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EFFECT OF TETRACAINE ON MODEL AND ERYTHROCYTE MEMBRANES BY DSC AND EPR

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DSC and EPR experiments were performed on human erythrocyte membranes and DPPC vesicles in order to study the effect of the anaesthetic drug tetracaine on structure and dynamics of the lipid region. Experiments using spin label technique showed that tetracaine induced fluidity changes of the lipid region in the environment of the fatty acid probe molecules incorporated into the membranes in the vicinity of the lipid-water interface. Similarly to EPR observations, DSC measurements reported decrease of the main melting and the pretransition temperature in comparison to control DPPC vesicles, which is the sign of destabilisation of the structure in the head group region of the lipids. Similar effect was observed in the case of erythrocytes where the protein conformation was also controlled in the presence of drug. A separated membrane melting with well distinguished membrane protein phase transition was found that was affected significantly by tetracaine.

These results suggest that tetracaine is able to modify not only the internal dynamics of erythrocyte membranes and produce destabilisation of the lipid structure, but the protein system as well. These might lead to further damage of the biological functions.

Keywords: *DPPC, DSC, EPR, erythrocytes, tetracaine, thermal denaturation*

Introduction

Many publications deal with the mechanisms of local anaesthesia, and their interaction with membrane lipids and proteins [1–5]. Although anaesthetics seem to act by modulating the activity of membrane proteins [6], the exact site and mechanism of anaesthetic action are largely unknown [7]. Local anaesthetics, in addition to block action potential on nerves, also influence various membrane events, such as dopamine uptake in synaptosomes, Ca^{2+} fluxes in synaptic vesicles, displacement of bound Ca^{2+} in human erythrocytes, activity of Na^+, K^+ ATPase in synaptosomes or in microsomes, inhibition of phosphatidylinositol transfer in microsomes as well as several others [8].

Local anaesthetics may interact with anaesthetic-specific receptors and/or with some non-specific hydrophobic sites of membrane proteins or the lipid part of membranes. Local anaesthetics were found to change various physical parameters of lipid or biological membranes [9], such as phase transition of lipid membrane, lipid polymorphism, order parameter of total brain lipid or synaptosomal membranes [10, 11]. There is good reason to suspect that anaesthetics may act through lipid bilayers.

There are many theories for the mechanism of action of local anaesthetics, from these the most frequently mentioned are the following two mechanisms: (*i*) local anaesthetics alter the function of sodium channels indirectly as a consequence of their perturbation of membrane lipid matrix [12]; (*ii*) the anaesthetics may block the channels directly or they could bind to the protein in such a way, as to perturb their conformation and close the channels [13]. The Meyer-Overton rule [14–19], which is stating that the potency of general anaesthetics correlates strongly with their solubility in olive oil, has long been considered as the basis of the first mentioned lipid theory of narcosis. It has been suggested that changes in the molecular organisation of membranes, ranging from an increase in fluidity to lateral phase separation and alteration of lipid-protein interaction (melting of the lipid annulus) are involved in the mechanism of anaesthesia [20]. This is consistent with the hypothesis that alterations in the organization of lipid bilayers are likely to constitute a general mechanism for modulation of membrane protein functions [21].

Indeed, many reports confirm the interaction of local anaesthetics with phospholipids bilayers [7, 20, 22, 23]. For reasons we thought it of interest to examine the binding affinity of tetracaine with cell membranes and its perturbing effects upon the phospholipid bilayer structures. With this aim we used human erythrocytes and dipalmitoyl-phosphatidylcholine multibilayers. Many different physical chemical techniques have been used to provide

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evidence that anaesthetic agents and similar drugs have a biophysical effect on cell membranes, which can often be described as a fluidizing or disordering action. In order to examine the effect of tetracaine on membrane proteins and lipid phase of the membrane we used differential scanning calorimetry (DSC) and electron paramagnetic resonance spectroscopy (EPR). With these techniques we were able to detect changes in both the lipid and the protein region of the membrane.

