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NMR investigation of the influence of procaine and its metabolites on the water exchange through human erythrocyte membranes

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The effect of procaine hydrochloride and its metabolites on the diffusional water exchange through erythrocyte membranes was investigated at 37 °C and at concentrations ranging between $5 \cdot 10^{-5}$ M and $5 \cdot 10^{-1}$ M by using the NMR manganese doping method. Procaine hydrochloride and 2-diethylaminoethanol have a moderate stimulating effect on the water exchange, of up to 20%, at concentrations ranging between 10^{-3} and 10^{-2} M, while an increasing inhibitory effect was found at higher concentrations. The *p*-aminobenzoic acid has no effect on the water exchange up to 10^{-2} M and, at higher concentrations, and apparent decreasing inhibition was noticed which is thought to be an artefact due to the uptake of Mn^{2+} by the cells. The temperature dependence studies suggest that procaine HCl enhances the uptake of Mn^{2+} by the cells. An opposite effect was found for rigid erythrocytes. The *p*-aminobenzoic acid and 2-diethylaminoethanol appeared to be more effective than procaine hydrochloride in increasing the uptake of Mn^{2+}.

Introduction

Water transport across biological and model membranes has been, and is, extensively studied but the process controlling water movement has not been fully elucidated [1,2]. However, the effects of various chemical agents have been investigated in order to understand the mechanisms of water permeability at cellular and/or molecular level [3].

The NMR original technique for measurements of water exchange through human erythrocyte membrane, based on the doping of blood samples with Mn^{2+} [4], gained wider use in the last decade [5]. The interaction of local anesthetics on cell membranes has been studied extensively in order to unravel its molecular mechanisms on whole organism [6,7].

Procaine, the first synthetic local anesthetic, is also used to prove the physical properties of the different regions of lipids interface because of its simple and well-differentiated NMR spectra [8]. Studies of interactions of procaine with erythrocyte membranes are particularly challenging, since the possibility has been considered that procaine and its metabolites are transported to the brain by means of the erythrocytes and are possibly released there by a subsequent transfer mechanism to initiate pharmacological activity [9–1].

Procaine is known to have various effects on

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erythrocyte membranes: increase of deformability at moderate concentrations and the increased cell adhesion [12], decrease of calcium binding on the intracellular site of the membrane [13], protection against osmotic hemolysis and increase of membrane fluidity [14], possible utility as antithrombic agent [15,16], inhibition of monoamino oxidase [17], cytochrome oxidase [18], and $(Na^+ + K^+)$-ATPase [19] limbic excitant [5].

This work reports for the first time information regarding the procaine effects on human erythrocyte membrane with the NMR doping method. We have performed NMR relaxation investigations of red blood cells water exchange as a function of procaine concentration at 37 °C as well as of the temperature dependence of the water exchange time at certain procaine concentrations.

Discussions of the data will consider the fact that the NMR manganese doping method provides two different kinds of information: (a) at physiological temperatures and generally in the vicinity of these temperatures the information is essentially related to the water lifetime within the cells which in turn is controlled by the diffusional water exchange through the membrane. The water exchange process may also be dominant over the entire 0–40 °C range of temperatures at low manganese doping [20–21]; (b) in the lower range of temperatures, i.e., below 25 °C, in certain experimental conditions related either to the doping manganese concentration or to the sample characteristics the information is related to the intracellular manganese concentration.

Material and Methods

Human blood was collected in heparinised tubes and processed within 4 h. Blood samples were obtained from apparently healthy male donors aged at most 45 years.

Preparation of washed erythrocytes samples and NMR measurements were carried out as previously described [20]. Some washed erythrocytes were rendered rigid through a 30 min in vitro incubation at 50 °C [21].

Procaine hydrochloride and its metabolites (*p*-aminobenzoic acid and 2-diethylaminoethanol) were dissolved to a concentration of 250 mM and appropriate volumes of these freshly made stock solutions were added to either whole blood, washed erythrocytes, or rigidified erythrocytes aliquots in order to obtain the desired final drug concentration in the blood samples.

Both controls and drug-containing samples were then incubated for 30 min at 37 °C and NMR measurements were subsequently performed as previously described [20]. Low concentrations of paramagnetic doping manganese ions in the aqueous extracellular environment (i.e., between 3.5 and 8.0 mM) were used throughout in order to avoid artefactual shortening of the water protons apparent transverse relaxation time in the low temperature range. NMR measurements of the relaxation time as a function of drug concentrations were performed independently at 37 °C and at room temperature (20 °C) and checked by authors working in two different laboratories.

