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Na-nitroprusside and $HgCl_2$ modify the water permeability and volume of human erythrocytes

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Abstract

The passage of water through the aquaporin-1 (AQP1) transmembrane channel protein of the human erythrocyte is known to be inhibited by organic mercurials such as *p*-chloromercuribenzoate (pCMB), which react with the free SH-group of the critical cysteine (Cys189) located near the constriction of the AQP1 water-specific channel. Sodium nitroprusside (SNP), which is known as a nitric oxide (NO) donor in interactions with SH-containing molecules, is shown here to suppress the diffusional water permeability (P_d) of the erythrocyte membrane, presumably as a result of reaction with the Cys189 of the human erythrocyte AQP1 water channels. Further, treatment of erythrocytes with $HgCl_2$ is found to result in a cell volume decrease that can be related to activation of membrane K^+-selective Gárdos channels and subsequent loss of intracellular K^+ and cell shrinkage. The variations in P_d and volume of the erythrocyte were deduced from induced variations in the measured proton (^1H) nuclear magnetic resonance (NMR) transverse (T_2) relaxation functions of water exchanging between diamagnetic intracellular and paramagnetic extracellular compartments of the 20–25% hematocrit samples. The extracellular solvent contained 10 mM membrane-impermeable paramagnetic Mn^{2+} ions. The ^1H-T_2 NMR technique allows determination of the time constant τ_{exch} (for exchange of the erythrocyte intracellular water) that is inversely proportional to the permeability coefficient P_d when the intracellular water volume is left unmodified, as in the case of SNP-treated erythrocytes. However, for $HgCl_2$-treated erythrocytes, this technique showed simultaneous variation of both τ_{exch} and the volume ratio V_{in}/V_{out} of intracellular and extracellular water in proportions suggesting that P_d was left unmodified. The $HgCl_2$ effect has been found to be partly reversible by the reducing activity of added mercaptoethanol.

Keywords: Human erythrocyte; Water permeability coefficient; Cell volume; Na nitroprusside; $HgCl_2$; Proton NMR

1. Introduction

One inevitable consequence of the fact that living processes take place in water as a universal biological solvent is the need of living cells to regulate its influx and efflux. Plasma membranes of all cell species are to some extent permeable to water as a result of the relatively slow diffusion of individual water molecules through the membrane lipid bilayer. However, plasma membranes of certain cell species, e.g., mammalian red blood cells and renal tubules are known to conduct large osmotically driven water fluxes that cannot be accounted for by transmembrane lipid-mediated water permeation alone. Therefore many years ago it was postulated that there must be a parallel – membrane protein-mediated – water permeation pathway (reviewed in [1]). Finally, in 1988, the first such specialized channel integral membrane protein of 28 kDa (CHIP28) was discovered in human red blood cells by Agre and coworkers [2], and subsequently identified as a water channel [3,4]. This membrane water channel, now called aquaporin-1 (AQP1), belongs to a large group of membrane water channel proteins named aquaporins, all related to the major intrinsic protein of eye lens fibres (MIP26).

AQP1 operates as a constitutively open, osmo-regulated, bidirectional and strictly selective membrane protein channel. It allows efficient, osmotically driven transmembrane water conduction but excludes – as a two-stage filter [5,6] – all other uncharged solutes and ions, including protons. Proton (or H_3O^+) exclusion from the water stream through the AQP1 pore is essential for maintaining the proton gradient across cellular

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and subcellular membranes which is a critical component of the bioenergetics of all living cells [7].

Before the discovery of AQP1, the channel-mediated transmembrane water passage of the human erythrocyte was shown to be inhibited by mercurials [8]; this effect is now known to be due to mercurial reaction with the SH-group of cysteine 189 located near the constriction of the AQP1 "hour-glass" water channel [9]. Although mercurials completely inhibit the AQP1 channels, they are not necessarily specific to this function since they react also with other accessible SH-groups in many membrane proteins such as AQP1, whose monomer contains a further three cysteines. Nevertheless, mercurials such as *p*-chloromercuribenzoate (pCMB) and *p*-chloromercuribenzene sulfonate (pCMBS) are the only known effective inhibitors of the AQP1 function.

