ELSEVIER

Biophysical Chemistry 87 (2000) 213–223

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Interaction of benzocaine with model membranes

Luciana de Matos Alves Pinto, Daniela Kiyoko Yokaichiya, Leonardo Fernandes Fraceto, Eneida de Paula*

Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (Unicamp), Sao Paulo, Brazil

Received 30 May 2000; accepted 24 July 2000

Abstract

We measured the absorption properties, water solubility and partition coefficients (P) between n-octanol, egg phosphatidylcholine (EPC) liposomes and erythrocyte ghosts/water for benzocaine (BZC), an ester-type always uncharged local anesthetic. The interaction of BZC with EPC liposomes was followed using Electron Paramagnetic Resonance, with spin labels at different positions in the acyl chain (5, 7, 12, 16-doxylstearic acid methyl ester). Changes in lipid organization upon BZC addition allowed the determination of P values, without phase separation. The effect of BZC in decreasing membrane organization (maximum of 11.6% at approx. 0.8:1 BZC:EPC) was compared to those caused by the local anesthetics tetracaine and lidocaine. Hemolytic tests revealed a biphasic (protective/inductive) concentration-dependent hemolytic effect for BZC upon rat erythrocytes, with an effective BZC:lipid molar ratio in the membrane for protection (Re^{PROT}), onset of hemolysis (Re^{SAT}) and 100% membrane solubilization (Re^{SOL}) of 1.0:1, 1.1:1 and 1.3:1, respectively. The results presented here reinforce the importance of considering hydrophobic interactions in the interpretation of the effects of anesthetics on membranes.

Keywords: Benzocaine; Partition coefficient; EPR; Membranes; Erythrocytes; Hemolysis

Abbreviations: BZC, benzocaine; C^{PROT}, drug concentration for maximal protection against hypotonic hemolysis, C^{SAT}, drug concentration for the onset of hemolysis; C^{SOL}, drug concentration for total lysis; DMSO, dimethylsulfoxide; EPC, egg phosphatidylcholine; EPR, electron paramagnetic resonance; Ht, hematocrit; LA, local anesthetic; MeSL, doxylstearic acid methyl ester spin probes; P, partition coefficient; Re, effective drug/lipid molar ratio in the membrane

* Corresponding author. Fax: +55-19-788-7840.

E-mail address: depaula@unicamp.br (E. de Paula).

0301-4622/00/$ - see front matter
PII: S0301-4622(00)00196-4

1. Introduction

Among the theories for the mechanism of action of local anesthetics (LA), particularly important is the one that explains the inactivation effect of LA on the voltage-gated sodium channel of axons [1] as well as those focusing on the effects of LA interaction with the lipid membrane phase [2]. Possibly the best indication of the importance of LA interaction with the lipid phase is the direct correlation between LA hydrophobicity and clinical potency [3–5]. The neutral form of the anesthetic molecules plays a special role in this interpretation since it is more hydrophobic than the charged species [1,3,6].

Benzocaine (BZC, Fig. 1), an ester-type LA, attracted our attention because — different from the other clinically used local anesthetics — it is always uncharged at physiological pH and causes no use-dependent inhibition of the voltage-gated Na^+ channel [7]. BZC is used for topical anesthesia, since its low water solubility limits infiltrative administration [3,6,8].

Although the effect of BZC on membrane excitability has been extensively studied [1,7], there are only a few papers describing the interaction of benzocaine with model membranes [9–11] or the fundamental physicochemical properties of BZC in this interaction [12–14].

The purpose of the present study was to describe the interaction of BZC with phospholipid and erythrocyte membranes, and to correlate its effect with LA hydrophobicity and location inside the bilayer as one step to understand local anesthesia.

2. Materials and methods

Benzocaine was purchased from Hoeschst Marion Roussel S.A. and used without further purification. Egg phosphatidyl choline (EPC) and MeSL spin labels (methyl ester of doxyl stearic acids) labeled at carbons 5, 7, 12 and 16 were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were of analytical grade.

Because of the low solubility of BZC, a dimethylsulfoxide PBS solution was used as solvent, both in EPR and hemolytic experiments. The amount of DMSO in the solutions did not exceed 8.5% v/v (1 M) and blank controls were prepared throughout, to rule out the effect of the solvent.

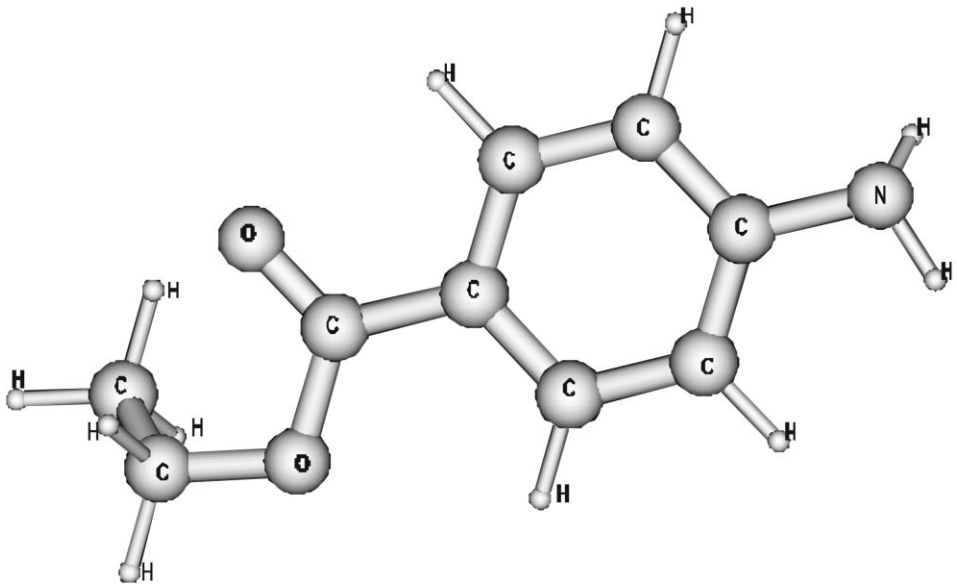

Fig. 1. Chemical structure of benzocaine (BZC).