All blood samples for NMR measurements were prepared under similar conditions and therefore effects of procaine and its metabolites were assessed only as relative values of water permeability with respect to control samples. For these reasons, we prefer to report here comparatively only the T'_{2a} values of the experimental apparent transverse relaxation time of paramagnetically doped blood samples rather than calculate τ_a values of the transmembrane water exchange time for each drug concentration.

Since the permeability coefficient is inversely related to τ_a and values of the apparent transverse relaxation time T'_{2a} were taken as estimates for the exchange time, then percent inhibition of water exchange with respect to control samples was calculated according to the formula:

$$\% \text{ inhibition} = \frac{1/T'_{2a} \text{ control} - 1/T'_{2a} \text{ sample}}{1/T'_{2a} \text{ control}} \times 100$$

Results

Water exchange time through washed erythrocytes membranes was investigated over a wide range of procaine hydrochloride and procaine metabolites concentrations (Fig. 1). Water exchange through the membrane was not changed significantly for procaine concentrations up to about 10^{-3} M which was followed by a minimum at 10^{-2} M. 2-Diethylaminoethanol produced a

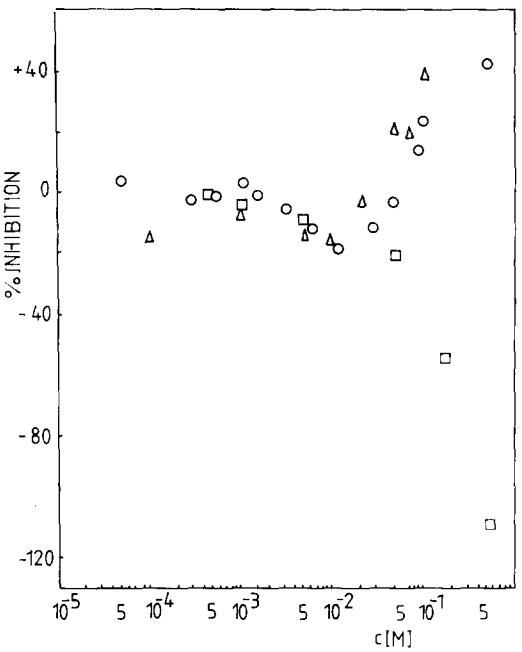

Fig. 1. The water exchange time through washed human erythrocytes at 37 °C as a function of the drug concentration: ○, procaine hydrochloride; △, 2-diethylaminoethanol; □, *p*-aminobenzoic acid.

10–20% decrease of inhibition. Further concentration increase above 10^{-2} produced a steep increase, similar to procaine. The *p*-aminobenzoic acid only produced a slight decrease of inhibition up to 10^{-2} M. At higher concentrations a sharp decrease of inhibition is evident. Hemolysis occurred at concentrations of about $5 \cdot 10^{-1}$ M for procaine while 2-diethylaminoethanol and *p*-aminobenzoic acid did not produce hemolysis.

NMR measurements of the water exchange time in whole blood samples were also performed in the presence of procaine, at concentrations between 10^{-4} and 10^{-2} M. The results were closely similar to those mentioned above for washed erythrocytes (data not shown).

The parameters describing the temperature dependence of the apparent water exchange in the presence of the above mentioned drugs are summarized in Table I. At low manganese concentrations, the Arrhenius plots for control samples of washed erythrocytes were linear over the entire temperature range between 5 and 40 °C, with slopes corresponding to activation energies of 21–25 KJ · mol^{-1} as previously shown [20]. At

TABLE I

ACTIVATION ENERGIES FOR THE WATER DIFFUSIONAL EXCHANGE ACROSS HUMAN ERYTHROCYTE MEMBRANES INCUBATED WITH PROCAINE HYDROCHLORIDE AND PROCAINE METABOLITES

No.	Sample	Mn^{2+} concentration in the extracellular environment (mM)	Discontinuity temperature (°C)	ΔG^* activation energy (kJ · mol^{-1})		
				overall activation energy	low temperature activation energy	high temperature activation energy
1.	Erythrocytes	5.0	–	21.0	–	–
	(control)	8.0	–	25.0	–	–
		18.0	26.5	–	2.1 ± 2.1	23.8 ± 1.7
2.	Erythrocytes and procaine HCl	8.0	17.0	–	15.6 ± 0.5	31.4 ± 1.5
	(10^{-2} M–$9 \cdot 10^{-2}$ M)	18.0	23.0	–	2.4	20
3.	Rigid erythrocytes	3.5	–	11.4	–	–
4.	Rigid erythrocytes and procaine HCl					
	($5 \cdot 10^{-2}$ M)	3.5	2	26.0	–	–
5.	Erythrocytes and 2-diethylaminoethanol					
	($6 \cdot 10^{-2}$ M)	3.5	27	–	6.5	45.6
6.	Erythrocytes and *p*-aminobenzoic acid					
	(10^{-1} M)	3.5	20	–	5.8	38.2