The aim of the present study was to examine the effects of SH-reacting Hg (II) (as $HgCl_2$), of SH-reacting NO from sodium nitroprusside (SNP for short) and, for comparison, also of pCMB, on the volume and diffusional water permeability (P_d) [1] of the human erythrocyte membrane. As far as effects of $HgCl_2$ are concerned, Hg^{2+} has been recently shown to induce activation of erythrocyte K^+-selective Gárdos channels, with subsequent loss of erythrocyte K^+, water efflux and cell shrinkage, as well as to stimulate exposure of erythrocyte phosphatidylserine to the cell surface [10]. Furthermore, $HgCl_2$ is known to induce membrane shape changes in human erythrocytes [11]. SNP is considered as a nitric oxide (NO) donor in interactions with SH-containing molecules [12], and in case that released NO interacts with the free SH-groups of the critical cysteine (Cys189) of the AQP1 water channels, suppression of erythrocyte P_d would be expected.

The influence of these compounds on cell volume and/or P_d can be deduced from induced variations in the measured proton (^1H) nuclear magnetic resonance (NMR) transverse (T_2) relaxation function $M(t)$ of water exchanging between the diamagnetic intracellular and paramagnetic extracellular compartments of erythrocyte suspensions whose extracellular water has been doped with membrane-impermeable paramagnetic Mn^{2+} ions [13–15]. As described previously [15,16], the application of this so-called ^1H-T_2 NMR technique, used also in this study, enables one to determine the time constant τ_{exch} for exchange of the erythrocyte intracellular water as well as the volume ratio V_{in}/V_{out} of the intracellular and extracellular water components of the erythrocyte suspension. Variations in the parameters τ_{exch} and V_{in}/V_{out}, induced by the reagents mentioned above, can be used to analyze the extent and nature of their effects on water P_d or cell water volume (and hence on cell volume) of the human erythrocyte. In particular, if for some of these effectors only τ_{exch} is increased while the ratio V_{in}/V_{out} (and hence the isotonic erythrocyte volume) remains unaffected, this is evidence that only membrane P_d – and not volume – of the red cell has been modified, implying that the AQP1 water permeability has been depressed. On the other hand, if both τ_{exch} and V_{in}/V_{out} are decreased by the action of a chemical agent, this implies (as discussed later) that it is the volume v of the intracellular water of the erythrocyte – and hence the cell volume – that has been modified, while P_d has remained unaffected. In this context it should be mentioned that the technique of ^1H NMR diffusion–diffraction of water [17] has been utilized both to characterize shape and size changes of human erythrocytes induced either by variations in osmolality of the suspension medium or by altering cell metabolism affecting the adenosine triphosphate (ATP) concentration level, and to study the disordered erythrocyte shape in patients with hereditary stomatocytosis and megaloblastic anaemia.

2. Materials and methods

2.1. Cell preparation

All erythrocyte samples were prepared from blood obtained by venipuncture from healthy adult male volunteers on the day of experiments. Fresh citrated blood was washed three times with isotonic TRIS buffer (pH 7.4) by centrifugation (5 min, 1500 ×*g*) at room temperature and two times with 330 mOsm Tris buffer (pH 7.4). For experiments, a 40% stock suspension of erythrocytes was prepared. In the samples for NMR measurements, the final concentration of red blood cells was ~20 to 30% and the concentration of added paramagnetic Mn^{2+} ions was 10 mM. The membrane effectors pCMB, sodium nitroprusside and $HgCl_2$ were examined at 0.25 mM concentrations (pCMB and $HgCl_2$ also at 1 mM concentrations). All measurements were performed on cells at isotonicity.