2.1. Membrane preparation

EPC multilamellar vesicles were prepared by evaporating stock chloroform EPC solutions under a stream of nitrogen. The samples were left under vacuum for at least 2 h at room temperature (22°C). Vesicles were obtained by the addition of phosphate buffered saline, PBS (5 mM phosphate, 150 mM NaCl, pH 7.4), and vortexing for 5 min.

2.2. Erythrocyte membranes

Freshly obtained rat blood was collected into Alsever's solution (27 mM sodium citrate, 72 mM NaCl, 114 mM glucose, 2.6 mM citric acid) and washed three times in PBS, with centrifugation at $260 \times g$ for 3 min. Human erythrocyte ghosts were prepared as described by Dodge et al. [15].

2.3. EPR experiments

The experiments were conducted at room temperature (22°C) using a Bruker ER-200 spectrometer, operating at 9 GHz (3·4 kGauss) and 0.2 ml flat cells. From the spectra of the MeSL spin labels incorporated into multilamellar EPC membranes to a 1% molar ratio, we determined $h_{+1}/h_{0,}$ the ratio of low to central-field heights of the nitroxide signal (Fig. 2). h_{+1}/h_0 measure changes in membrane organization [16,17], and is expressed on a percent basis relatively to the control (without LA), according to the following formula:

$$\%\text{Effect} = \frac{(h_{+1}/h_0)\text{sample} - (h_{+1}/h_0)\text{ control}}{(h_{+1}/h_0)\text{ control}} \times 100 \quad (1)$$

This parameter comprises the effect of order and molecular mobility in the bilayer. The difference between the low-field (h_{+1}) and the mid-field (h_0) height is high in the bilayer because of the anisotropy and slow molecular motion of the probe [16]. As membrane organization decreases, h_{+1}/h_0 approaches zero and % Effect increases.

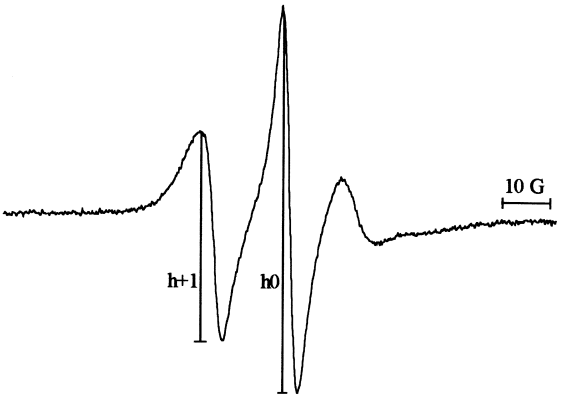

Fig. 2. EPR spectra of 5-MeSL (1 mol%) in EPC multilamellar vesicles showing the measurement of the h_{+1}/h_0 ratio: [EPC] = 8 mM, PBS buffer, pH 7.4, room temperature.

2.4. Partition coefficient between octanol and water (Poct)

PBS and *n*-octanol solutions were pre-equilibrated overnight. After BZC addition the mixture was vortexed for 5 min and incubated for an additional 15 min before centrifugation at 1000 × g for 5 min *P*oct was optically determined at 284 nm (ε_M BZC = 15 850).

The amount of drug bound to the lipid phase was obtained by subtracting the supernatant concentration from the total drug concentration, measured before phase mixing. The partition coefficient, *P*, was determined according to Eq. (2):

$$P = \frac{n_m/V_m}{n_w/V_w}, \quad (2)$$

where n denotes the number of BZC moles, V is the volume and the subscripts m and w refer to the membrane (or octanol) and aqueous phase, respectively.

2.5. Partition coefficient determination by phase separation

A known amount of BZC in PBS buffer was added to the membranes, followed by shaking and 10 min incubation at room temperature. After ultra centrifugation at $120\,000 \times g$ for 2 h, the

BZC concentration in the supernatant was optically detected at 284 nm, against a control (membrane in PBS). Partition coefficients were determined by this procedure for multilamellar EPC vesicles ($P_{\text{p-s}}$) and erythrocyte ghosts (P_{ghost}).

2.6. Partition coefficient determination by spectroscopic methods

The partition coefficient of BZC between EPC vesicles and PBS buffer was also determined by EPR without phase separation. Changes in the 5-MeSL spectra due to BZC partition and membrane saturation were used to determine P_{epr} and P_{sol}, as described before [17,18].

Briefly, in the first approach, the effect of BZC on membrane organization was determined at different (4–10 mM) EPC concentrations. For a given effect, plots of BZC total concentration, n_t — where $n_t = n_w + n_m$ in Eq. (2)-vs. membrane volume (taken from the lipid concentration, assuming density = 1 g/ml) give straight lines that allow direct P determination (P_{epr}) from the slope/intercept ratio [18].

The second method takes into account the limited solubility of BZC, leading to a fixed n_w value in Eq. (2). The hyperbolic EPR curves for the effect of BZC on membrane organization were used to determine n_t for membrane saturation (inflexion of Fig. 3b). As $n_t = n_m + n_w$, and n_w is equal to the water solubility of BZC [17], we calculated n_m and the partition coefficient, P_{sol}, for BZC between EPC/water according to Eq. (2).