Materials and methods

Chemicals

5-(4'4-Dimethyloxazolidine-N-oxyl) stearic acid spin label (5-SASL), also named as 5-doxyl stearic acid, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and other chemicals were of analytical grade and purchased from Sigma (Germany) and Fluka Co. (Germany). 3 mg mL^{-1} stock solutions were prepared from the 5-SASL probe molecule in ethanol, and the solution was kept until use at –18°C. The tetracaine was dissolved in phosphate buffered saline (PBS: 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 145 mM NaCl, 5 mM KCl, pH 7.4), the final concentration of tetracaine was 2 mg mL^{-1} in each sample. Tetracaine stock solution was always freshly prepared immediately before experiments.

Preparation of samples and spin labelling

The experiments were carried out on intact erythrocytes and erythrocyte ghosts isolated from freshly drawn venous blood, which was collected in vacutainer tubes containing EDTA. The blood was centrifuged (1000 x g, 30 min) and washed three times in PBS. The buffy coat and the top of the pellet were removed after each centrifugation by aspiration to remove all white blood cells. The red blood cells were used for the experiments on the same day of the isolation. Erythrocyte ghosts were prepared according to Dodge and co-workers [24].

The erythrocyte membranes were spin labelled by the following method: 13 μL of the stock solution of the spin label was added to 1 mL of cell suspension and the mixture was gently shaken for 3 min at room temperature to facilitate spin probe incorporation. Thereafter, the erythrocyte suspension was centrifuged for 10 min at 1000xg and washed twice with PBS. Under these conditions, no isotropic triplet arising from unincorporated spin probes was detected.

In order to model the effect of tetracaine on the lipid region, experiments were made on liposomes as well. DPPC vesicles were prepared as follows: 10 mg of lipid was dissolved in ethanol, and the ethanol-lipid mixture was dried under a stream of CO_2 and then kept overnight in an exsiccator. On the second day 1 mL of physiological saline was added to the lipid film. The mixture was vortexed and sonicated for 3x10 min to obtain vesicles. The incubation of the samples with the tetracaine lasted for 5 min before measurements.

EPR measurements

Electron paramagnetic resonance spectra were recorded with an ESP 300E spectrometer (Bruker BioSpin, Germany) equipped with a 412 VT temperature regulator. The EPR spectra of 5-SASL incorporated into the membranes were taken in the temperature range from 0 to 44°C on both control and treated samples. WG 808-Q sample cell (Wilmad Co., USA) or capillary tubes, which was sealed to prevent water loss, were used to measure the EPR spectra. The conventional EPR spectra were obtained using 20 mW microwave power and 100 kHz field modulation with an amplitude of 2 G. The field scan was 10 mT with field centre of 348.5 mT. 1024 data points were recorded with a time constant of 20 ms and the scan rate was 11.4 min. The spectra were scaled to the same peak-to-peak amplitude or normalised to an identical double integral. In biological membranes molecular ordering exists. Usually, the order parameter (*S*) is calculated from the spectra to describe the effect of different substances on membranes [25, 26]. The spectra at lower temperatures did not give resolved turning points from which the inner hyperfine splitting could be estimated. Therefore, the calculation of the order parameter *S* proposed by Israelachvili *et al.* [27] was used, which is particularly useful in cases where the inner hyperfine splitting is not measurable from the experimental spectra:

$$S = (3(A'_{zz} - A_{\perp})/(A_{zz} - A_{\perp}) - 1) \quad (1)$$

where $2A'_{zz} \approx 2A'_{\parallel}$ is the outer hyperfine splitting measured in the experimental spectrum, $A_{zz} \approx A_{\parallel}$ and $A_{\perp} = (A_{xx} + A_{yy})$, where A_{xx}, A_{yy} and A_{zz} are the principal values of the A tensor. The order parameters were calculated by using the values $A_{\parallel} = 3.36$ mT and $A_{\perp} = 0.605$ mT [28]. A computer algorithm based on a non-linear least squared fitting, developed in our laboratory, calculated the hyperfine splitting constants of the experimental spectra. In order to determine the phase transition temperature of the lipid region, the break point of the order parameter *vs*. temperature relationship was calculated according to Jones and Molitoris [29].