2.2. NMR measurements and data analysis

The influence of $HgCl_2$, sodium nitroprusside $Na_2Fe(CN)_5NO$ and *p*-chloromercuribenzoate (pCMB), on the diffusional water permeability (P_d) and cell water volume (v) of the erythrocyte was studied by means of an ^1H-T_2 NMR method [13–15]. Our measurements were performed at 21 °C on ~0.3 ml samples with 20–30% erythrocytes in the buffer solution containing 10 mM paramagnetic $MnCl_2$. As the method requires also information about the intracellular diamagnetic water proton NMR transverse relaxation time $T_{2,in}$, this was determined separately on tightly packed cells in the erythrocyte buffer without manganese. The $T_{2,in}$ value of 90±5 ms was obtained. All NMR experiments were performed at a proton resonance frequency of 100 MHz on a Bruker Biospec System (Bruker, Rheinstetten, Germany), equipped with a superconducting magnet (Oxford Instruments Ltd., England). The radiofrequency π-pulse separation in the Carr–Purcell–Meiboom–Gill (CPMG) sequence was $\Delta t = 0.6$ ms for the determination of the relaxation functions $M(t)$ and $\Delta t = 6$ ms for that of $T_{2,in}$.

Because of the two essentially different NMR relaxation compartments of water protons in Mn^{2+}-doped erythrocyte suspensions, i.e., the paramagnetically doped extracellular medium (site b) and the diamagnetic cellular interior (site a), the observed transverse water proton NMR signal $M(t)$ is typically a superposition of two exponentially decaying contributions. The component characterized by a short effective proton NMR spin–spin relaxation time T'_{2b} (of ≤1 ms in our case) corresponds to water protons in the paramagnetic extracellular solution, and the other one characterized by a longer effective NMR relaxation time T'_{2a} due to the intracellular water proton NMR signal as modified

by transmembrane water exchange. Namely, because of the relatively long intracellular water proton NMR spin–spin relaxation time ($T_{2,\mathrm{in}} \approx 90$ ms), T'_{2a} is largely determined by the mean life time (~ 14 ms) of water inside the erythrocyte. Of course, when translocated by equilibrium exchange into the paramagnetic external medium, water protons are brought in contact with paramagnetic Mn^{2+} ions and relax in a much shorter time (≤ 1 ms) because of the strong quenching action of Mn^{2+}.

Due to equilibrium diffusional exchange of water between the paramagnetic extracellular (b) and diamagnetic intracellular (a) sites under isoosmotic conditions of the red cell suspension, the proton NMR transverse or spin–spin magnetization relaxation behaviour can be described by the set of coupled rate equations [13] as

$$\mathrm{d}(\Delta m_a)/\mathrm{d}t = -(\Delta m_a/T_{2a}) - (\Delta m_a/\tau_a) + \Delta m_b/\tau_b, \tag{1a}$$

$$\mathrm{d}(\Delta m_b)/\mathrm{d}t = -(\Delta m_b/T_{2b}) - (\Delta m_b/\tau_b) + \Delta m_a/\tau_a, \tag{1b}$$

where Δm_a and Δm_b are deviations from the equilibrium transverse spin magnetization in the two sites, τ_a and τ_b are the mean lifetimes of the water molecules in each site, and T_{2a} and T_{2b} are the respective spin–spin relaxation times of water protons in the two sites. The solution of these two simultaneous equations gives the normalized transverse relaxation curve of the biexponential form [13] as

$$M(t) = P'_a \exp(-t/T'_{2a}) + P'_b \exp(-t/T'_{2b}). \tag{2}$$

All the observed $M(t)$ curves of $MnCl_2$-doped red cell samples exclusively exhibited such a biexponential character.

By analyzing the experimental transverse NMR relaxation curve $M(t)$ in terms of Eq. (2) only three independent parameters are available: the effective relaxation times T'_{2a} and T'_{2b}, and the effective fraction P'_a of the total $M(t)$ signal, while $P'_b = 1 - P'_a$. However, the unknown parameters available, as they are analytically related to T'_{2a}, T'_{2b} and P'_a within the theoretical framework of Ref. [13], are: P_a (the actual fraction of the NMR signal due to intracellular water protons), τ_a, T_{2a} and T_{2b} while $P_b = 1 - P_a$ and $\tau_b = (P_b/P_a)\cdot\tau_a$, by detailed balancing. It is therefore necessary to measure one of these four parameters independently, and T_{2a} was chosen. T_{2a} was separately measured on tightly packed erythrocytes (without extracellularly added $MnCl_2$), so T_{2a} is identified with $T_{2,\mathrm{in}}$ mentioned before.

