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# SPIN LABEL STUDY OF THE PERTURBATION EFFECT OF THE LOCAL ANAESTHETICS TETRACAINE AND DIBUCAINE ON SYNAPTOSOMES AT PHARMACOLOGICAL CONCENTRATIONS

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**Abstract**—The method of electron spin resonance spectroscopy of spin probes was used to determine the lowest concentrations of the local anaesthetics dibucaine and tetracaine exerting perturbations on synaptosome membranes. The perturbation depends on the temperature and the membrane depth, as well as on the concentration and the structure of the anaesthetics. Using spin labelled stearic acid at the 5th carbon position a negligible effect of the anaesthetics on the order parameter was found in the membrane both at 1° and 22°, within the buffer concentration 0.01–10 mmol/l, but at 37° and concentrations higher than 0.1 mmol/l the disordering effect was significant and of comparable efficiency for dibucaine and tetracaine. Employing stearic acid labelled at the 16th carbon position, disordering of the hydrocarbon core of the membrane caused by tetracaine or dibucaine was detected at 1° and 22°, as well as at 37°. The disordering effect occurred at buffer concentrations higher than 0.01 mmol/l for dibucaine, and higher than 0.1 mmol/l for tetracaine. At equal anaesthetic membrane concentrations and at the 16th carbon membrane depth, dibucaine was approximately twice as effective as tetracaine in perturbing synaptosomes. Tetracaine induced nonlamellar phases in the rat brain lipid membrane as detected by $^{31}P$ NMR spectroscopy. The dynamic and structural perturbation effects of the local anaesthetics was found in that concentration range at which the anaesthetics influence various activities of biological membranes.

There are important unsolved problems concerning the understanding of the biological response of drugs at molecular level. Local anaesthetics, in addition to block action potential on nerves, also influence various membrane processes, such as dopamine uptake in synaptosomes [1], $Ca^{2+}$ fluxes in synaptic vesicles [2], displacement of bound $Ca^{2+}$ in human erythrocytes [3], activity of $Na^+$, $K^+$ ATPase in synaptosomes [4] or in microsomes [5], inhibition of phosphatidylinositol transfer in microsomes [6] or phospholipase $A_2$ in monolayer [7] as well as several others. The mode of these local anaesthetic effects is not fully understood.

Local anaesthetics may interact with anaesthetic-specific receptors [8] and/or with some non-specific hydrophobic sites of membrane proteins [9, 10] or the lipidic part of membranes [11]. Local anaesthetics were found to change various physical parameters of lipid or biological membranes, such as phase transitions of lipid membrane [12], lipid polymorphism [13], order parameter of total brain lipid or synaptosomal membranes [14–17]. The perturbation effects have mostly been studied either with the anaesthetic concentrations being several times higher than pharmacological ones, or without determining the final anaesthetic concentrations in the buffer after equilibration with the membrane.

The aim of the present work was both to investigate the disordering (perturbation) effect of the local anaesthetics dibucaine and tetracaine on synaptosomal membranes at pharmacological concentrations and to compare their relative "intrinsic efficiency" to perturb the membrane at different depths.

## MATERIALS AND METHODS

Dibucaine and tetracaine were from Medika Čsl. 3 (Czechoslovakia). Stearic acid spin probe I(12,3),§ and I(1,14) were from Syva (Palo Alto, CA). All other chemicals were of analytical grade from commercial sources.

### *Sample preparation and ESR measurements*

The synaptosomes were isolated according to the method described by Krueger *et al.* [18] with a slight modification so that synaptosomes in 0.8 mol/l sucrose solution were diluted by buffer containing (in mmol/l) NaCl 145, KCl 5, Hepes·HCl 5, EDTA 0.1, pH 7.4. The synaptosomes were centrifuged at 12,000 *g* for 20 min, resuspended in the above-

§ Abbreviations used: ESR, electron spin resonance; NMR, nuclear magnetic resonance; I(12,3), stearic acid labelled with the dimethyloxazolidinyl (doxyl) group at the 5th carbon; I(1,14), stearic acid labelled with the dimethyloxazolidinyl (doxyl) group at the 16th carbon.

mentioned buffer at the ratio of 10 mg protein/ml and stored for 2–7 days at −25°.

