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Ethanol- and acetonitrile-induced inhibition of water diffusional permeability across bovine red blood cell membrane

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Abstract

The effect of 0–3% (v/v) ethanol and acetonitrile on water diffusional permeability of bovine and chicken red blood cells (RBCs) was studied using a pulse ^{1}H-T_2 NMR technique. Transmembrane water diffusional exchange times, τ_{exch}, of 9.2 ± 0.46 ms and 18.3 ± 1.0 ms were determined for bovine and chicken erythrocytes at 27.5° C, respectively. Arrhenius activation energies E_a of water diffusion were 20.4 and 35.8 kJ mol^{-1}. Ethanol, and acetonitrile being 2-fold more effective, markedly increased both τ_{exch} and E_a in bovine RBC as compared to the well-known mercurial inhibitor of water channels, *p*-chloromercuribenzene sulfonate. Chicken RBCs that have no protein water channels, were found to be completely insensitive for either agent. It was suggested that ethanol and acetonitrile partitioning into the lipid phase of bovine RBC membrane affects the permeability of CHIP28 water channel but not the lipid confined water diffusion. The results suggest that the inhibition of transmembrane movement of water via CHIP28 channels might be involved in the anti-hemolytic action of anaesthetics such as ethanol.

Keywords: Ethanol; Acetonitrile; Anesthetic; Red blood cell; Water channel; Water diffusional permeability; NMR, ^{1}H-

1. Introduction

The protective role of anaesthetics in hypotonic lysis of RBCs has been extensively described. At concentrations higher than used clinically, anaesthetics were, however, found to be lytic. The concentration of ethanol, a weak anaesthetic, required for a 50% reduction of hypotonic hemolysis, has been estimated to be around 500 mM [1]. While 3–4 M ethanol has been reported to cause hemolysis by the formation of membrane pores with a diameter of approx. 1.3 nm [2], the mechanism of the anti-hemolytic activities of ethanol and other anaesthetics is still not fully understood. Partition of such compounds into the membrane, followed by a membrane surface expansion and increased membrane fluidity, have been invoked to explain the increased resistance of RBCs to lysis [1]. An impetus for the present study was the observation that anaesthetics, including long chain alkanols, increased the hydraulic (osmotic) permeability coefficient L_p of the human erythrocyte membranes [3]. But controversial results were observed with 100–400 mM ethanol which apparently inhibited the human RBC water permeability in a hyperosmolar medium. After correcting the observed hydraulic permeability coefficient of the ethanol-treated cells for osmolality effects, the conclusion was drawn [3] that ethanol increased L_p as had been obvious for higher alkanols. Since Farmer and Macey [4] claimed L_p to be independent of cell size and osmolality of the medium, a fact also confirmed in other Refs. [5,6], the correction of L_p [3] seems misleading. Very recently Preston et al. [7] showed that the expression of the CHIP28 water channel in *Xenopus* oocytes increased their susceptibility to hypoosmotic lysis. This provided another clue to re-investigate the ethanol effect on transmembrane water flow in RBCs.

In order to avoid eventual structural changes of RBC membranes originating from osmolality stress, such as for example transmembrane redistribution of phospholipids [8], and inferred effects on water movement across the cell

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membrane, we preferred to re-investigate the effects of ethanol on the transmembrane water diffusional permeability under iso-osmotic conditions. In addition, acetonitrile, a non-hydroxyl and non-hydrogen bonding species, was compared to ethanol. The water diffusional permeability of bovine (BRBCs) and chicken red blood cells (CRBCs) were determined using a pulse ^{1}H-T_2 NMR technique [9]. Applying this method showed that solely in the case of BRBCs, both ethanol and acetonitrile increased the diffusional exchange time and the Arrhenius activation energy. Such effects have been shown for mammal RBCs when treated with *p*-chloromercuribenzene sulfonic acid (*p*-CMBS), a well-known inhibitor of water diffusional permeability [10,11]. In contrast, the low water diffusional permeability of CRBCs is well known to be *p*-CMBS insensitive [12], which we found to be also true for ethanol and acetonitrile.

