:EYPC = 0.4. The solvent was removed in a stream of nitrogen with subsequent evacuation at about 10^{-3} Pa in the presence of P_2O_5 for several hours. The dry samples were then weighed as quickly as possible and hydrated in the nitrogen atmosphere with relative water vapour pressure of 81%. After 48 h of hydration the samples were weighed again and the amount of water in the sample was calculated as a weight difference of hydrated and dry sample. After that, the samples were kept in the nitrogen atmosphere with relative water vapour pressure of 81% and their weight was checked repeatedly at 24-h intervals. It was stable in the range of experimental error for several days.

ESR spectra of spin probes in the oriented samples were measured by an ERS 230 X-band ESR spectrometer (ZWG AdW DDR, Berlin, Germany) using the 100 kHz modulation technique. Spectrometer was equipped with a N°24 GX goniometer (Radiopan, Poland), which allows a precise positioning of the sample within the microwave cavity of ESR spectrometer with defined angle θ of the plate normal (director) with respect to the applied magnetic field. Typical instrumental settings were 5 mW microwave power, modulation amplitude less than one half of the peak–peak width of the central line in the spectra, and the rate of magnetic field sweep 0.25 G/s at 0.5 s time constant. All measurements were made at temperature 20 ± 1°C.

3. Results and discussion

The oriented multilayers of EYPC + CnA containing 12-DPPC or 16-DPPC spin probe displayed typical triplet ESR spectra with hyperfine splitting dependent on the sample orientation in the magnetic field. An example of the angular dependence of the hyperfine splitting $A(\theta)$ is depicted in Fig. 1, where θ is the angle of the director (normal to the plate surface) orientation relative to the static magnetic field B. The linear coarse of the $A^2(\theta) = f[\sin^2(\theta)]$ dependence was found for all samples used in this work and serves as evidence of sample orientation. From the angular dependence of $A(\theta)$

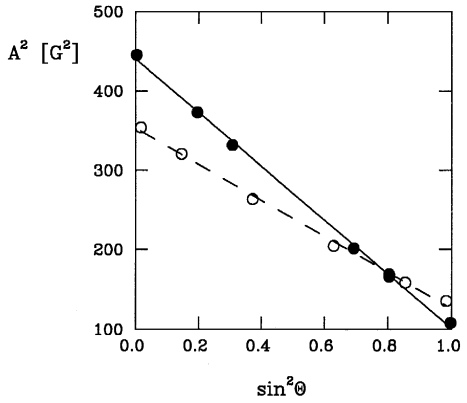


Fig. 1. The dependence of $A^2(\theta) = f[\sin^2(\theta)]$ for the 12-DPPC spin probe in EYPC bilayers (full symbols) and bilayers of EYPC + C_2A mixture (open symbols), EYPC:C_2A = 1:0.4 (mol/mol).

$$A(\theta)^2 = A_{\parallel}^2 \cos^2\theta + A_{\perp}^2 \sin^2\theta \quad (1)$$

the elements of the averaged hyperfine splitting tensor parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) to the static magnetic field were obtained using the least-squares method. The order parameter S_z was calculated then using the following expression (see Marsh [28])

$$S_z = \frac{f_a(A_{\parallel} - A_{\perp})}{\left[A_{zz} - 0.5(A_{xx} + A_{yy})\right]} \quad (2)$$

where f_a is a polarity correction factor

$$f_a = \frac{(A_{xx} + A_{yy} + A_{zz})}{(2A_{\perp} + A_{\parallel})} \quad (3)$$

and A_{xx}, A_{yy} and A_{zz} are the principle values of the hyperfine tensor in the nitroxide frame of axis. The order parameter is a measure of the mean square deviation of the molecular frame z-axis from the director. The values of A_{xx}, A_{yy} and A_{zz} obtained by

Lange et al. [29] were used in the evaluation of our data.

For hydration, the dry oriented samples were placed in the surroundings with relative humidity 81%. Fig. 2 shows examples of the order parameter time course during the process of hydration. The constant value of the order parameter in the time region 20–50 h confirms that the hydration process was completed in this time interval.