*Preparation of samples for ESR measurements.* The synaptosomes were incubated with the buffer containing the local anaesthetic dibucaine hydrochloride or tetracaine hydrochloride (1 mg protein/5 ml) at 37° for 45 min. The samples were centrifuged at 12,000 *g* for 20 min at 30–37° and the pelleted synaptosomes were incubated with spin probe at the ratio 2 μg probe/mg protein. The method of Lowry *et al.* [19] was used for protein determination, with human serum albumin as the standard. To determine the disordering propensities of the anaesthetics at the same membrane concentration the synaptosomes with spin probe at the protein/buffer weight ratio of 1:10 were added to the dry local anaesthetic. The mixture was equilibrated by mixing and then subjected to freeze-thaw cycles for several times. ESR spectra were recorded by an BRUKER ER 200 D-SRC spectrometer in glass capillary (i.d. 1 mm) at 10 mW microwave power, modulation amplitude 0.2 mT and 0.05 mT, on using probe I(12,3) and I(1,14), respectively. On the spectra with the I(1,14) spin probe, in addition to the spectrum arising from the probe incorporated into a mobile fluid lipid membrane region, a small immobilised spectral component was also seen. We evaluated only the mobile part of the spectra [20, 24]. To assess the efficiency of the anaesthetics in perturbing the synaptosomal membranes the parameters evaluated from the ESR spectra, inner splitting $A_\perp$, outer splitting $A_\parallel$ and order parameter $S$ calculated according to Marsh [21], were used. Decrease of the order parameter $S$ or the parameter $A_\parallel$, or increase of the parameter $A_\perp$ indicates higher disorder or dynamics of the hydrophobic part of the membrane [22]. The concentrations of the local anaesthetics reported in this paper refer to those in the buffer phase immediately after drug addition.

*Disordering effects expressed in the temperature scale*

Since spin probes I(12,3) and I(1,14) possess a different motion and order in the membrane, the three ESR parameters ($A_\parallel$, $A_\perp$ and $S$) were used to evaluate the disordering effect of the anaesthetics. In order to compare the disordering propensities of the anaesthetics detected by different spin probes at various membrane depths and temperatures the following parameters were introduced: $P_S = \Delta S/(\Delta S_0/\Delta t)$, $P_\parallel = \Delta A_\parallel/(\Delta A_{\parallel 0}/\Delta t)$ and $P_\perp = \Delta A_\perp/(\Delta A_{\perp 0}/\Delta t)$. $\Delta S$, $\Delta A_\parallel$ and $\Delta A_\perp$ are changes of the parameters after addition of the anaesthetics. $\Delta A_{\parallel 0}/\Delta t$, $\Delta A_{\perp 0}/\Delta t$ and $\Delta S_0/\Delta t$ are temperature gradients of the control sample parameters. The parameters $P_i$ express the temperature effect necessary to reach the same values of the ESR parameters in the control sample as in the sample with fixed temperature but with the local anaesthetics.

*Partition coefficient determination*

The apparent volume partition coefficient ($K_p$) of the anaesthetics into the lipid part of synaptosomes was determined using u.v. spectroscopy. The sample of 1 mg synaptosomal protein per 0.5 ml of the buffer containing anaesthetic was equilibrated at 37° for 45 min and centrifuged at 12,000 *g* for 20 min. The anaesthetic supernatant concentration was determined by absorption at 310 nm and 238 nm for tetracaine and dibucaine, respectively. Lipid concentration of the synaptosomes was calculated by assuming 1 μl of lipids/l mg of protein [23].