Also, as the parameter τ_a represents the mean lifetime of water molecules in the intracellular space before they are transferred to the extracellular space, τ_a is identified as the time constant τ_{exch} introduced before ($\tau_a \equiv \tau_{\mathrm{exch}}$). Of course, the transmembrane water transfer refers both to lipid- and AQP1 channel-mediated pathways of the erythrocyte membrane. Therefore, the overall kinetics of this transfer, as characterized by the measured time constant τ_{exch}, results from water diffusion both through the AQP1 channels and across the lipid portion of the red cell membrane.

Regarding the channel-mediated contribution, the diffusional efflux J_{ch} of labelled water through a single channel with a uniform cross section of area a and of length Δx is, according to Fick's first law, given as

$$J_{\mathrm{ch}} = -aD_w(c_{\mathrm{out}} - c_{\mathrm{in}})/\Delta x, \quad c_{\mathrm{out}} < c_{\mathrm{in}}, \tag{3}$$

where D_w is the diffusion constant of water within the channel pore, and c_{in} and c_{out} are the intra- and extracellular concentrations of labelled water (in units mol/cm^3). Analogously, if there are n such water channels per unit membrane area, the channel-mediated efflux of labelled water per unit membrane area will be

$$J_{w,\mathrm{ch}} = n\ a\ D_w(c_{\mathrm{in}} - c_{\mathrm{out}})/\Delta x = P_{d,\mathrm{ch}}(c_{\mathrm{in}} - c_{\mathrm{out}}), \tag{4}$$

where

$$P_{d,\mathrm{ch}} = n\ a\ D_w/\Delta x \tag{5}$$

represents the channel part of the membrane diffusional water permeability coefficient (in units cm/s).

The diffusional water permeability coefficient of the erythrocyte membrane due to its AQP1 water channels, $P_{d,\mathrm{AQP}}$, is analogously introduced via the channel-mediated diffusional efflux $J_{w,\mathrm{AQP}}$ of labelled water per unit membrane area:

$$J_{w,\mathrm{AQP}} = P_{d,\mathrm{AQP}}(c_{\mathrm{in}} - c_{\mathrm{out}}). \tag{6}$$

In this case, however, because of its hourglass channel profile, the AQP1 water pore consists of the cone-shaped extracellular and intracellular region and the central narrow channel through which the water must pass in single file [18].

As water diffuses also across the lipid bilayer part of the red cell membrane (by the so-called "kink mechanism" [19]), the corresponding diffusional water permeability coefficient, $P_{d,\mathrm{lip}}$, is defined via the lipid-mediated water diffusional efflux per unit membrane area:

$$J_{w,\mathrm{lip}} = P_{d,\mathrm{lip}}(c_{\mathrm{in}} - c_{\mathrm{out}}). \tag{7}$$

In this case water crosses the membrane by a solubility-diffusion mechanism so that $P_{d,\mathrm{lip}}$ is directly proportional to the diffusion constant of water within the lipid moiety of the membrane and to the partition coefficient of H_2O between lipid and water phase, and inversely proportional to the thickness of the lipid bilayer [1].

The total efflux of labelled water is the sum of both contributions

$$J_{w,\mathrm{tot}} = J_{w,\mathrm{AQP}} + J_{w,\mathrm{lip}} = P_d(c_{\mathrm{in}} - c_{\mathrm{out}}), \tag{8}$$

where

$$P_d = P_{d,\mathrm{AQP}} + P_{d,\mathrm{lip}} \tag{9}$$

represents the overall diffusional water permeability coefficient of the membrane.