2.7. Hemolytic assay under hypotonic conditions

Rat erythrocytes (hematocrit, Ht = 0.15%) were incubated in hypotonic PBS (5 mM sodium phosphate, 66 mM NaCl, pH 7.4) to induce approximately 50% hemolysis. BZC (0–21 mM) was added and the samples were incubated for 40 min. After centrifugation at $260 \times g$ for 3 min, released hemoglobin was measured in the supernatant at 412 nm. Results were expressed on a Relative Absorbance (RA) scale ranging from < 1 (protection) to > 1 (hemolysis). RA = 1 indicates 50% hemolysis induced by the 66 mM saline control. Each experiment was run in triplicate and RA values represent the mean of three independent experiments.

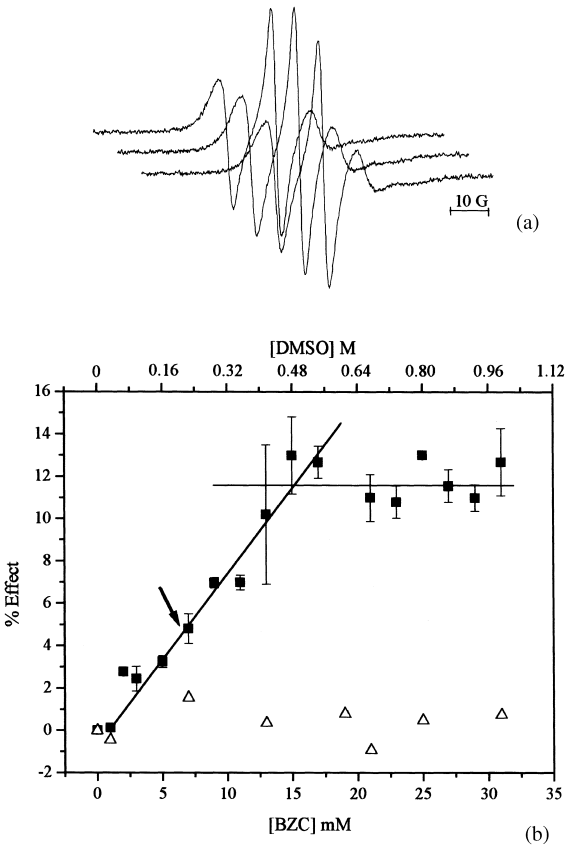

Fig. 3. (a) EPR spectra of 5-MeSL (1 mol%) in 8 mM EPC multilamellar vesicles (middle spectrum) in the presence of 0.5 M DMSO (lower) or 15 mM BZC (upper) solubilized in DMSO:PBS buffer, pH 7.4, room temperature. (b) BZC effect, as registered by the EPR spectra of 5-MeSL, on the lipid organization of EPC multilamellar vesicles (■). The effect of DMSO, used as a solvent for BZC, is also shown (Δ). The arrow indicates BZC concentration for Re = 0.33:1 (see text).

2.8. Isotonic hemolytic assay

Erythrocytes (Ht = 0.15%) in isotonic PBS solution were incubated with different BZC concentrations (0–25 mM) and the samples left at room temperature for 40 min before centrifugation at $260 \times g$ for 3 min. Hemoglobin released into the supernatant was detected at 412 nm. The hemo-

lytic effect, measured as Relative Hemolysis (RH), was determined on the basis of released hemoglobin [19]:

$$\mathrm{RH} = \frac{A_s - A_{c1}}{A_{c2} - A_{c1}}, \quad (3)$$

where A is the absorbance, s the sample, c_1 the mechanical hemolysis control (erythrocytes in PBS) and c_2 the 100% hemolysis (erythrocytes in water) control. Each experiment was run in triplicate and RH values represent the mean of three experiments.

Lichtenberg defined C^{SAT} and C^{SOL} as the amount of surfactant drugs needed for initial (membrane saturation) and total membrane solubilization, respectively [20]. Although BZC is not a surface active compound, we borrowed this definition and considered *solubilization* as the 100% release of hemoglobin into the supernatant. C^{SAT} and C^{SOL} were determined in the hemolytic experiments allowing the calculation of *Re*, the effective drug/lipid molar ratio in the membrane, for initial (Re^{SAT}) and total hemolysis (Re^{SOL}).

2.9. Re (drug / lipid ratio) calculation

Since the P value is known, *Re* can be calculated from the ratio between drug in the membrane [*nm* in Eq. (2)] and lipid membrane concentration, assuming a lipid density of 1 g/ml [19,21].

3. Results and discussion

Table 1 shows the partition coefficients of BZC between octanol (P_{oct}), erythrocyte ghosts (P_{ghost}) and multilamellar EPC vesicles ($P_{\mathrm{p\text{-}s}}$) and water, determined at pH 7.4 by phase separation. Organic solvent–water systems have been used as models of membranes for the study of partition coefficients, although the absolute P_{oct} values rarely coincide with those found in the anisotropic lipid bilayers or biological membranes [18,22,23]. Nevertheless, a good correlation exists between P_{oct} values and P values from model membranes/water within homologous series of anesthetic compounds [5]. P_{oct} indicates that BZC is a mild hydrophobic LA; in the ester family it is not so hydrophilic as procaine, nor so hydrophobic as tetracaine [12,14].