The interactions of heptacaine C7A and its homolog C10A with EYPC bilayers at low hydration level were studied in our earlier papers [30,31]. The fluidity, detected as the correlation time of methyl esters of stearic acid spin probes labeled on the 12-th or 16-th carbon atom, increased with increasing concentration of local anesthetic up to the molar ratio CnA:EYPC ≤ 0.5. A phase separation was observed at molar ratio C7A:EYPC ≥ 0.5 for samples with low hydration level in X-ray diffraction experiments (unpublished). In the present paper we wish to observe the highest possible effect of CnA molecules on EYPC bilayers but to avoid phase separation. Therefore, the molar ratio CnA:EYPC = 0.4 is used.

The presence of CnA in EYPC oriented bilayers at molar ratio of CnA:EYPC = 0.4 causes a decrease of the order parameter S_z for both spin probes compared to the control sample without CnA molecules (Fig. 3). The molecular frame z-axis is parallel to the long molecular axis when the spin labelled acyl chain of the m-DSA spin probe is in *all-trans* configuration

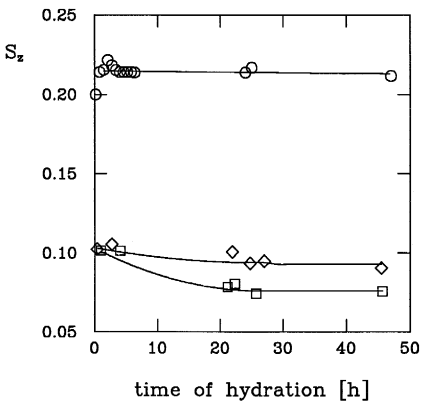


Fig. 2. The dependence of the 16-DPPC order parameter S_z on the time of hydration in oriented multilayers with different composition. Circles, EYPC; diamonds, EYPC + C_8A; squares, EYPC + C_2A. EYPC:CnA = 1:0.4 (mol/mol).

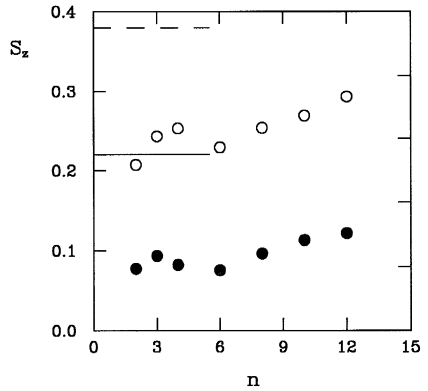


Fig. 3. The dependence of the order parameter S_z on the alkyloxy chain length n of CnA admixture in EYPC oriented multilayers. Full symbols, 16-DPPC spin probe; open symbols, 12-DPPC spin probe. Full and dashed horizontal lines show the order parameter S_z in control sample without CnA for 16-DPPC and 12-DPPC, respectively. Each point represents the mean ± S.D. of 2–5 measurements of one sample after 20–30 h of hydration.

[28]. Due to *trans-gauche* isomerisation the values of order parameter from different positional isomers of m-DPPC decrease when m moves from the lipid-water interface towards the center of the bilayer. The values of the 12-DPPC order parameter are always higher than that of the 16-DPPC spin label which is in accordance with the well known 'flexibility gradient' in the lipid bilayer [28,29,32–34].

The decrease of the order parameters S_z in the presence of CnA indicates a perturbation of the bilayer structure. This is in agreement with the results obtained in ESR spin label studies of interaction of amphiphilic amines (local anesthetics and beta blockers), alkylamine N-oxides and alkylammonium ions (bactericides) with various model and biological membranes [3,8,30,31,35–41]. The polar (charged or not charged) part of these drugs interacts with lipid polar fragments and their lipophilic parts insert in the hydrophobic region of the bilayer consisting of the lipid acyl chains. The interaction of CnA with the lipid bilayer should be the same. We have found earlier [42] that the average length of the EYPC acyl chain is 17.8 carbon atoms with 1.2 double bonds. Due to the mismatch between the lengths of the EYPC acyl chains and the CnA alkyloxy chain, the intercalation of CnA into EYPC bilayer will create a free volume in the bilayer hydrophobic region. Sup-

posing a constant surface area of the bilayer at the constant CnA:EYPC molar ratio, the free volume will be a linear function of the CnA alkyloxy chain. We have supposed that this free volume is filled-in by *trans-gauche* isomerisation of neighbouring EYPC acyl chains which should lead to the decrease of the order parameter. This model suits well for $n = 6 \div 12$, where the order parameter increases with increasing n but fails for the shorter n. For $n = 2 \div 4$ smaller values of S_z were expected.