*Sample preparation and $^{31}$P NMR measurements*

Total lipids were isolated from rat brain according to Folch *et al.* [25]. The lipids and tetracaine were dissolved in chloroform–methanol and the solvent was evaporated in a stream of nitrogen followed by evacuation. The samples were hydrated with 100 mmol/l NaCl, 2 mmol/l EDTA, 50 mmol/l Pipes·HCl in $D_2O$ at pH 6.0. The lipid/tetracaine molar ratio in the sample was calculated assuming the lipid molecular weight to be 775. The final (lipid + tetracaine)/buffer weight ratio in the sample was 1/2. In order to attain equilibration of tetracaine in the lipids, the samples were subjected to freeze–thaw–vortex cycles for several times, homogenised by centrifugation, and equilibrated at room temperature for 24 hr before measurement. $^{31}$P NMR spectra were recorded on a BRUKER HX 90 spectrometer with a home-built proton 600 W decoupling unit at 25°.

## RESULTS

*Partition coefficient*

The measured apparent partition coefficient $K_p$ for tetracaine was 130 ± 16 and 115 ± 16 at the 0.25 mmol/l and 0.8 mmol/l buffer concentrations, respectively. The $K_p$ coefficient for dibucaine was 427 ± 80 and 230 ± 30 at 0.17 mmol/l and 0.65 mmol/l buffer concentrations, respectively.

*Perturbation effects detected by I(12,3) spin probe*

The relative perturbation propensities of the anaesthetics at the 5th carbon membrane depth depended on the temperature. Dibucaine and tetracaine did not significantly influence the parameter $A_\parallel$ at 1° (Fig. 1) and 22°, but decreased the parameter $A_\parallel$ as well as the parameter $S$ at 37° (Fig. 2). The disordering effect of the anaesthetics at 37° was significant at buffer concentrations higher than

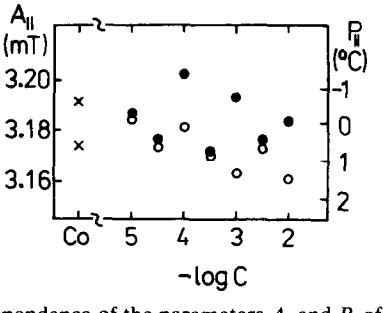


Fig. 1. Dependence of the parameters $A_\parallel$ and $P_\parallel$ of I(12,3) spin probe in synaptosomes on the buffer concentration (C in mol/l) of dibucaine (full circles) and tetracaine (open circles). Co—control sample. Temperature 1°. The temperature gradient of $\Delta A_{\parallel 0}/\Delta t = -14.1\ \mu T \cdot °C^{-1}$ was used.

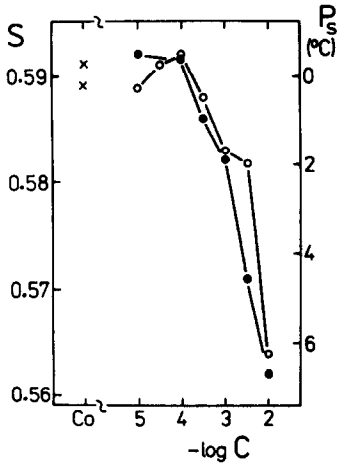


Fig. 2. Dependence of the parameters $S$ and $P_S$ of I(12,3) spin probe in synaptosomes on the buffer concentration (C in mol/l) of dibucaine (full circles) and tetracaine (open circles). Co—control sample. Temperature 37°. The temperature gradient of $\Delta S_0/\Delta t = -0.00417°C^{-1}$ was used.

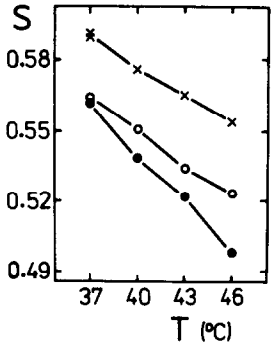


Fig. 3. Temperature dependence of the parameter $S$ of I(12,3) spin probe in synaptosomes: ×, Control, ○, tetracaine (10 mmol/l); ●, dibucaine (10 mmol/l).