DSC measurements

The phase transition of the DPPC and red blood cell samples was monitored by a SETARAM Micro DSC-II calorimeter. All experiments were done from 0 to 100°C. The heating/cooling rate was 0.3 K min^{-1}. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μL sample volume in average. PBS was used as reference sample. The sample and reference vessels were balanced with a precision of ±0.1 mg. There was no need to do any correction for the heat capacity of the sample and reference vessels during the evaluation of experimental scans. The samples were denatured during each cycle. The repeated scan of the recooled DPPC samples showed that the phase transition processes were reversible, while red blood cells underwent to an irreversible denaturation. Calorimetric enthalpy change was calculated from the area under the heat absorption curve using two-point setting SETARAM peak integration. For graphic presentation of the results the Origin 6.0 program (SPSS Corporation) was used.

Results and discussion

Thermal denaturation experiments

When the temperature is increased all the systems try to populate higher energy states. The change in energy state distribution is very sharp if the system undergoes to a cooperative structural change. Biologically interested thermotropic transitions e.g. gel to liquid transition of lipids [30], unfolding of proteins [31] are highly cooperative changes over a narrow temperature range [32]. Any structural change caused by any intervention will appear as a shift of the melting temperature due to the change of the entropy of state and broadening of DSC curve, which means decreased cooperativity [32].

Local anaesthetics involving tetracaine can influence both the membrane lipids and proteins of human erythrocytes. To separate these phenomena from each other, the same experiments on dipalmitoylphosphatidylcholine multibilayers were performed as well. As it can be seen in Fig. 1a, DPPC has an extraordinarily sharp main gel to liquid crystalline transition at T_m=41.7°C with a gel to rippled phase pretransition at T_p=34.4°C. After 2 mg mL^{-1} tetracaine treatment the melting peak has shifted to 37.5°C with a simultaneous broadening and at 19.4°C very probably the pretransition appeared (Fig. 1b). However, only a small change of enthalpy was obtained (0.525 J g^{-1} for untreated and 0.498 J g^{-1} after treatment with tetracaine). The half width of main transition for DPPC was ~1°C, while for sample treated with tetracaine enlarged to ~2°C. Figures 2a and b show the thermograms of the same samples during the cooling phase evidencing that the effect of tetracaine is almost reversible (enthalpy changes were 0.506 J g^{-1} for untreated and 0.501 J g^{-1} after treatment).

It is known that thermotropic behaviour of liposomes is very sensitive to alterations of the lipid packing within the bilayers [32]. When the packing is interrupted, that is the 'internal order' is reduced after the treatment, the entropy of system increases and therefore the phase transition is shifted to lower temperature according to the equation $T_m=\Delta H/\Delta S$, where ΔH and ΔS are the change of enthalpy and entropy. These facts might indicate that tetracaine distributes preferentially into liquid-crystalline regions in the plan of the membrane. A similar effect was reported for the anaesthetic agent dibucaine [33].