It can easily be shown (see, e.g., Ref. [20]) that in our case the time evolution of the concentration difference $(c_{\mathrm{in}} - c_{\mathrm{out}})$ of magnetically labelled water across the erythrocyte membrane,

for either of the permeation pathways concerned above (Eqs. (6)–(8)), is governed by the rate equation

$$\frac{\mathrm{d}}{\mathrm{d}t}(c_{\mathrm{in}}-c_{\mathrm{out}}) = -s\,P\left(\frac{1}{v_{\mathrm{in}}}+\frac{1}{v_{\mathrm{out}}}\right)(c_{\mathrm{in}}-c_{\mathrm{out}}), \tag{10}$$

where P is the permeability coefficient referring to either $P_{\mathrm{d,AQP}}$, $P_{\mathrm{d,lip}}$ or P_{d}. In Eq. (10), s is the membrane surface area of a single erythrocyte, v_{in} is the intracellular water volume of a single erythrocyte (about 0.70 of its total isotonic cell volume [21]), and v_{out} is its corresponding extracellular water volume in the cell suspension, i.e., for the red cell sample of cytocrit percentage h, hence $v_{\mathrm{out}}=[(1-h)/0.7h]v_{\mathrm{in}}$. The product of constants on the right-hand side of Eq. (10) is identified as the inverse time constant for the concentration difference time decrease due to either of the transport pathways mentioned above, i.e.,

$$1/\tau = s\,P\left(\frac{1}{v_{\mathrm{in}}}+\frac{1}{v_{\mathrm{out}}}\right). \tag{11}$$

As far as our experiment is concerned, the CPMG pulse NMR technique was used in which the initial radiofrequency $\pi/2$-pulse reorients water proton spins of the sample perpendicular to the direction of the external static magnetic field. The resulting transverse proton spin magnetization serves as a magnetic label of water molecules in the cell suspension that is detected by NMR. The paramagnetic Mn^{2+} ions added in the extracellular space represent a fast permanent sink of the water proton transverse magnetization in this compartment, thus effectively leaving the magnetically labelled water molecules only in the intracellular space. With regard to Eq. (10), this situation effectively corresponds to the one of a very large extracellular volume v_{out} (i.e., $v_{\mathrm{out}} \gg v_{\mathrm{in}}$) that contains no labelled water ($c_{\mathrm{out}}=0$), in which case from Eq. (11)

$$1/\tau = (s\,P)(1/v_{\mathrm{in}}). \tag{12}$$

Ignoring the slow natural NMR relaxation of the transverse water proton magnetization in the intracellular space, and recalling that the experimentally determined time constant τ_{exch} results from both the AQP1 and lipid-mediated water diffusion contributions, i.e., $P \equiv P_{\mathrm{d}}$, the τ value of Eq. (12) is identified as τ_{exch}. Designating $v_{\mathrm{in}}=v$, according to Eq. (12) therefore

$$P_{\mathrm{d}} = (v/s)(1/\tau_{\mathrm{exch}}), \tag{13}$$

which is the result as quoted in [14,21–23]. Recall that in Eq. (13) the parameter v represents the intracellular water volume of a single erythrocyte (about 0.70 of its total isotonic cell volume [21]), and s is its membrane surface area.

The experimental transverse NMR relaxation functions $M(t)$ were fitted to a theoretical biexponential curve (Eq. (2)) as calculated for the effects of two-site equilibrium exchange and of natural proton NMR spin–spin (T_2) relaxation of water in paramagnetically doped erythrocyte suspensions on the CPMG decay [13,14] to obtain values for the exchange time constant τ_{exch} and the intracellular/extracellular water volume ratio $V_{\mathrm{in}}/V_{\mathrm{out}}$ ($=P_{\mathrm{a}}/P_{\mathrm{b}}$) of the erythrocyte suspensions. The experimental error of determining the two parameters was ±7%. It is worth mentioning that along with the known room-temperature ratio v/s of 4.56×10^{-5} cm for the isotonic human erythrocytes [21] and the experimental control τ_{exch} value of 14.0 ms (see Fig. 1), the permeability coefficient P_{d} of 3.3×10^{-3} cm/s is obtained for the native human erythrocyte membrane. This value is well within the P_{d} range of $(3–4)\times10^{-3}$ cm/s most often quoted in the literature (see reviews [1] and [21]).