P_{ghost} reveals that BZC partition inside ghosts membranes is very similar to that in the zwitterionic EPC bilayers ($P_{\mathrm{p\text{-}s}}$). This result is quite peculiar for BZC since the high cholesterol content (30% in weight) of erythrocyte membranes [24] should restrict drug partition into them in comparison to the fluid EPC vesicles. The slightly higher P_{ghost} value may reflect the preferential binding of membrane proteins for the uncharged species of amphiphilic compounds, since dibucaine (an aminoamide LA) and trifluoperazine (a tricyclic phenothiazinic antipsychotic agent) which have an amine group with pK just above pH 7.4 show smaller P_{ghost} than $P_{\mathrm{p\text{-}s}}$ [19,21].

Table 1 also shows values of P_{epr} and P_{sol}, taken from the EPR experiments, without phase separation. Although both methodologies provided similar results, the standard deviation for P_{epr} was smaller, justifying its adoption for the *Re* calculation (see below). Using nine different aminoester and aminoamide local anesthetics, we have shown before that there is a good correlation between the partition coefficient values determined by EPR (P_{epr} and P_{sol}) and those determined by phase separation ($P_{\mathrm{p\text{-}s}}$) in EPC/water systems [17]. However, the values of P_{epr} and P_{sol}

Table 1
Partition coefficient for BZC (pH 7.4; room temperature) between octanol/water (P_{oct}), erythrocyte ghosts/water (P_{ghost}) and EPC liposomes/water obtained by phase separation ($P_{\mathrm{p\text{-}s}}$) or spectrophotometrically (P_{epr}, P_{sol}). The results are the mean of at least five experiments

Benzocaine	P_{oct}	P_{ghost}	$P_{\mathrm{p\text{-}s}}$	P_{epr}	P_{sol}
Mean ± S.D.	37.8 ± 6.1	287 ± 76	253 ± 43	115 ± 18	106 ± 40

for BZC determined here were lower than $P_{p\text{-}s}$, as explained by the fact that the first experiments were run in the presence of 0.5 M DMSO (DMSO:PBS buffer) to increase the water solubility of BZC (4.4 mM) and to reach suitable BZC:EPC ratios inside the membrane in the EPR measurements. We measured an increase in the water solubility of BZC in the presence of 0.5 M DMSO (11.3 mM), which explains the proportional decrease in the lipid hydrophobicity of the compound as detected by the P_{epr} and P_{sol} values.

In fact we are not the first to use DMSO to solubilize BZC [7,25]. DMSO is a water-miscible solvent, it forms hydrogen bonds with water molecules, shrinking the solvation shell of phospholipid membranes [26] and stabilizing their structure at low temperatures [27].

The ester linkage of benzocaine has good chemical stability in comparison to other ester-type LA [12,28] and no hydrolysis was detected at pH 7.4 up to 4 h (data not shown).

3.1. EPR experiments

Fig. 3a shows the EPR spectra of 5-MeSL inside EPC multilamellar vesicles with and without BZC. A plot of the effect on membrane organization [Eq. (1)] vs. BZC concentration is given in Fig. 3b. The figure shows that BZC decreased the membrane organization of phospholipid membranes until membrane saturation was reached and addition of the LA was not accompanied by further changes in fluidity. The maximum effect (11.6%) was reached at a benzocaine concentration of 15.4 mM (n_t), corresponding to a BZC:lipid molar ratio, *Re*, inside the membrane equal to 0.8:1. *Re* was calculated from Eq. (2) using P_{epr} (Table 1). For this we first calculated n_m, the amount of BZC in the membrane and then the ratio of n_m/ [lipid] gave *Re*, as explained in Section 2.

This behavior and the extent of maximal membrane perturbation detected with 5-MeSL was comparable to those determined for aminoester LA such as tetracaine, procaine and chloroprocaine and was more pronounced than those caused by the cyclic aminoamide LA mepivacaine and bupivacaine [17]. Fig. 3b highlights BZC's effect at 0.33:1 BZC:lipid molar ratio (arrow) and shows that DMSO, used to solubilize BZC in water, did not cause any change in membrane organization.

The values of P_{sol} in Table 1 were determined from plots like that illustrated in Fig. 3b, assuming a limiting water solubility, as described in Section 2 and [17]. Also, from plots like that illustrated in Fig. 3b, but using at least four different membrane concentrations, we determined P_{epr}, by the method of Lissi et al. [18].

Using different paramagnetic probes we could monitor the phospholipid acyl chain to detect the regions most affected by the presence of BZC. Fig. 4 is a plot of the effect (decrease in membrane organization) of BZC on EPC bilayers, as detected by 5, 7, 12 and 16-MeSL. Stearic acid spin labels do monitor different depths of the acyl chain, as demonstrated by Godici and Landsberger [29] using ^{13}C NMR relaxation times. The two curves represent the maximum perturbing effect and the effect at a fixed *Re* (0.33:1 BZC:EPC) inside the membrane. One can see that the disturbing effect of BZC is more evident when the probe monitors intermediate–C_5–C_7–positions in the acyl chain.

To understand these differential effects we compared the effect of BZC on membrane orga-

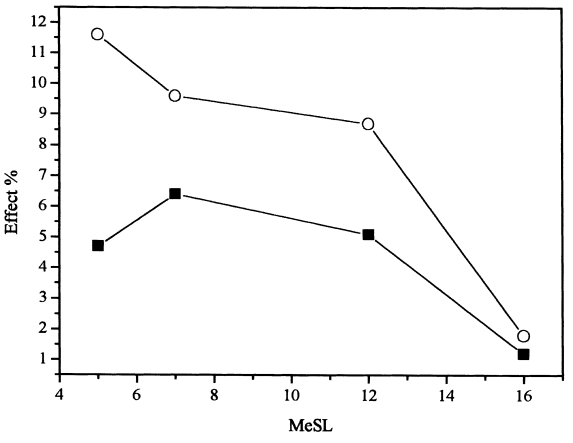

Fig. 4. Maximum effect of BZC on membrane organization (○) and effect at a fixed, *Re* = 0.33:1, BZC:EPC molar ratio in the membrane (■), as detected with 5, 7, 12 and 16-MeSL spin probes.