Performing similar experiments on treated and untreated erythrocytes (Figs 3a and b) a complex DSC trace was obtained. Tetracaine affected the

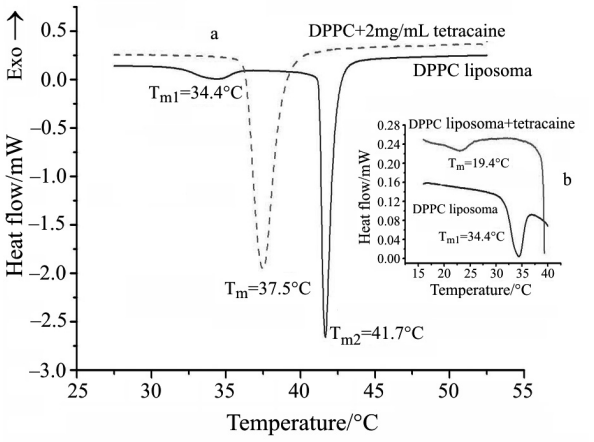

Fig. 1 The main thermal denaturation of DPPC vesicles in the absence (solid line) and presence of tetracaine (dotted line) (a) as well as their pretransitions (b)

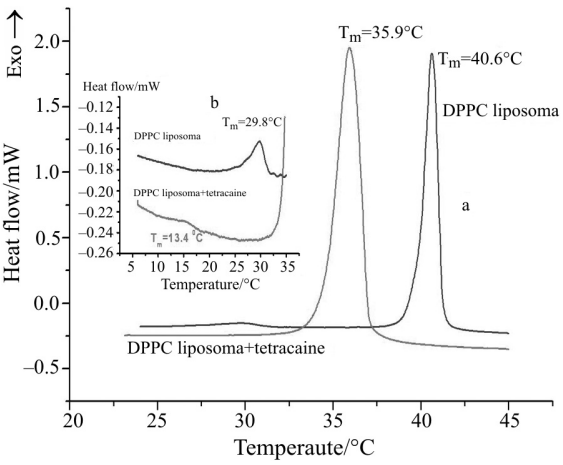

Fig. 2 Thermal transitions of DPPC sample in cooling phase (symbols are the same as in Fig. 1.)

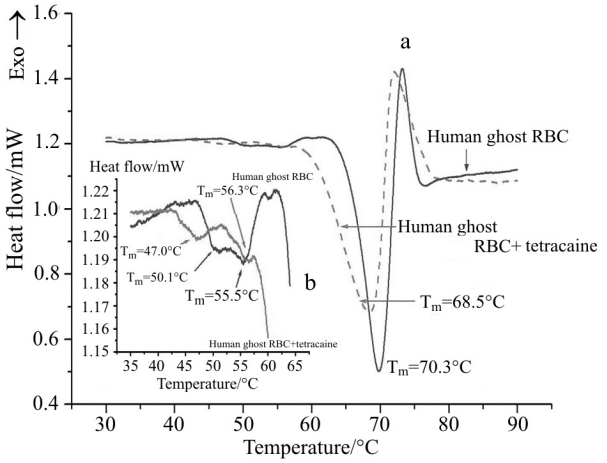

Fig. 3 DSC curves of untreated and treated erythrocytes (symbols are the same as in Fig. 1. Endotherm reactions are deflected downwards)

lipids and the proteins of the membrane, and as well as the haemoglobin in the inside of the cells. The two lower thermal transitions could be assigned to the components of the membrane: T_{m1}=50.1°C and T_{m2}=55.5°C for untreated and T_{m1}=47°C and T_{m2}=56.3°C for erythrocytes treated with tetracaine (Fig. 3b). It can be suggested that the transition at about 55–56°C can be assigned to membrane proteins, the much larger transition at about 68–70°C originates from the melting of haemoglobin. Both DSC and EPR experiments support the view that tetracaine produces only moderate shift of transition temperature in protein systems, whereas the packing of lipids is strongly affected. The thermal denaturation of haemoglobin is irreversible; the large exotherm process arises from aggregation of the protein. The melting characteristic for membrane lipids has changed significantly similar to DPPC vesicles, the shift of T_m~3°C due to the change of fluidity. The effect of tetracaine seems to be less pronounced for membrane proteins monitored by the melting temperature, because almost no decrease was detected in T_m. Only a moderate change was calculated for enthalpy changes (ΔH changed from 0.0191 to 0.0158 J g^{-1} after treatment). It seems that tetracaine modifies only moderately the global conformation of membrane proteins, but its effect on dynamics should be dominant. The effect of tetracaine on the structure of haemoglobin is similar to its effect on the main transition of DPPC. In untreated case we detected a co-operative melting with T_{hm}=70.3°C, while for treated sample we measured a transition at 68.5°C approximately with about 50% larger half width. It means that tetracaine could modify the global structure of haemoglobin with no simultaneous decrease in enthalpy. In both cases we observed a definite exothermic process superimposed on the melting which is the sign of protein aggregation.