Eq. (13) enables one to analyze effects of chemical agents on the red cell membrane. Thus, if a reagent induces an increase of the exchange time constant from τ_{exch} to τ^{*}_{exch}, and does not affect the ratio $V_{\mathrm{in}}/V_{\mathrm{out}}$ (i.e., v is left unmodified), the corresponding degree of permeability inhibition (reduction of P_{d} to P^{*}_{d}) is calculated from Eq. (13) as

$$(P_{\mathrm{d}}-P_{\mathrm{d}*})/P_{\mathrm{d}} = 1-(\tau_{\mathrm{exch}}/\tau^{*}_{\mathrm{exch}}) \tag{14}$$

On the other hand if, for a different type of membrane effector, the parameters τ_{exch} and $V_{\mathrm{in}}/V_{\mathrm{out}}$ are decreased, it will be shown that it is not P_{d} but rather v and hence the red cell volume that is modified.

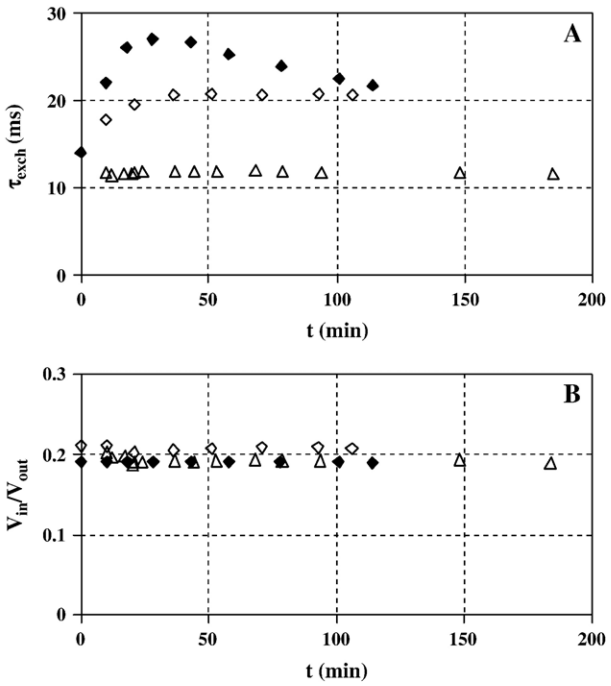

Fig. 1. pCMB-induced inhibition of diffusional water permeability of human erythrocytes, expressed as (A) increase of the time constant τ_{exch} for cell water exchange and (B) the constant volume ratio $V_{\mathrm{in}}/V_{\mathrm{out}}$ of sample intra- and extracellular water components. *Symbol*: ♦ (1 mM pCMB); ◇ (0.25 mM pCMB); △ (control).

3. Results

Inhibition data of erythrocyte diffusional water permeability (P_{d}) obtained with 1 mM and 0.25 mM pCMB, exhibited as an increase in τ_{exch} at constant volume ratio $V_{\mathrm{in}}/V_{\mathrm{out}}$ (i.e., at unmodified intracellular water volume v) are shown in Fig. 1. The τ_{exch} value increased from 13.8 ms to 27.3 ms after