Our results suggest that both ethanol and acetonitrile inhibit water transmembrane transport via the function of the *p*-CMBS sensitive pathway, rather than the diffusion of water through the lipid part of the cell membrane.

2. Materials and methods

Citrated bovine and chicken blood were obtained from a local slaughter-house. RBCs were centrifuged at 3000 rpm for 5 min to remove the buffy coat and plasma. The cells were washed four times with a 3–4-fold volume excess of 130 mM NaCl, 20 mM Tris-HCl (pH 7.4) by centrifugation. The next two washes were performed with 130 mM NaCl, 30 mM sucrose, 20 mM Tris-HCl (pH 7.4). This suspending solution was used throughout the experiments. The RBCs were prepared daily and used immediately.

All chemicals used were analytically graded.

2.1. Estimation of RBC volume changes

The effects of paramagnetically doping $MnCl_2$ stock solution, and of either ethanol or acetonitrile on changes of cell volume, were estimated using a turbidimetric method originally designed for hemolytic assays and osmotic protection experiments [13,14]. Washed bovine or chicken RBCs were suspended in 130 mM NaCl, 30 mM sucrose, 20 mM Tris-HCl, pH 7.4, (further RBC-buffer) to give an apparent absorbance of 0.5 at 700 nm with a 1 cm-length path. At 27° C, 120 μl of 0.5 M $MnCl_2$ in the RBC-buffer were added to 3 ml of an RBCs suspension. The final concentration of $MnCl_2$ was 19.2 mM. After 1 min, 30 μl of ethanol (Merck, Germany) or alternatively acetonitrile (Kemika, Croatia) were mixed into the suspension to give a final 1% (v/v) concentration, that is, 171 mM ethanol or 190 mM acetonitrile. Changes in the apparent absorbance at 700 nm were monitored on a Pye Unicam UV/VIS spectrophotometer (UK).

2.2. ^{1}H-T_2 NMR measurements

0.5 ml of washed bovine or chicken RBCs with 27–30% hematocrits in RBC-buffer were taken as samples for NMR measurements. Immediately before NMR measurements, the samples were paramagnetically doped using stock 0.5 M $MnCl_2$ in RBC-buffer to obtain a final 19.2 mM manganese. The effect of up to 3% (v/v) ethanol or acetonitrile, and 2 mM *p*-chloromercuribenzene sulfonic acid (Sigma, USA) on the water permeability was studied by measuring the water diffusional exchange time τ_{exch}. The ^{1}H-T_2 NMR method of Conlon and Outhred [9] was used as described previously [15]. Appropriate volumes of either absolute ethanol, acetonitrile or stock 100 mM *p*-CMBS (dissolved in aqueous 130 mM NaCl by NaOH titration) were added to paramagnetically doped RBC samples to achieve the final concentrations. In some experiments, cells were successively treated with 1% (v/v) ethanol or acetonitrile, and 5–10 s later with 2 mM *p*-CMBS.

The water proton transverse NMR relaxation measurements were performed on a Bruker B-KR 322 s pulsed NMR spectrometer at a proton resonance frequency of 42.44 MHz. The 180°-pulse separation in the Carr-Purcell-Meiboom-Gill (CPMG) sequence was 0.6 ms [16,17]. The temperature dependence of the diffusional water exchange time was measured from 20–41° C by raising the temperature at intervals of 5° C. It was controlled by streaming thermostated nitrogen over the sample glass tube. Settlement and RBC rouleaux formation [18] were prevented by occasional stirring. The intracellular water proton transverse relaxation time T_{2a} was measured separately between 20–41° C at intervals of 5° C using tightly packed BRBCs or CRBCs in the RBC-buffer without manganese. In this case the 180°-pulse separation in the CPMG sequence was 6 ms. The observed NMR transverse relaxation curves $M(t)$ (see Fig. 1 for example) were fitted by the minimisation of a sum of squares on a VAX-11/750 computer. The theoretical background and modelling of the water diffusion in a two-compartment system employed by us has been extensively described elsewhere (see Refs. [9,18,19] for formal considerations).