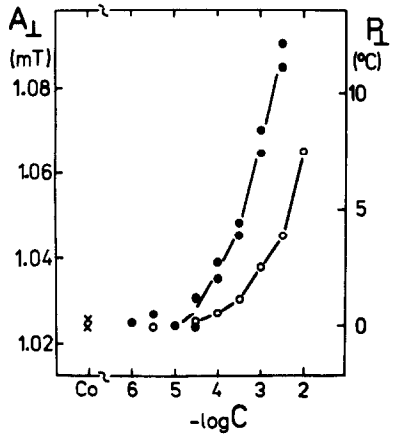


Fig. 4. Dependence of the parameters $A_\perp$ and $P_\perp$ of I(1,14) spin probe in synaptosomes on the buffer concentrations (C in mol/l) of dibucaine (full circles) and tetracaine (open circles). Co—control sample. Temperature 1°. The temperature gradient of $\Delta A_{\perp 0}/\Delta t = 5.47\,\mu T \cdot °C^{-1}$ was used.

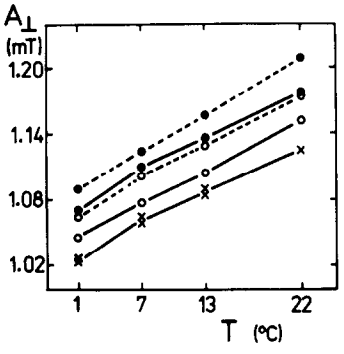


Fig. 5. Temperature dependence of the parameter $A_\perp$ of I(1,14) spin probe in synaptosomes: ×, control; ○, tetracaine at buffer concentrations of 3.16 mmol/l (full line) and 10 mmol/l (broken line); ●, dibucaine at buffer concentrations of 1 mmol/l (full line) and 3.16 mmol/l (broken line).

0.1 mmol/l, and their efficiency kept increasing with increasing temperature, with dibucaine showing a more pronounced effect than tetracaine (Fig. 3).

*Perturbation effects detected by I(1,14) spin probe*

The local anaesthetics increased the parameter $A_\perp$ within the studied temperature range from 1° to 37°, at buffer concentrations higher than 0.01 mmol/l and 0.1 mmol/l for dibucaine and tetracaine, respectively (Figs 4–6). The disordering effect was proportional to the anaesthetic buffer concentrations. The disordering propensities of dibucaine were several times higher than that of tetracaine within the temperatures studied.

*Disordering effect expressed in the temperature scale*

The comparison of the $P_S$, $P_\parallel$ and $P_\perp$ parameters for evaluating the disordering effect of the anaesthetics in the temperature scale is shown in Fig. 7. The following temperature gradients of the control sample labelled by the probe I(12,3) within the range

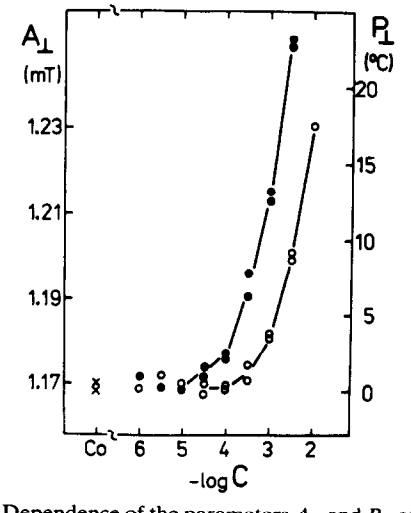


Fig. 6. Dependence of the parameters $A_\perp$ and $P_\perp$ of I(1,14) spin probe in synaptosomes on the buffer concentrations (C in mol/l) of dibucaine (full circles) and tetracaine (open circles). Co—control sample. Temperature 37°. The temperature gradient of $\Delta A_{\perp 0}/\Delta t = 3.6\,\mu T \cdot °C^{-1}$ was used.

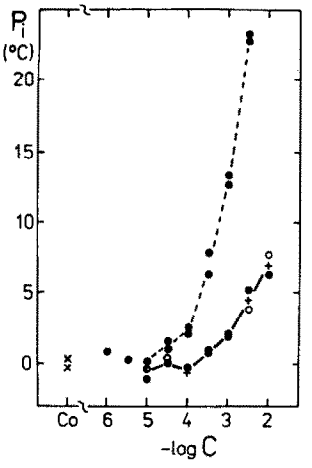


Fig. 7. Dependence of the parameters $P_i$ of spin probes I(12,3) (full line) and I(1,14) (broken line) in synaptosomes on the buffer concentrations (C in mol/l) of dibucaine. Co—control sample; +, $P_S$; ○, $P_{\|}$; ●, $P_{\perp}$. Temperature 37°.