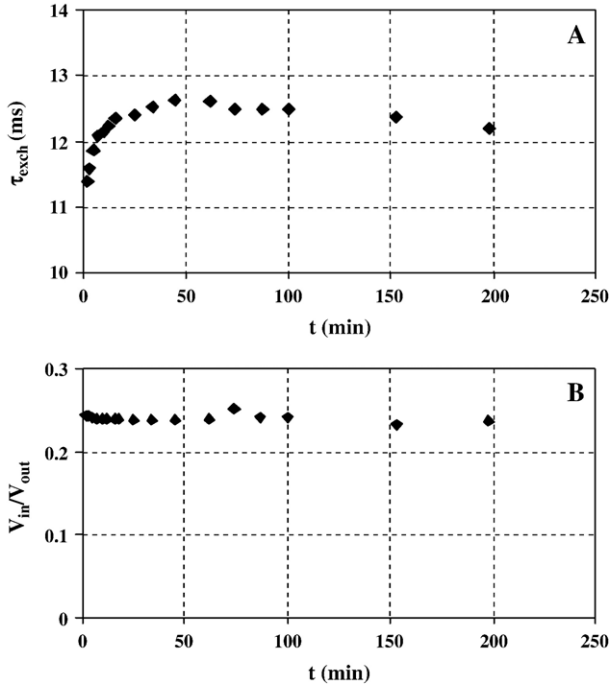

Fig. 2. Inhibition of diffusional water permeability of human erythrocytes by 0.25 mM sodium nitroprusside, expressed as (A) lengthening of the time constant τ_{exch} for cell water exchange at (B) constant volume ratio V_{in}/V_{out} of sample intra- and extracellular water components.

incubation with 1 mM pCMB. Eq. (14) then implies suppression of P_d by ~50%, in excellent agreement with the quoted result [1]. As the membrane lipid portion and the AQP1 channels contribute almost equally to total membrane P_d of human erythrocytes [1], the observed degree of P_d inhibition implies that the 1 mM pCMB concentration was high enough to block all the erythrocyte AQP1 channels present in a 20% hematocrit sample. The degree of P_d inhibition obtained with 0.25 mM pCMB was ~33%.

Fig. 2 shows the effect of 0.25 mM sodium nitroprusside (SNP) on erythrocyte P_d. The effect is qualitatively comparable to that obtained by pCMB. SNP also induces an increase of τ_{exch} at constant V_{in}/V_{out} ratio. However, the degree of P_d inhibition reached with 0.25 mM SNP (as calculated from Eq. (14)) was ~11%, about one third of that obtained with 0.25 mM pCMB (~33%). This indicates that, compared to the same concentration of pCMB, SNP is an inherently weaker inhibitor of erythrocyte P_d (Treatment of the erythrocyte sample with 1 mM SNP resulted in the same degree of P_d inhibition – data not shown – as that obtained with 0.25 mM SNP). One reason for the lower inhibitory potency of SNP with respect to that of pCMB could be that, when pCMB binds to the SH-group of the Cys189 residue located near the constriction of the AQP1 channel, it completely blocks the channel aqueous pathway [9]. Since the SH-reacting nitroprusside (SNP), $Na_2Fe(CN)_5NO$, as an NO-group donor, can also react with this cysteine residue to form a labile R–S–NO product [12] at the AQP1 channel constriction, the bound NO-group appears to impose a less efficient barrier to water permeation than the larger carboxyphenyl mercuryl residue of pCMB.

Fig. 3 shows that 1 mM $HgCl_2$ influences both the time constant τ_{exch} of diffusional exchange of the erythrocyte intracellular water and the volume ratio V_{in}/V_{out} of intra- and extracellular water fractions in the erythrocyte suspension; both parameters decrease with time until, after ~100 min, they attain constant values τ^*_{exch} and V^*_{in}/V^*_{out}. On addition of an excess of the reducing agent, 3 mM mercaptoethanol, into a parallel sample of the erythrocyte suspension preincubated for 65 min with 1 mM $HgCl_2$, both parameters increased again but did not attain their initial (control) values, showing that the reaction with $HgCl_2$ is not fully reversible. As neither haemolysis was observed in the $HgCl_2$-treated sample, nor presence of paramagnetic Mn^{2+} ions was detected in the erythrocyte intracellular space, the possibility of $HgCl_2$-induced membrane leakage could be excluded as a cause of the observed decrease of τ_{exch} and V_{in}/V_{out}. Also, as proved later, the AQP1-mediated transmembrane water passage was not affected by $HgCl_2$. Therefore, the observed $HgCl_2$-induced decrease of τ_{exch} and V_{in}/V_{out} is attributable to a reduction in cell volume – and hence to a decrease of the intracellular water volume from v to v^* – due to erythrocyte shrinkage. Indeed, in a most recent study [10], Hg^{2+} has been shown to trigger activation of K^+-selective Gárdos channels of the erythrocyte membrane, with subsequent loss of erythrocyte K^+, water efflux and cell shrinkage. Of course, it is assumed that this change does not affect the cell membrane surface area s.