The Arrhenius activation energies E_a for water diffusion across the membranes of RBCs treated with either 3% (v/v) ethanol or acetonitrile, and 2 mM *p*-CMBS as well as the controls were calculated from the slope of $\log\tau_{exch}$ vs. $1/T$ plots. Data were analysed by a *Fig. P.* package (BIOSOFT, UK). Modal steady-state values of τ_{exch} obtained upon the treatment of RBCs with either effector were taken into consideration.

3. Results and discussion

To study the effects of ethanol on water diffusional permeability (WDP) we chose bovine and chicken RBCs

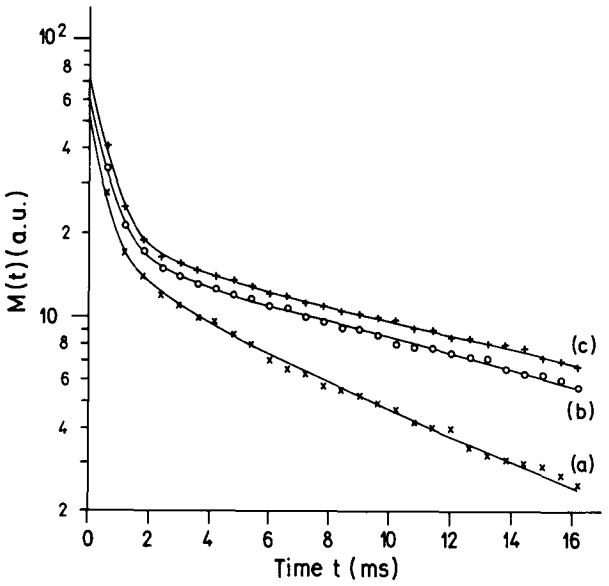

Fig. 1. Examples of the computer fit (line) to the measured water proton transverse (T_2) NMR relaxation curve $M(t)$ as determined by the echo amplitudes (symbols) in the Carr-Purcell-Meiboom-Gill pulse sequence applied to the 28% (v/v) suspensions of BRBCs in 130 mM NaCl, 30 mM sucrose, 20 mM Tris-HCl (pH 7.4), and paramagnetic 19.2 mM $MnCl_2$. Temperature was 27.5° C. (a) control BRBC suspension without additive; (b) 3 min after addition of 3% (v/v) acetonitrile; (c) 17 min after addition of 2 mM *p*-CMBS to the BRBC suspension.

for the following reasons. On the one hand, the WDP of bovine RBCs has been characterised and previously shown to be very similar to that of human RBCs if one considers the value of τ_{exch}, and the Arrhenius activation energy E_a, and the inhibition of the WDP by *p*-CMBS [15]. This reflects closure of specialised protein water channels [7,20]. On the other hand, chicken RBCs have been reported to hemolyse slowly in a hypotonic medium as compared to human or bovine BRCs [21]; they possess a low water osmotic permeability (WOP) insensitive to *p*-CMBS. The results of Farmer and Macey [10] implied that the osmotic permeability coefficient for inward water flow in CRBCs is about 30-fold lower than that in BRBCs. The WDP ($P_d = 1.35 \cdot 10^{-3}$ cm/s) was also lower, very similar to that through a non-porous lipid bilayer, and unaffected by the mercurial reagent. The WDP was characterised by a higher activation energy of 42 kJ/mol [12] than that in human RBCs of 20–24.5 kJ/mol [22,23]. Our results presented in Table 1 are in reasonable agreement with the published data.