higher manganese concentrations an apparent thermal transition is evident [21]. The temperature dependence is changed significantly when procaine HCl is added. At low manganese doping a change of slope occurs at 17° C, while no break is evident for controls. The apparent transition is not influenced significant by the drug concentration within the range 10^{-2} M–$9 \cdot 10^{-2}$ M. At higher manganese doping the break is shifted from 26.5° C for controls to 23° C for procainized samples.

When washed erythrocytes were rigidified in vitro the Arrhenius plot of the water exchange remained linear but its activation energy decreased sharply to 11.4 KJ $\cdot$ mol^{-1}. Following incubation of rigidified erythrocytes with $5 \cdot 10^{-2}$ M procaine HCl, a linear Arrhenius plot was obtained showing no breaks. These plots were closely similar to those corresponding to control samples of normal washed cells, procaine HCl incubated.

Both *p*-aminobenzoic acid and 2-diethylaminoethanol produced discontinuities in the Arrhenius plot compared to controls of close manganese doping. Also, both metabolites increased significantly the apparent activation energies in the higher temperature range. *p*-Aminobenzoic acid appeared to have a milder effect than 2-diethylaminoethanol. Procaine metabolites, mainly 2-diethylaminoethanol, had comparatively a stronger effect than procaine HCl alone as seen by higher activation energies and a higher temperature of the discontinuity.

Discussion

Procaine is known to be a cationic local anesthetic which interacts with both lipids and proteins in biomembranes. It has been suggested that this drug is retained and interacts preferentially with lipid molecules in the inner half of the lipid bilayer [23].

The intact normal human erythrocytes (discocytes) become crenated cells (echinocytes) following washing procedures [12]. The echinocytes are converted to discocytes when procaine is added in low or moderate concentrations. Therefore, the normal erythrocyte morphology is restored in the presence of procaine. Further increase of procaine concentration in the blood samples results in discocyte expansion which is followed by conversion to stomatocytes and finally to spherocytes [12]. The water exchange measurements possibly reflects changes in the membrane functional characteristics and cell volume associated to this morphological sequence.

Some recent papers have also pointed out that procaine also influences red blood cell membrane deformability, probably owing to its interaction with the proteins on the inner side of the cell membrane, which forms the cytoskeleton and maintains the normal biconcave disc shape and rheological properties of erythrocytes [12].

Bearing these facts in mind, an interpretation of erythrocyte water exchange time dependence on procaine concentration could be attempted as follows: the slight decrease of relaxation time occurring at drug concentrations between 10^{-3} and 10^{-2} M could be linked to the conversion of echinocytes into discocytes. Since this process does not result in any significant changes of cell volume in this concentration range [12], the decrease of T'_{2a} might be ascribed to changes resulting in a true increase of water diffusional exchange which is associated with this morphological change. However, the above-mentionted lack of cell volume increase is only documented in the literature for a 15 mM concentration. At higher amounts of procaine an increase of cell volume was noticed [24]; this process was actually found to start around 10 mM, and became significant at 30 mM. The increase of cell volume is known to shift the water exchange time towards greater values [25]. Actually, cell swelling results in an increase of the relative fraction of intracellular water molecules with respect to extracellular water. The immediate consequence of this fact is an increase of the water exchange time, τ_a, (and of the apparent relaxation time, T'_{2a}) according to the formula:

$$P_a/P_b = \tau_a/\tau_b$$

where P_a and P_b are the water molecule populations and τ_a and τ_b are the mean lifetime of a water molecule inside and outside the cell, respectively. The sharp increase of T'_{2a} at procaine concentrations higher than 10^{-2} M closely follows cell swelling [24]. Therefore, this should be regarded only as an apparent inhibition. The activation energy in the upper range of temperatures is,

however, likely to be true activation energies representing the permeability process [20,21]. The increased value of 31.4 $kJ \cdot mol^{-1}$ compared to control values of 23.8 $kJ \cdot mol^{-1}$ may suggest that in addition to an apparent inhibition there is also a true inhibition due to hindering of the water molecules passage through the membrane.

The above discussion also holds true for procaine effects on water exchange of heat-rigidified erythrocytes, except that at high drug concentrations an 'inhibition' of only 15% was noticed as compared to 40% for normal unmodified red cells. This could be consistent with the fact that swelling of rigid cells takes place with more difficulty at similar procaine concentrations.