of 37°–46° were used: $\Delta S_0/\Delta t = -0.00417°C^{-1}$; $\Delta A_{\perp 0}/\Delta t = 2.811\,\mu T\cdot°C^{-1}$ and $\Delta A_{\|0}/\Delta t = -8.0\,\mu T\cdot°C^{-1}$. Figure 7 shows that the disordering effect of dibucaine as detected by I(12,3) spin probe at 37° expressed in the temperature scale was similar for all the ESR parameters used. However, despite equal $P_i$ parameters, the shapes of the ESR spectra of synaptosomes containing anaesthetics were not the same as the spectra of the control sample at higher temperatures. For comparison, Fig. 7 depicts also the effect of dibucaine at 37° on the parameter $A_{\perp}$ of the probe I(1,14) expressed also in the temperature scale. The temperature gradient $\Delta A_{\perp 0}/\Delta t = 3.6\,\mu T\cdot°C^{-1}$ of probe I(1,14) was used. Dibucaine was about 5–7 times more efficient to perturb the membrane at the hydrocarbon membrane core compared to the 5th carbon depth. A similar effect was found for tetracaine which was about 3 times more efficient at the hydrocarbon membrane core than at the 5th carbon membrane depth.

*Perturbation effect at the same dibucaine and tetracaine membrane concentration*

Using the measured partition coefficients for dibucaine and tetracaine and the values of their perturbation effects at 37° (Figs 2–7), tetracaine could be calculated to have approximately 1.5 times higher disordering propensities than dibucaine as detected by spin probe I(12,3), and dibucaine to have approximately 2.5 times higher disordering propensities than tetracaine as detected by spin probe I(1,14) at the same membrane concentration. Similar results were obtained by direct measurement (Fig. 8) where the perturbation of dibucaine detected by spin probe I(1,14) was about 2.1 times higher than the effect of tetracaine at the same membrane concentrations.

*Structural perturbation effect detected by $^{31}P$ NMR*

The influence of tetracaine on polymorphism of the rat brain lipid membrane is shown in Fig. 9.

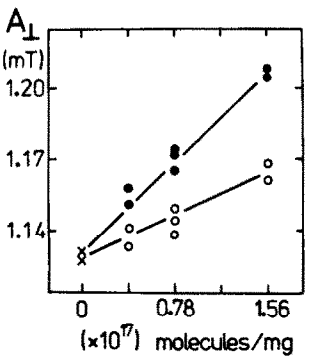


Fig. 8. Dependence of the parameter $A_{\perp}$ of I(1,14) spin probe in synaptosomes on concentration of dibucaine (full circles) and tetracaine (open circles) in synaptosomes. The concentration is expressed in number of anesthetic molecules per 1 mg protein of synaptosomes. Temperature 22°.

The control sample exhibits asymmetric $^{13}P$ NMR spectrum, characteristic mostly of the lamellar lipid phase (L$\alpha$). When the sample contained tetracaine (molar ratios 10:1, 8:1 and 4:1) in addition to lamellar phase signal, new isotropic ($\sigma_{iso}$) and hexagonal ($H_{\|}$) signals appeared in the spectrum. The NMR spectra were thus a superposition of the lamellar and nonlamellar signals, i.e. phase separation occurred in the sample. The lamellar NMR signal is seen also from the sample with the lipid/tetracaine molar ratios of 2:1 and 1:1.