For both water osmotic and diffusional permeability determination, it is essential to control cell volume to resolve the possible effects of modifiers on either the water permeability coefficient or the rate constant which is inversely proportional to the exchange time [20]. Using the ^{1}H-T_2 NMR method, Outhred and Conlon [19] clearly showed that there was a linear relationship between τ_{exch} and cell volume. For this reason it is a prerequisite to check volume changes of RBCs during the ethanol and

Table 1
Effect of *p*-chloromercuribenzenesulfonic acid (*p*-CMBS), ethanol and acetonitrile on bovine (BRBC) and chicken (CRBC) red blood cell water diffusional permeability characterised by τ_{exch} and E_a

Cells	Treatment	[b] τ_{exch} (ms) (27.5° C)	E_a (kJ/mol) (20–41° C)
BRBC	none	9.2±0.5	20.4
	p-CMBS, 2 mM	19.6±1.0	43.7
	ethanol, 3% (v/v)	13.4±0.8	27.8
	acetonitrile, 3% (v/v)	17.1±0.9	36.6
CRBC	none	18.3±1.0	35.8

[a] No significant changes in τ_{exch} and E_a were observed after treatment of CRBC with either effector.
[b] Values are means and S.E. of three or four replicates. See Materials and methods for experimental details.

acetonitrile treatment despite using sucrose as a protectant [2] routinely in the RBC-buffer. The addition of $MnCl_2$ and final 1% (v/v) ethanol or acetonitrile to BRBCs (shown in Fig. 2A) rapidly reduced transmitted light by only 1.6 and 1.2%, respectively. It is very likely that these

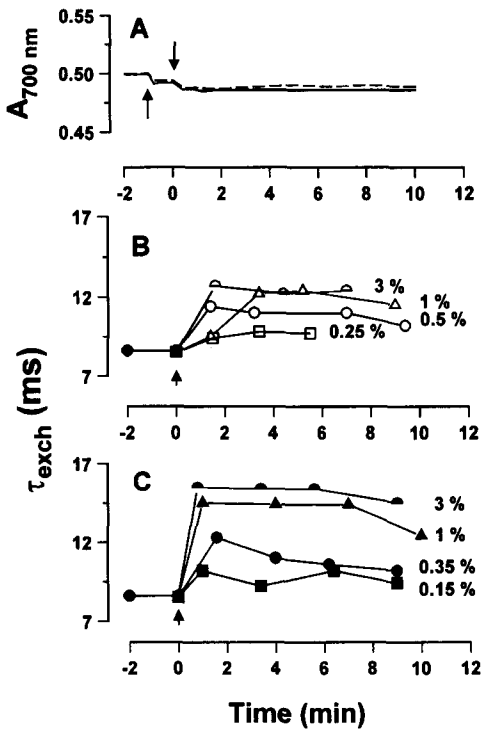

Fig. 2. Effect of ethanol and acetonitrile on the bovine RBC volume changes estimated in terms of apparent absorbance (A), and on the RBC water diffusional exchange time τ_{exch} (B, C). Panel A: Arrows indicate the successive additions of either chemical at a concentration also used in NMR measurements, i.e., 19.2 mM $MnCl_2$, followed by either 1% (v/v) ethanol (solid line) or acetonitrile (dashed line) to approx. 0.05% (v/v) BRBCs in the RBC-buffer. Representative time courses of increase of τ_{exch} are shown upon addition (indicated by arrow) of either ethanol (panel B) or acetonitrile (panel C) to BRBCs with a 27–30% hematocrit in the RBC-buffer. Final concentrations are indicated. All experiments were done at 27.5° C.

observed changes were due to a slightly increased extracellular tonicity and to a concomitant dilution caused by the addition of manganese solution. With ethanol and acetonitrile which caused only a negligible decrease in tonicity, the turbidity decrease should be merely ascribed to the dilution effect (0.93%), since on the basis of their P_d values one might assume an equilibration of ethanol and acetonitrile across the RBC membranes in a short time. P_d of ethanol in human RBCs was found to be $2.1 \cdot 10^{-3}$ cm s^{-1}, and the rate of self-exchange was within a few tens of ms for 1–500 mM ethanol [24]. To ensure additionally that only small volume changes were observed turbidimetrically, we also checked ethanol and acetonitrile treated RBCs under a light microscope. No observable changes of RBCs volume were seen after treatment with 3% (v/v) ethanol or acetonitrile. Hence, we assume that both ethanol and acetonitrile, at concentrations used, can not increase the τ_{exch} (Fig. 2B and C) by increasing RBC volume. Also, a decreased cell volume should rather result in a shorter τ_{exch}, opposite to what we observed in the study.