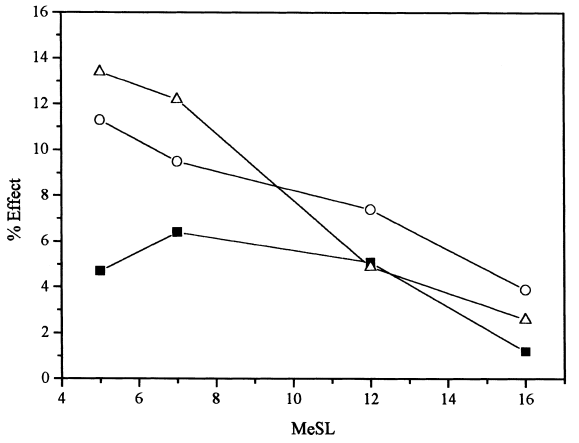

Fig. 5. Decrease in membrane organization caused by the local anesthetics benzocaine (■), lidocaine (○) and tetracaine (Δ) when present at a fixed molar ratio, $Re = 0.33{:}1$, in the membrane. Data obtained from the EPR spectra of 5, 7, 12 and 16-MeSL spin probes incorporated (1 mol%) into 8-mM EPC vesicles.

nization with that of lidocaine and tetracaine (Fig. 5) in their uncharged form, pH 10.5, and present at the same *Re* in the membrane. Lidocaine is a less hydrophobic LA that seems to lie preferentially in the glycerol neighborhood of EPC bilayers (Pinto, Fraceto, Spisni, Schreier and de Paula, in preparation). Tetracaine is a more hydrophobic LA whose uncharged species seems to position its benzenoid ring mainly at the level of C_2 in the acyl chain of phosphatidylcholine membranes, extending its *p*-butyl group up to C_8–C_{10}, as revealed by ^{2}H-NMR experiments [30].

Even considering that 16-MeSL experiences a more mobile/less ordered environment, due to the profile of the acyl chain dynamics [29–32] and order in the bilayers [33,34] — that could explain the low sensitiveness of the probe at that position, these results clearly show that BZC disturbs mainly the acyl chain core ($5 < 7 > 12$ MeSL), in a different manner than tetracaine ($5 \approx 7 > 12$ MeSL) and lidocaine ($5 > 7 > 12$).

Desai and coworkers used the intrinsic fluorescence of LA molecules to determine the equivalent dielectric constants of their environment when inserted into micelles [11]. Inside SDS micelles they found a polarity of approximately 35 (Dielectric Constant) for BZC, comparable to that of uncharged tetracaine in the same micelles, indicating that the aromatic ring of both LA were located in a region of the micelles with a polarity equivalent to that of the interface glycerol/carbonyl region of phospholipid membranes (Dielectric Constant = 30 according to [35]).

In a previous work we have shown that lidocaine and tetracaine disturb the overall membrane organization to a 26.5% and 15% extent, respectively, as monitored by 5-MeSL in EPC vesicles [17]. Here we determined a maximum effect of 11.6% for BZC. The reason for this less pronounced effect of BZC on membrane organization may be due to the steric features of the molecule since benzocaine has a small van der Waals volume (147.4 Å^3) in comparison to lidocaine and tetracaine (227.3 and 250.8 Å^3, respectively) [5]. Besides, BZC lacks a polar amine group in the hydrophilic domain of the molecule (opposite to the aromatic ring) that would be able to establish either electrostatic (above pK) or hydrogen binding [14]. As a consequence, BZC is not anchored at the interface glycerol/polar head-group region and its effect seems to be more discrete, although deeper (in position) than the other LAs studied.

We obtained other indications of the discrete effect and not preferential positioning of BZC with ^{1}H-NMR ROESY experiments, since no LA:lipid intermolecular NOE peaks were detected for BZC in unilamellar EPC vesicles (unpublished data); lidocaine, tetracaine and other aminoamide LA established detectable cross-peaks with both the polar head group and acyl chain protons of the phospholipid membranes.

3.2. Hemolysis experiments

Seeman described the biphasic effect of many amphiphilic compounds upon erythrocyte membranes: at lower concentrations they protect the membranes against hypotonic hemolysis, while at higher concentrations they induce lysis [36]. Tertiary amine LA such as procaine, tetracaine, lidocaine [37] and dibucaine [21,37] present this biphasic behavior, although just tetracaine and

dibucaine have been shown to be surface active compounds [38]. Protection against hypotonic hemolysis is believed to result from the amphiphilic partition into the membrane, increasing the membrane area/volume ratio of the cell and thereby the critical hemolytic volume of the erythrocyte.

The effect of BZC on biological membranes was evaluated in hemolytic experiments under normal and hyposmotic condition. Fig. 6a shows a typical hemolytic curve obtained after 40 min incubation of BZC with rat erythrocytes under isotonic conditions. From the amount of drug for membrane saturation at the beginning of the hemolytic curve ($C^{SAT} = 14.1$ mM) and for complete membrane solubilization ($C^{SOL} = 17.3$ mM) at 100% hemolysis, we calculated *Re* values, using P_{epr} values, as explained before. P_{epr} was used since BZC partition into ghosts and EPC membranes are very similar (Table 1) and also because DMSO was used in the BZC solutions employed in the hemolytic tests. For BZC in a Ht = 0.15% (13.05 μM lipids [15,21]), we obtained Re^{SAT} and Re^{SOL} of 1.1:1 and 1.3:1, respectively.