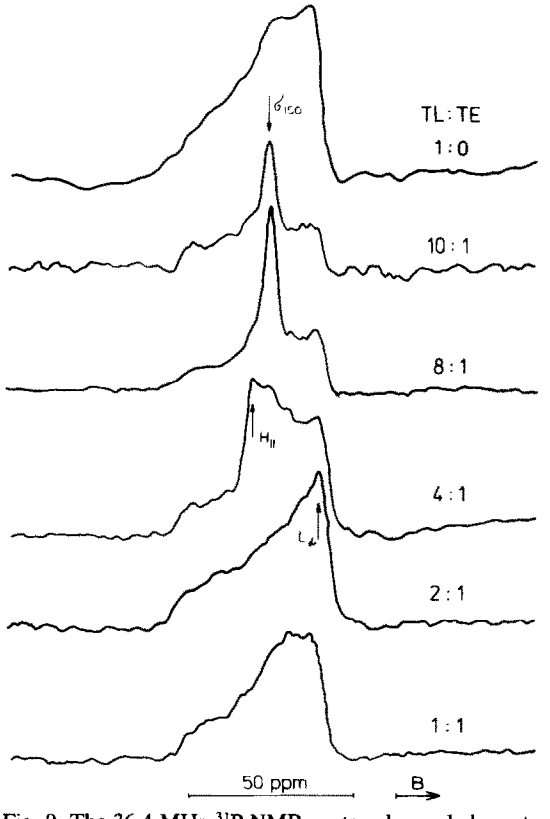


Fig. 9. The 36.4-MHz $^{31}P$ NMR proton-decoupled spectra of dispersion of different molar ratios of rat brain total lipids (TL): tetracaine (TE).

## DISCUSSION

### *Partition coefficient*

The $K_p$ values obtained for dibucaine and tetracaine in synaptosomes were lower in comparison to their partition coefficients in octanol/buffer system, where $K_p$ values of 25,000 and 5000 for dibucaine and tetracaine, were determined [26], but they were of the same order as those obtained in lipid–buffer systems [27, 28]. From the measured $K_p$ values for tetracaine the lipid/tetracaine molar ratio of 14:1 and 40:1 can be estimated in synaptosomal membranes at tetracaine buffer concentrations of 0.8 mmol/l and 0.25 mmol/l, respectively. These ratios are close to those at which tetracaine induced nonlamellar membrane phases in the lipid membranes (Fig. 9).

### *Perturbation effect at the same dibucaine and tetracaine membrane concentration at different membrane depths*

The increase of the membrane dynamics by the local anaesthetics may be the result of the intercalated drug interrupting hydrogen-bonds spanned between adjacent headgroups of lipids, as was suggested for phospholipid membranes [29], and interrupting hydrogen-bonds between lipids and proteins [30, 31]. The dependence of the perturbation effect of the anaesthetics on the membrane depth was suggested to be the result of the structural incorporation of the drug in the membrane [32, 17, 20]. The higher anaesthetic perturbation effect at the hydrocarbon membrane core than found at the 5th carbon membrane depth is in agreement with the results obtained by Boulanger *et al.* [32] on phosphatidylcholine bilayers as well as with our previous findings. We found that local anaesthetic disordering efficiency increased towards the methyl terminal of the lipid acyl chains in rat brain total lipid liposomes and in synaptosomal membranes [17, 20]. Our results support also the findings of Boulanger *et al.* [32] and Coster *et al.* [33] that the polarisable part of amphiphile local anaesthetics is located in the polar membrane part, with the apolar tail penetrating into the hydrocarbon core parallel to the lipid acyl chains. It has been suggested [32, 17] that such drug incorporation may create "free volume" in the hydrophobic part of the membrane. This is supported by our results on the higher disordering effect of dibucaine and tetracaine at the 16th carbon depth compared to the 5th one, and also by the finding that the dibucaine disordering "intrinsic efficiency" is about two times higher than that of tetracaine in the hydrocarbon lipid membrane core. The latter finding may depend on the different molecular shape of dibucaine and tetracaine incorporated in membrane as schematically depicted in Fig. 10, where "free volume" under dibucaine is higher than under tetracaine. Such incorporation of the drug changes membrane elastic energy, destabilizes lamellar membrane structure, as shown for tetracaine (Fig. 9), and thus may affect protein function [34, 35, 36].