Investigation of the dependence of the apparent relaxation time values on the concentration of procaine metabolites revealed that 2-diethylaminoethanol effects were quite similar to those of procaine: it produced a slight increase of the exchange at low concentrations, followed by an apparent inhibitory effect, which was also probably due to cell swelling at high concentrations. On the other hand *p*-aminobenzoic acid produced no permeability changes at therapeutical doses, but the relaxation time decreased dramatically at higher concentrations. It should be noted that *p*-aminobenzoic acid is negatively charged at physiological values of pH and it has no effect on red cell shape [13].

It has been suggested that band 3 proteins of the cell membrane are involved in the diffusional and osmotic water transport [26]. However, there is no conclusive evidence of the location of the channels which transport water but most likely they are separated from the intermembrane part of the protein interacting with transported anions [27]. A photoaffinity labeling of procaine-binding sites in normal erythrocyte membranes also revealed that procaine is incorporated into both band 3 protein (60–70%) and lipid (40–30%) components of the membrane [28]. The same report specifically indicates that procaine binding "to band 3 protein occurs at a locus different than that involved in anion translocation process" [28]. Our data may suggest that procaine is a moderate stimulator of the water diffusional exchange in the 10^{-3}–10^{-2} M range of concentrations, as a result of interaction with membrane proteins involved in the water transport.We shall further discuss the results in terms of manganese uptake by the cells. This kind of information is evident from the temperature dependence investigation of the water proton relaxation data. Breaks are interpreted as evidence for increased uptake of Mn^{2+} by the cells. The higher the temperature of the break the higher the intracellular Mn^{2+} concentration [21]. There is also the possibility that no breaks are evident. However, any apparent activation energies lower than the value corresponding to the water self diffusion may be considered as evidence for the presence of a significant amount of intracellular divalent manganese ions [21].

The present data show that procaine hydrochloride induces either a break at lower manganese doping, or a shift of the break at lower temperatures and higher manganese doping. These data suggest that procaine HCl triggers an increased uptake of manganese ions compared to controls in the lower range of manganese doping (5–8 mM). At higher manganese doping (18 mM) there seems to be a reversal of the effect, control samples appearing to take up more manganese than procaine-incubated samples. On the other hand, the uptake of manganese in procaine-treated cells is less sensitive to the extracellular manganese concentration.

Increased manganese doping tends to shift the discontinuity from lower to higher temperatures (Table I, entries nos. 1 and 2). It should be kept in mind that cases without discontinuities in the 0–40 °C range have in fact discontinuities at lower temperatures corresponding to lower intracellular manganese concentration [21]. The fact that both procaine metabolites (at lower manganese doping) show higher discontinuity temperatures than procaine-treated samples clearly suggests an increased Mn^{2+} influx.

The rigid erythrocytes show an abnormal low value of the apparent activation energy which is lower than for water self diffusion. Therefore, this is evidence for the presence of a significant amount of Mn^{2+} within the cells so that the water proton relaxation inside the cells is controlled by the electron spin–nuclear spin dipolar coupling of Mn^{2+} with water protons. The break corresponding to this relatively high intracellular Mn^{2+} concentrations probably occurs at higher tempera-

tures falling outside the range of temperatures which is normally explored for living cells. The addition of procaine HCl to rigid erythrocytes has a protective effect against manganese penetration, the temperature dependence being similar to normal controls. This is an opposite effect compared to normal washed cells treated with procaine HCl at low manganese doping.

It should be pointed out that the temperature dependence of the NMR data only offers a demiquantitative picture of the Mn^{2+} uptake. These data are in fact a byproduct of the NMR water exchange experiment and the two phenomena should be clearly delimited. A temperature dependence investigation is always necessary in order to establish whether a water exchange or a nuclear relaxation process is dominant. However, the NMR method could be used to monitor the manganese uptake by measuring the value of T'_{21} at temperatures below the break.

In conclusion, procaine HCl at concentrations between 10^{-3} and 10^{-2} M, i.e., in the range of therapeutical doses, increases the rate of water exchange and the uptake of Mn^{2+}. At higher concentrations, a composed inhibition effect is noticed on the water exchange due to both the swelling of cells and an alteration of the membrane structure. Procaine HCl has an inhibitory effect on the uptake of Mn^{2+} by rigid erythrocytes. Both procaine metabolites seem to have a more inhibitory effect on the water exchange than procaine HCl, as judged from the values of the activation energy. Also, the metabolites appear to increase Mn^{2+} uptake by the erythrocytes as compared to procaine HCl.

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