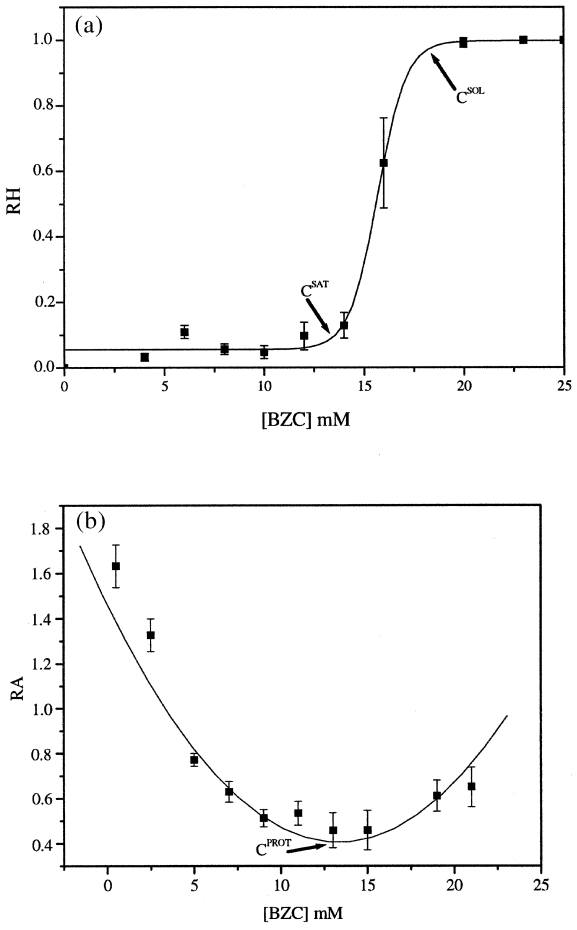

Fig. 6. (a) BZC-induced isotonic hemolysis; C^{SAT} and C^{SOL} (see text) determinations are shown. (b) BZC-induced protection against hypotonic hemolysis: Ht = 0.15%, PBS buffer, pH 7.4, incubation time: 40 min, room temperature.

For Dibucaine, a more hydrophobic LA, we have determined Re^{SAT} and Re^{SOL} of 0.34 and 0.69:1 [21], and for trifluoperazine Re^{SAT} and Re^{SOL} of 0.43 and 1.5:1 [19], ratios that suggest saturation of the membrane phase with the amphiphiles [2,39] leading to membrane solubilization.

Fig. 6b depicts the protective BZC effect on erythrocyte hemolysis under hypotonic conditions. The maximal protective BZC concentration (C^{PROT}) for a Ht = 0.15% was 13 mM, giving an Re^{PROT} value of 1.0:1 BZC:lipid.

Since there are some reports in the literature relating methemoglobin (MetHb) formation to BZC administration [40,41], we performed a control experiment using purified Hb solution and incubated it for 40 min with increasing amounts of BZC (Fig. 7). No significant increase in MetHb content was detected after BZC incubation up to 20 mM, excluding the possibility of the hemolytic effect being affected by Hb oxidation. The oxidative effect of BZC is related to its structural similarity to *p*-amine propyophenone (PAPP), a strong MetHb inducing agent [42].

4. Conclusions

The *P* values determined by EPR in Table 1 were smaller than that determined by phase separation ($P_{p\text{-}s}$), reflecting the lower partitioning of BZC into EPC bilayers when dissolved in DMSO:PBS solvent. In fact, the use of DMSO to solubilize BZC turns the water phase more attractive for the anesthetic, decreasing P_{epr} and P_{sol} values.

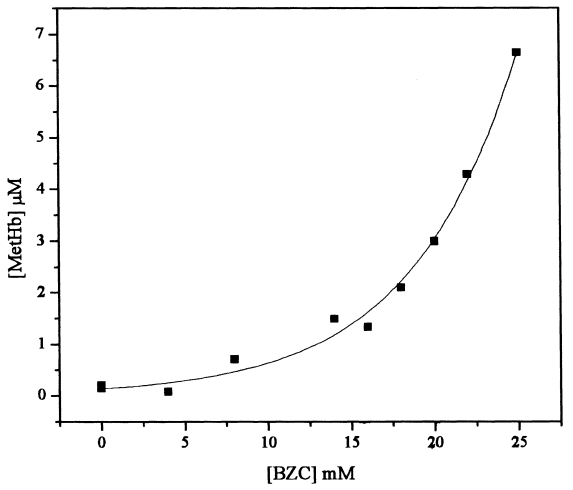

Fig. 7. MetHb formation induced by BZC in a purified Hb solution in PBS buffer, pH 7.4, at room temperature.

Since we knew the real partition coefficient for BZC in the EPR experiment, we could compare the effect of BZC with that of other commonly studied LA such as lidocaine and tetracaine. BZC decreased the overall membrane lipid organization, inserting itself into a deeper position in the bilayer, although its effect on membrane organization was less pronounced than the other LA, probably due to its smaller molecular size.

Knowledge of the P value allowed also the quantitative study of the hemolytic effect of benzocaine. As seen for other LA [36], BZC showed a biphasic (protective/inductive) concentration-dependent hemolytic effect on erythrocytes. The BZC:lipid molar ratio in the membrane for protection (Re^{PROT}) was lower than those required for lysis (Re^{SAT} and Re^{SOL}). On average, ca. 1:1 is the BZC:lipid ratio for hemolysis, a quite reasonable value for a true saturation of the membrane phase [39,43].