Table 1. Comparison of potencies of local anaesthetics in biological membrane activities and membrane perturbation

| Activity | System | Concentration (mmol/l) Te[a] | Di[b] | Ref. |
|---|---|---|---|---|
| Hydrophobic membrane perturbation[c] | Synaptosomes | >0.1 | >0.01 | |
| Dopamine uptake (inhibition 50%) | Synaptosomes | 0.027 | 0.0018 | 1 |
| Block of nerve conduction (minimum) | Sciatic nerve | 0.01 | 0.005 | 44 |
| $Ca^{2+}$ fluxes (inhibition 50%) | Synaptic vesicles | 1.8 | 0.46 | 2 |
| Displacement of bound $Ca^{2+}$ (half maximal) | Erythrocytes | 1.7 | 0.4 | 3 |
| $Na^+$, $K^+$-ATPase (inhibition 50%) | Synaptosomes | 10 | 3.6 | 4 |
| $Mg^{2+}$-ATPase (inhibition 50%) | Synaptosomes | 7.9 | 2.9 | 4 |
| Acetylcholinesterase (inhibition 50%) | Synaptosomes | 0.18 | 0.63 | 4 |
| $Na^+$, $K^+$-ATPase (inhibition 50%) | Microsome | 2.8 | 0.5 | 5 |
| $K^+$-NPPase (inhibition 50%) | Microsome | 1.7 | 0.5 | 5 |
| $Na^+$-ATPase (inhibition 50%) | Microsome | 0.3 | 0.6 | 5 |
| Phosphatidylinositol transfer (inhibition 50%) | Microsome | 0.96 | 0.3 | 6 |
| Phospholipase $A_2$ (inhibition 50%) | Monolayer | 0.52 | 0.15 | 7 |
| Virus infection (inhibition 50%) | BHK cells | 0.2 | 0.025 | 45 |

Te[a]—tetracaine, Di[b]—dibucaine, c—data taken from Fig. 6.

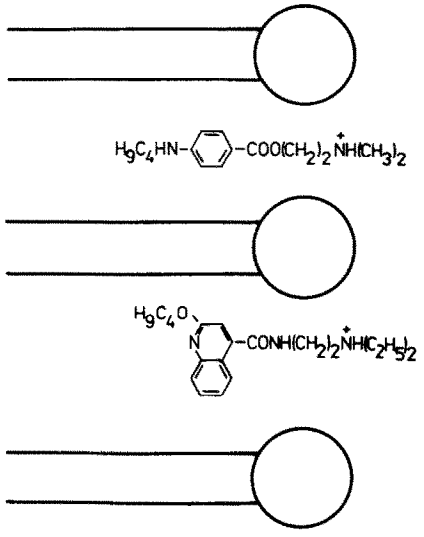


Fig. 10. Schematical incorporation of tetracaine (left) and dibucaine (right) between lipids in synaptosomes.

The inequality of the membrane perturbation ratio of dibucaine/tetracaine ratio at the different membrane depths and different temperatures clearly indicates that the anaesthetics perturb the membrane in a structure-dependent manner, i.e. that each induces a specific rather than nonspecific membrane perturbation.

*Effective concentrations of the anaesthetics*

Both local anaesthetics tested in this study perturb the synaptosomes at concentrations that are within the range of most of their biological membrane activities. They have, for example, been reported to affect the activity of cytochrome oxidase [37, 38] and phospholipases $A_2$ [26], phosphorylation of vinculin [39], hypotonic haemolysis [40], calcium signals in frog skeletal muscle fibres [41], nerve growth in culture [42], and spontaneous oscillation of membrane potential in L cells [43]. We compared the membrane perturbation effect of the anaesthetics studied with some of their biological membrane activities, as shown in Table 1. Only the anaesthetic concentrations required for dopamine uptake inhibition and blocking of nerve conduction are lower than their perturbation concentrations. The spin probes I(12,3) and I(1,14) reflect an overall membrane perturbation; however, if the anaesthetic perturbation effect is a noncooperative phenomenon, each anaesthetic molecule may at any lower concentration be presumed to induce local perturbation in membrane which may not be detected by ESR spectroscopy, yet may be detected by target membrane system.

Our results support the hypothesis that some local anaesthetic membrane activities may be mediated, at least to some extent, through their specific dynamic and structural perturbation effect on membranes.

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