As neither of the chemicals significantly affected chicken RBC WDP, further detailed studies were performed on bovine RBCs. With both ethanol and acetonitrile, particularly at higher concentrations, the increase of the τ_{exch} was rather high, with a maximum τ_{exch} reached within 15 s (see Fig. 2B and C). This time was necessary to place the sample into the NMR probe, and therefore the kinetics of the permeability changes could not be resolved. Although we noticed variability in the τ_{exch} values inherent in different day RBC batches, the results were highly consistent within a single experimental set. In Fig. 2B and C, a rapid increase in the τ_{exch} values, that is a decrease of water diffusional permeability, is noteworthy and comparable to the *p*-CMBS effect (see also Fig. 4). After reaching a steady-state, a moderate decrease of the τ_{exch} was regularly observed. A change in RBC volume can be excluded as a reason for this apparent higher water permeability. Also, the highest concentration of ethanol used in our study was lower than concentrations reported as inducing morphological changes of the RBCs, and more than an order of magnitude below those causing hemolysis [25]. This phenomenon might result in markedly decreased apparent τ_{exch} values [26]. However, it is more likely that ethanol, and acetonitrile, render the RBC membrane leaky for Mn^{2+}. Intracellular manganese apparently diminishes the values of τ_{exch} [23].

The steady-state effect of ethanol and acetonitrile on τ_{exch} is presented in Fig. 3. A saturation type curve could be suggested; however, a saturation maximum can not be determined definitively. Higher concentrations of either agent were omitted as potentially lytic (see [25]). It appears that acetonitrile is about twice more effective in inhibiting bovine RBC WDP than ethanol.

The observed differences between the WDP of BRBCs and CRBCs, as well as activation energies, strongly suggest that the *p*-CMBS sensitive permeation, however not a

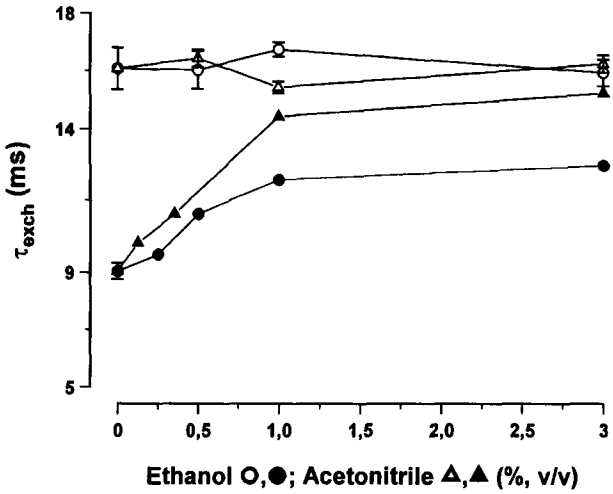

Fig. 3. Dependence of the steady-state water diffusional exchange time τ_{exch} across BRBC (solid symbols) and CRBC (open symbols) membranes on ethanol and acetonitrile concentration. Single points indicate representative τ_{exch} values measured on the same day. Vertical bars are means ± S.E. from three or four independent measurements at 27.5° C. See Materials and methods for experimental details.

lipid pathway of water transport, was affected by either additive. Therefore, we combined *p*-CMBS and ethanol or acetonitrile as shown in Fig. 4. Since neither combination of the effectors significantly affected the CRBC values of τ_{exch}, the treatment of CRBCs with 2 mM *p*-CMBS is presented in Fig. 4 for clarity only. With BRBCs, a typical *p*-CMBS inhibition of WDP was accelerated and strengthened by the presence of ethanol or acetonitrile. However,