The order parameter of the m-DPPC spin label samples in the first approximation the motion of the label paramagnetic group due to *trans-gauche* isomerization around C-C bonds of the labeled acyl chain as well as the motion of this acyl chain as a whole. We have further tested if the smaller values of S_z for $n = 2 \div 4$ might be caused by a combination of these motions. The known values of S_z order parameter determined with two different position spin labels 12-DPPC and 16-DPPC enable to extract information about the probability of *gauche* conformations p_g and about effective energy difference E_g between *trans* and *gauche* conformations in the liquid-crystalline phase of the lipid. Supposing that the rotations around a single C-C bonds are interdependent and the first segment is immovable, Seelig [32,33] derived for the order parameter of the m-th segment $(S_z)_m$

$$(S_z)_m = S_\sigma^{m-1} \cdot S_0 \tag{4}$$

where m is the number of C-C single bonds between the dimethyloxazolidinyl and carbonyl groups of the m-DPPC spin probe, S_σ is the order parameter for a single C-C bond and S_0 the order parameter of the m-DPPC molecule as a whole. Supposing that S_σ is approximately constant between C_{12} and C_{16} carbons, S_σ can be calculated from the S_z values obtained with 12-DPPC and 16-DPPC spin probes. Provided that intramolecular and intermolecular interactions disfavour combinations $g^{\pm}g^{\mp}$ and $g^{\pm}g^{\pm}$, Marsh [28] derived the following equation for the temperature dependence of S_σ

$$1 - S_\sigma^2 = \frac{9\sigma}{\left[1 + 8\sigma + (1 + 8\sigma)^{1/2}\right]} \tag{5}$$

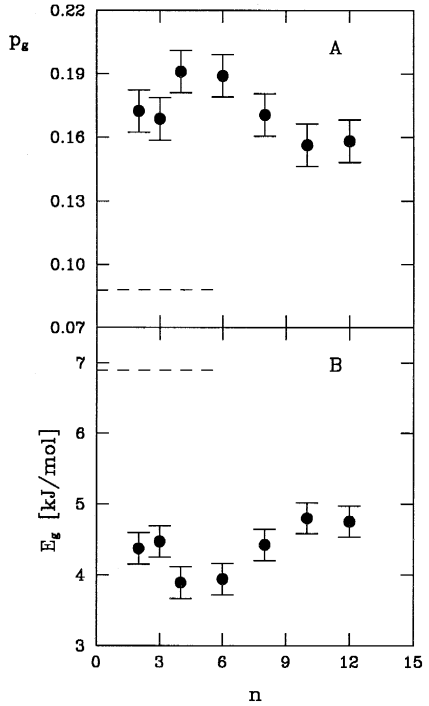


Fig. 4. The dependence of the probability p_g of *gauche* conformers (A) and of the effective energy difference E_g between the 12-th and 16-th carbons of the m-DPPC spin probe (B) on the alkyloxy chain length n of CnA admixture in oriented EYPC bilayers. Horizontal lines, control sample without CnA. For error bars see Fig. 3.

together with the equation for $p_g = p_{g+} + p_{g-}$

$$p_g = 0.5\left[1 - (1 + 8\sigma)^{-1/2}\right] \tag{6}$$

The effective energy difference E_g between *gauche* and *trans* conformation at the absolute temperature T is given by

$$\sigma = \exp\left(\frac{-E_g}{RT}\right) \tag{7}$$

where R is the molar gas constant.