EPR experiments

The effect of tetracaine was followed by the EPR technique as well. For the experiments human erythrocytes were used and the effect of tetracaine was examined in the outer lipid region of the membrane by the 5-SASL probe. It is known that the 5-SASL reporter molecules are located in the hydrophobic region of the membranes in the vicinity of the lipid-water interface, and are undergone anisotropic rotation about their longer axis in a moderately immobilising environment, especially at lower temperature. The conventional EPR spectra of erythrocyte membranes at two temperatures are shown in Fig. 4. The spectra indicate that the labels were located almost exclusively in the lipid region of the membrane, aqueous lines attributable to unincorporated spin label were not observed, or the amount was estimated to be only a few percents of the total absorption. From the EPR spectra 5.726±0.025 mT was obtained for the hyperfine splitting constant ($2A'_{zz}$) at room temperature. The order parameter S calculated from values of $2A'_{zz}$ according to equation (1) shows the timeaveraged orientation of the nitroxide z axis relative to the normal of the membrane. A slow cycle of temperature from 0 upto 44°C, and back to 0°C did not result in a hysteresis of parameters. In contrast with DPPC vesicles, no exact melting temperature could be determined from the order parameter *vs.* temperature plot. An approximate analysis resulted in an average of melting points of membrane lipids at about 20°C, which agrees well with data published earlier [34]. The melting temperatures determined either by EPR or DSC differ from each other. It can be explained by the fact that the dif-

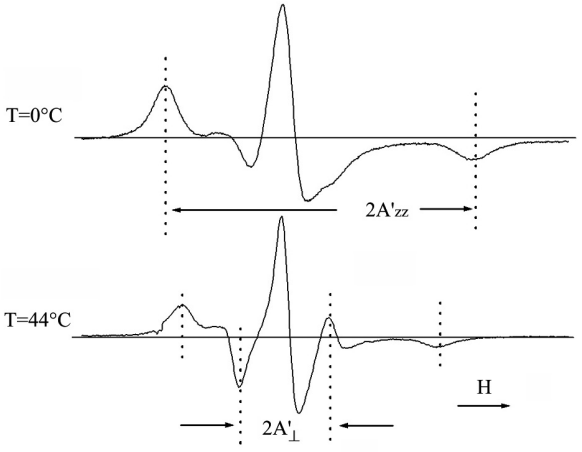

Fig 4 EPR spectra of the 5-SASL spin labelled erythrocytes at 0 and 44°C. Arrows show the spectral parameters, which were used to evaluate the spectra

ferent regions of the membrane are not accessible for the probe molecules and/or the solubilities of the fatty acid labels are different in these regions.

Tetracaine produced significant decrease of the distance between the outermost hyperfine extrema and consequently in the order parameter evidencing the mobilizing effect of the drugs upon the lipid phase, where the labels were located (Fig. 5). This fluidizing effect of tetracaine could be detected in the whole temperature range investigated. The hyperfine splitting constant changed at a given temperature with increasing concentration of drugs, and attained to a constant level at about 3 mg mL^{-1} concentration (final concentration). Above 3 mg mL^{-1}, only insignificant variations in the hyperfine splitting were observed. A gradual increase in $2A'_{zz}$ could be achieved as the