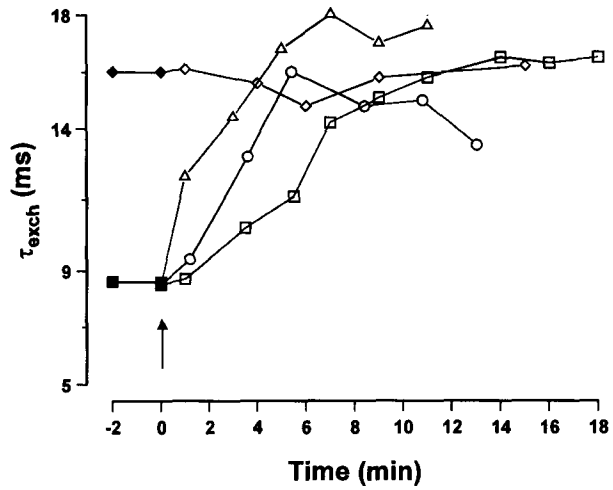

Fig. 4. Changes of water diffusional exchange time τ_{exch} across red blood cell membranes upon treatment with combined inhibitors. Bovine RBCs were treated with 2 mM *p*-CMBS (squares), 1% (v/v) ethanol and 2 mM *p*-CMBS (circles), 1% (v/v) acetonitrile and 2 mM *p*-CMBS (triangles); chicken RBCs were treated with 2 mM *p*-CMBS (diamonds). Closed symbols denote values before inhibitor treatment. Arrow indicates addition of the effectors. When combined, *p*-CMBS immediately followed the other effector. NMR measurements were performed at a 27% hematocrit and 28.5° C as described in Materials and methods. Representative time-courses are presented.

the observed combined inhibition did not significantly exceed that of maximal *p*-CMBS inhibitions. These, in addition to the results in Table 1, again reinforce the suggestion that ethanol and acetonitrile reduce the *p*-CMBS sensitive portion of the BRBC WDP.

Our study suggests that ethanol at anti-hemolytic concentrations reduces water flow through *p*-CMBS sensitive water channels. It leaves, however, the lipid confined diffusion of water intact. Inhibition of the human RBC WOP by amides (urea, substituted ureas, propionamide, valeramide), but not creatinine, has been interpreted as the binding of amides to a common regulatory element that might control the transport of urea, ethylene glycol and water [27]. Our results obtained using an alkanol and nitrile compound, put in question the specificity of amides. There may be another explanation of the modification of the water channel permeability by low molecular compounds. As observed previously [27], the inhibition potency of amides correlates with their decreasing polarity, a fact which may parallel the partition of these effectors into the surface of a lipid bilayer [28] resulting in modified membrane dipole potentials, hydration forces, and ordering of water at the membrane surface [29]. For example, ethanol at concentrations used in our study has been reported to induce an interdigitated gel phase in phosphatidylcholine vesicles [30]. The observed lipid phase transitions induced by ethanol and the resulting effects on membrane architecture were interpreted in terms of configurational entropy [31], and depression and widening of the temperature of the main lipid phase transition [32]. Additives such as ethanol and acetonitrile can induce changes in lipid microdomains also affecting the membrane protein subtle conformation or could even cause dissociation of multimeric proteins [33]. A direct perturbation of the protein water channel by ethanol or acetonitrile, however, can not be decisively excluded at present. It is well known that ethanol may directly modify the function of membrane proteins. For example, a different mode of action of short and long chain alkanols upon nicotinic acetylcholine receptor channels has been reported [34].

To conclude, our study suggests that at least low molecular solutes partitioned into the cell membrane may decrease water permeation through protein water channels. Anti-hemolytic activities of anaesthetics and tranquillizers have been mainly ascribed to the increase of critical hemolytic volumes of RBCs [1,21]. Meanwhile, the assumption has usually been made that water permeability of the RBC membrane, which also influences the rate of RBC lysis (see Ref. [21]), was not changed by either cell swelling or the presence of effectors. However, our results suggest that the decrease of membrane water permeability might also contribute to the well-known anti-hemolytic effect of ethanol, and probably other anaesthetics. A body of biophysical evidence growing for two decades for specialised mercurial-sensitive protein water channels in human RBCs was recently proved by the isolation of the CHIP28 protein belonging to aquaporins [35–37], its reconstitution in liposomes [38], and the expression of its functional form in *Xenopus* oocytes [7]. In particular, future studies on liposome reconstituted water channels may provide additional information on the role of anaesthetics in water diffusional and osmotic permeability.

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