As can be seen from Fig. 4, p_g is higher in the presence of CnA than in pure EYPC bilayers which indicates the perturbation effect of CnA. The hypothesis of free volume can be applied again only for $n = 6 \div 12$, where a decrease of p_g is observed for $n > 6$, but not for smaller values of n. Analogous information in units of energy renders the dependence of effective energy difference E_g between *trans* and *gauche* conformations between the C_{12} and C_{16} carbon atoms of spin labels according to Eq. (7). The

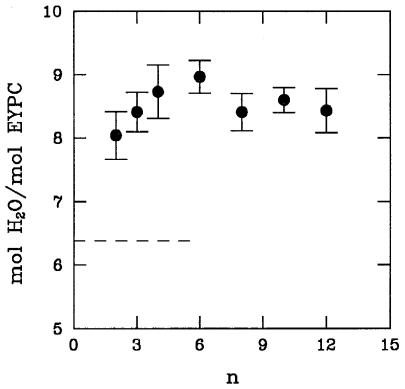


Fig. 5. The dependence of the degree of hydration on the alkyloxy chain length *n* of CnA admixture in EYPC bilayers. Horizontal line, control sample without CnA. Each point represents the mean ± S.D. of 6 measurements of one sample after 2–8 days of hydration.

values of E_g are all larger than the 2–3 kJ/mol value found for the rotational potential in liquid hydrocarbons [43]. As noted by Marsh [28], the value of E_g must be considered a pseudopotential containing contributions from intermolecular interactions as well as the intrinsic intramolecular rotational potential.

Up to now we have supposed that the decrease in free volume with increasing *n* is caused by the prolongation in the alkyloxy chain length whereas the surface area of bilayer at the constant CnA:EYPC molar ratio remained constant. It is known that this area and the level of lipid hydration are interdependent. Increasing hydration leads to expansion of the area per one lipid molecule on the lipid–water interface or vice versa [44–47]. We have shown earlier [26] that increasing hydration of EYPC in oriented multilayers induces a decrease in the order parameter and an increase in the probability of *gauche* conformers as detected by m-DPPC spin labels. Therefore, we have checked gravimetrically the level of hydration in multilayers at molar ratio of CnA:EYPC = 0.4. The results in Fig. 5 show that in the presence of CnA a higher level of hydration is achieved compared to the control sample. In these experiments we have confirmed earlier findings that heptacaine increases the amount of motionally restricted water molecules in EYPC bilayers as detected by ^{2}H-NMR spectroscopy of ^2H_2O [48] and of non-freezable water molecules in DPPC bilayers as found by differential scanning microcalorimetry [49]. It is further seen from Fig. 5 that the number of water molecules per EYPC molecule in the presence of CnA rises with *n* for $n = 2 \div 4$ and then slightly decreases or remains constant in the range of experimental error. In case of shorter acyl chains, the lower hydration induces probably smaller expansion of the surface area per one lipid molecule at the lipid–water interface and vice versa. The consequence would be smaller free volume created in the central region of the lipid bilayer. This might explain a different coarse of the of $S_z = f(n)$, $p_g = f(n)$ and $E_g = f(n)$ dependencies for CnA with shorter alkyloxy chains ($n = 2 \div 4$) compared to longer alkyloxy chains ($n > 6$).

We have found recently using small-angle X-ray scattering (SAXS) that the surface areas of 1,4-butanediaminium-N,N′-dialkyl-N,N,N′,N′-tetramethyl dibromides [50] and N,N-dimethyl-N-alkylamine N-oxides (Karlovská, Degovics, Lohner, Devínsky, Lacko and Balgavý, unpublished) in EYPC bilayers depend on the surfactant acyl chain length reaching a maximum at intermediate lengths. The same tendency has been observed for CnA anesthetics in the liquid-crystalline DPPC [51]. The results of the present paper are in accord with these observations. Besides the *trans-gauche* isomerization, an interdigitation of hydrocarbon chains in the bilayer could also eliminate the free volume in the bilayer. The result of interdigitation would be an increased value of the bilayer surface area. It has been observed that the local anesthetics tetracaine [52] and heptacaine [53] induce interdigitation of hydrocarbon chains in the gel phase and liquid crystalline phase of DPPC, respectively. The inderdigitation results in the increased rigidity in the hydrophobic center of the bilayer and the flexibility gradient as detected by spin labels is abolished [54]. This was not observed in the experiments in the present paper, so that the interdigitation as a possible cause of the surface area, hydration, p_g and E_g extremes at intermediate CnA alkyloxy chain lengths seems to be excluded. In conclusion, the results of the present paper indicate differences in the interactions of short and long alkyloxy chain homologs of local anesthetic heptacaine with phosphatidylcholine bilayers. These differences must be taken into account as an important factor in the mechanism of cut-off effect if caused by the local anesthetic–lipid bilayer interactions.

Acknowledgements

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