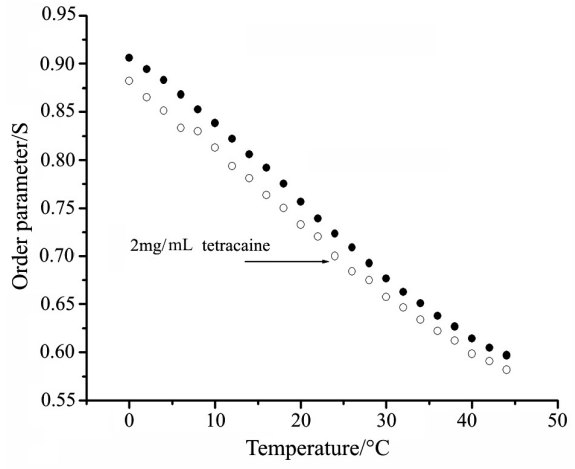

Fig 5 Temperature dependence of the order parameter of the 5-SASL labelled erythrocytes membranes (● – control, ❍ – treated with tetracaine)

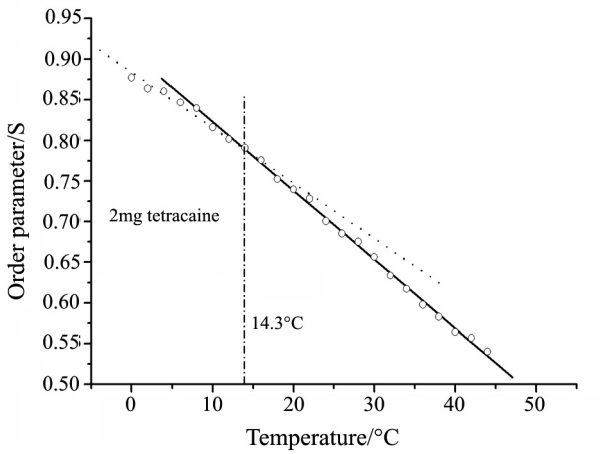

Fig 6 Temperature dependence of the order parameter of DPPC labelled with 5-SASL in the presence of 2 mg/mL tetracaine. The straight lines were fitted with least squared procedure, which resulted in the transition temperature as well

temperature is lowered, its limit value was 6.013±0.025 *mT*. The measured value suggests a decrease of the rotational correlation time of the spin label roughly by 20% after incubation with 3 mg mL^{-1} tetracaine. More dramatic changes could be detected in the presence of tetracaine in DPPC vesicles. The transition temperature, which was 19.5°C in the absence of tetracaine, decreased by about 5°C (T_m=14.3°C) after addition of 2 mg mL^{-1} tetracaine to the sample (Fig. 6). The hyperfine splitting constants for DPPC samples were 6.044 and 5.867 *mT*, respectively. The limit value of $2A'_{zz}$ was 6.396±0.004 *mT*. Calculation of the rotational correlation times resulted in 27.8 *ns* for control sample and 16.0 *ns* for sample in the presence of 2 mg mL^{-1} tetracaine. It corresponds to a decrease by a factor of 2 after incubation of the drug.

In order to interpret the changes of the protein components obtained on red blood cell by DSC, model experiments were performed on haemoglobin. Haemoglobin prepared from red blood cells was spin-labelled with maleimide spin label at the Cys-93 residue of the β-chain. Preliminary experiments showed that tetracaine produced local conformational change in the environment of the Cys-93 residue of the β-chain. The decrease of hyperfine splitting can be interpreted as a local loosening of the protein structure.

To sum up we can say that in the absence of proteins tetracaine can cause a change of wider range only in the fluidity. It can be explained by the homogeneity of membrane and with the absence of proteins or those molecules which are embedded into or associated with the membrane components. Its effect in liposomes can be mainly the perturbation of the liquid-crystalline regions in the plain of the membrane (change in the fluidity), while it modifies the global three dimensional structure of proteins in erythrocytes (including the membrane proteins too), which could be manifested in the alterations of biological functions.

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