Re for maximum change in phospholipid membrane organization (0.8:1 BZC:lipids) is close to Re^{PROT} (1:1) in erythrocyte membranes, revealing that hydrophobic interaction rules both phenomena.

The importance of the hydrophobic parameters for anesthesia (potency and toxicity) justifies the study of the neutral LA interaction and effects on membranes and the results presented here reinforce this hypothesis, since the extent of changes in membrane organization (EPR) and hemolysis was determined by the hydrophobic interaction of the BZC molecule with the bilayer.

Besides, site-directed mutagenesis has revealed the existence of a hydrophobic binding site for LA inside Na^+ channels [44,45], reinforcing the relevance of uncharged (hydrophobic) LA species for the mechanism of anesthesia.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant 96/1451-9) and Coordenação de Apoio ao Ensino Superior. L.M.A.P. (Grant. 96/9786-0), D.K.Y (Grant 97/02395-8) and L.F.F. (Grant 98/84-8) were the recipients of fellowships from FAPESP. We acknowledge Dr S. Schreier (Institute of Chemistry, University of São Paulo) for the access to the EPR facility and CENAPAD for the use of Moldem/Gamess Molecular Simulation programs.

References

[1] J.F. Butterworth, G.R. Strichartz, Molecular mechanisms of local anesthesia: a review, Anesthesiology 72 (1990) 711–734.
[2] E. de Paula, S. Schreier, Molecular and physicochemical aspects of local anesthetic-membrane interaction, Braz. J. Med. Biol. Res. 29 (1996) 877–894.
[3] B.G. Covino, H.G. Vassalo, Local anesthetics: mechanisms of action and clinical use, Grune and Stratton, New York, 1976.
[4] K.R. Courtney, Structure-activity relations for frequency-dependent sodium channel block in nerve by local anesthetics, J. Pharmacol. Exp. Ther. 213 (1980) 114–119.
[5] S.P. Gupta, Quantitative structure-activity relationship studies on local anesthetics, Chem. Rev. 91 (1991) 1109–1119.
[6] R.H. de Jong, Local anesthetics, Mosby, St. Louis, 1994.
[7] C. Quan, W.M. Mok, G.K. Wang, Use-dependent inhibition of Na^+ currents by benzocaine homologs, Biophys. J. 70 (1996) 194–201.
[8] G.R. Strichartz, J.M. Ritchie, in: G.R. Strichartz (Ed.), Local anesthetics, Handbook of Experimental Pharmacology, 81, Springer-Verlag, Berlin, 1987.
[9] A.G. Lee, Interactions between anaesthetics and lipid

mixtures amines, Biochim. Biophys. Acta 448 (1976) 34–44.
[10] P. Schlieper, L. Michaelis, Interaction of local anesthetics with small phospholipid vesicles investigated by proton NMR spectroscopy, Biophys. Struct. Mech. 10 (1983) 1–9.
[11] S. Desai, T. Hadlock, C. Messam, R. Chafetz, G. Strichartz, Ionization and adsoption of a series of local anesthetics in detergent micelles: studies of drug fluorescence, J. Pharmacol. Exp. Ther. 271 (1994) 220–228.
[12] G.R. Strichartz, V. Sanchez, R. Arthur, R. Chafetz, D. Martin, Fundamental properties of local anesthetics. II. Measured octanol:buffer partition coefficient and pKa values of clinically used drugs, Anesth. Analg. 71 (1990) 158–170.
[13] R.J.E. Grouls, E.W. Ackerman, H.H.M. Korsten, L.J. Hellebrekers, D.D. Breimer, Partition coefficients (*n*-octanol/water) of N-butyl-p-aminobenzoate and other local anesthetics measured by reversed-phase high-performance liquid chromatography, J. Chromatogr. B 694 (1997) 421–425.
[14] H. Matsuki, K. Shimada, S. Kaneshima, H. Kamaya, I. Ueda, Difference in surface activities between uncharged and charged local anesthetics: correlation with their anesthetic potencies, Coll. Surf. B-Biointerf. 11 (1998) 287–295.
[15] J.T. Dodge, C. Mitchell, D.J. Hanahan, The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes, Arch. Biochem. Biophys. 100 (1963) 131–139.
[16] S. Schreier, W.A. Frezzatti Jr., P.S. Araujo, H. Chaimovich, I.M. Cuccovia, Effect of lipid membranes on the apparent pK of the local anesthetic tetracaine. Spin label and titration studies, Biochim. Biophys. Acta 769 (1984) 231–237.
[17] E. de Paula, S. Schreier, Use of a novel method for determination of partition coefficient to compare the effect of local anesthetics on membrane structure, Biochim. Biophys. Acta 1240 (1995) 25–33.
[18] E. Lissi, M.L. Bianconi, A.T. AmaraL, E. de Paula, L.E.B Blanch, S. Schreier, Methods for the determination of partition coefficients based on the effect of solutes upon membrane structure, Biochim. Biophys. Acta 1021 (1990) 46–50.
[19] S.V.P. Malheiros, E. de Paula, N.C. Meirelles, Contribution of trifluoperazine/lipid ratio and drug ionization to hemolysis, Biochim. Biophys. Acta 1373 (1998) 332–340.
[20] D. Lichtenberg, Characterization of the solubilization of lipid bilayers by surfactants, Biochim. Biophys. Acta 821 (1985) 470–478.
[21] S.V.P. Malheiros, N.C. Meirelles, E. de Paula, Pathways involved in trifluoperazine-, dibucaine- and praziquantel-induced hemolysis, Biophys. Chem. 83 (2000) 89–1000.
[22] S. Roth, P. Seeman, The membrane concentrations of neutral and positive anesthetics (alcohols, chlorpromazine, morphine) fit the Meyer-Overton rule of anesthesia; negative narcotics do not, Biochim. Biophys. Acta 255 (1972) 207–219.
[23] F.A.P.C. Gobas, J.M. Lahittete, G. Garofalo, W.Y. Shiu, D. Mackay, A novel method for measuring membrane-water partition coefficients of hydrophobic organic chemicals: comparison with 1-octanol-water partitioning, J. Pharm. Sci. 77 (1988) 265–272.
[24] H.P Duwe, E. Sackmann, Bending, elasticity and thermal excitations of lipid bilayer vesicles — modulation by solutes, Phys. Acta 163 (1990) 410–428.
[25] Y.–M. Li, D.E. Wingrove, H.P. Too, M. Marnerakis, E.R. Stimson, G.R. Strichartz, J.E. Maggio, Local anesthetics inhibit substance P binding and evoked increases in intracellular Ca^{2+}, Anesthesiology 82 (1995) 166–173.
[26] S.N. Shashkov, M.A. Kiselev, S.N. Tioutiounnikov, A.M. Kiselev, P. Lesieur, The study of DMSO/water and DPPC/DMSO/water systems by means of the X-ray, neutron small angle scattering, calorimetry and IR spectroscopy, Phys. B 271 (1999) 184–191.
[27] Z.-W. Yu, P.J. Quin, Solvation effects of dimethyl sulphoxide on the structure of phospholipid bilayers, Biophys. Chem. 70 (1998) 35–39.
[28] A. Brosowski, F. Müller, Temperature-dependance of the pH-value and effects on the kinetics of the hydrolytic decomposition of benzocaine, Eur. J. Pharm. Biopharm. 40 (1994) 96–100.
[29] P.E. Godici, F.R. Landsberger, The dynamic structure of lipid membranes. A ^{13}C nuclear magnetic resonance study using spin labels, Biochemistry 13 (1974) 362–368.
[30] Y. Boulanger, S. Schreier, I.C.P. Smith, Molecular details of anesthetic-lipid interaction as seen by deuterium and phosphorus-31 nuclear magnetic resonance, Biochemistry 20 (1981) 6824–6830.
[31] Y. Kuroda, K. Kitamura, Intra- and intermolecular ^{1}H-^{1}H nuclear overhauser effect studies on the interactions of chlorpromazine with lecithin vesicles, J. Am. Chem. Soc. 106 (1984) 1–6.
[32] J.F. Ellena, S.J. Archer, R.N. Dominey, B.D. Hill, D.S. Cafiso, Localizing the nitroxide group of fatty acid and voltage-sensitive spin-labels in phospholipid bilayers, Biochim. Biophys. Acta 940 (1988) 63–70.
[33] A. Seelig, J. Seelig, Bilayers of dipalmitoyl-3-sn-phosphatidylcholine conformational difference between the fatty acyl chains, Biochim. Biophys. Acta 406 (1975) 1–5.
[34] D.A. Driscoll, S. Samarasinghe, S. Adamy, J. Jonas, A. Jonas, Pressure effects on dipalmitoylphosphatidylcholine bilayers measured by ^{2}H nuclear magnetic resonance, Biochemistry 30 (1991) 3322–3327.
[35] R.F. Flewelling, W.L. Hubbell, Hydrophobic ion interactions with membranes. Thermodynamic analysis of tetraphenylphosphonium binding to vesicles, Biophys. J. 49 (1986) 531–540.
[36] P. Seeman, The membrane actions of anesthetics and tranquilizers, Pharmacol. Rev. 24 (1972) 583–655.
[37] P. Seeman, Erythrocyte membrane stabilization by local anesthetics and tranquilizers, Biochem. Pharmacol. 15 (1966) 1753–1766.

[38] H. Matsuki, S. Hashimoto, S. Kaneshima, Surface adsorption and volume behavior of Local Anesthetics, Langmuir 102 (1994) 1882–1887.
[39] A.G Lee, S. Schreier, in Entrapment of drugs and other materials (Liposome Technology, Vol. II), C.R.C. Press, Boca Raton, 1993.
[40] A.T. Guertler, W.A. Pearce, A prospective evaluation of benzocaine-associated methemoglobinemia in human beings, Ann. Emerg. Med. 24 (1994) 626–630.
[41] F.D. Ellis, J.G. Seiler, M.M. Palmore, Jr., Methemoglobinemia: a complication after fiberoptic orotracheal intubation with benzocaine spray, J. Bone Jt. Surg. Amer. 77 (1995) 937–939.
[42] D.G. Martin, C.E. Watson, M.B. Gold, C.L. Woodard Jr., S.I. Baskin, Topical anesthetic-induced methemoglobinemia and sulfhemoglobinemia in macaques: a comparison of benzocaine and lidocaine, J. Appl. Toxicol. 15 (1995) 153–158.
[43] S. Schreier, S.V.P. Malheiros, E. de Paula, Surface active drugs: self-association and interaction with membranes and surfactants. Physicochemical and biological aspects, Biochim. Biophys. Acta (in press).
[44] D.S. Ragsdale, J.C. McPhee, R. Scheuer, W.A. Catterall, Molecular determinants of state-dependent block of Na^+ channels by local anesthetics, Science 265 (1994) 1724–1728.
[45] D.S. Ragsdale, J.C. McPhee, R. Scheuer, W.A. Catterall, Common molecular determinants of local anesthetic, antiarrhytmic, and anticonvulsant block of voltage-gated Na^+ channels, Proc. Natl. Acad. Sci. USA 93 (1996) 9270–9275.