In order to determine whether or not AQP1-mediated water passage across the erythrocyte membrane was itself influenced by $HgCl_2$, the value P^*_d of membrane water permeability for $HgCl_2$-treated erythrocytes must be compared to the value for

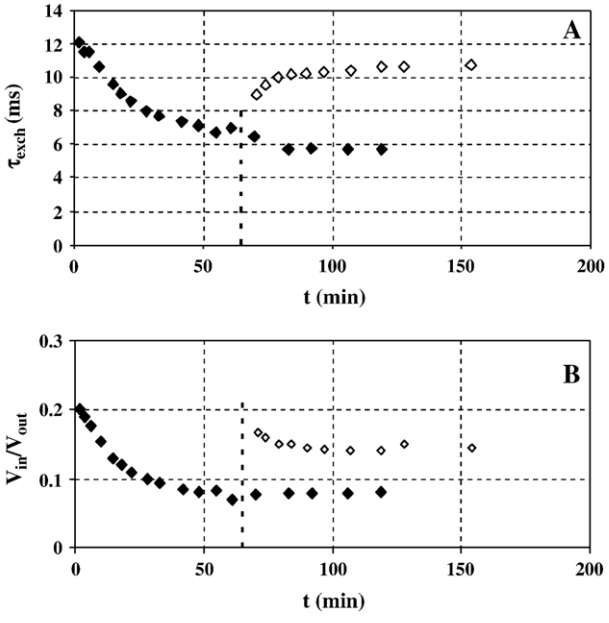

Fig. 3. $HgCl_2$-induced simultaneous decrease of (A) the time constant τ_{exch} for cell water exchange and of (B) volume ratio V_{in}/V_{out} of sample intra- and extracellular water fractions, respectively. This effect of $HgCl_2$-treatment can be partly reversed by adding excess reducing agent mercaptoethanol. *Symbol*: ♦ (effect of 1 mM $HgCl_2$ on τ_{exch} and V_{in}/V_{out}); ◇ (partial reversal of $HgCl_2$ effect observed upon addition of 3 mM mercaptoethanol (vertical dotted line) into a parallel sample preincubated for 65 min also with 1 mM $HgCl_2$).

untreated erythrocytes. In doing so, the corresponding ratios v^*/τ^*_{exch} and v/τ_{exch}, that (according to Eq. (13)) determine the two permeability values, are needed. While τ_{exch} and τ^*_{exch} are measured directly (see Fig. 3A), the volume ratio v^*/v of the intracellular water of the erythrocyte in its treated and control state is obtained from the V_{in}/V_{out} data of Fig. 3B as (see the Appendix):

$$v^*/v = \frac{\eta^*(1+\eta)}{\eta(1+\eta^*)}, \tag{15}$$

where $\eta^* = V^*_{in}/V^*_{out}$ and $\eta = V_{in}/V_{out}$ designate the modified (η^*) and unmodified (η) volume ratios of suspension intra- and extracellular water.

4. Discussion

Sodium nitroprusside (SNP) is known as a vasodilator and as such among the most often studied nitric oxide (NO) donors, as its vasodilator effects are believed to be mediated by NO released from SNP in interactions with sulfhydryl-containing species. Thus, in interactions of SNP with cysteine, *S*-nitrosothiol is formed and is considered as the storage and transporter of NO in vivo [12]. Our observation that the diffusional water permeability of SNP-treated erythrocytes is suppressed therefore suggests that this is due to sterically hindered water passage through the erythrocyte membrane AQP water channels, caused by NO from SNP upon binding to Cys189 located near the channel constriction. However, the bound NO-group appears to impose a less efficient barrier to the channel water permeation pathway than the larger carboxyphenyl mercuryl residue of pCMB.

An important aspect of Hg^{2+} reactivity with erythrocyte membrane components is its high affinity for protein sulfhydryl groups. The formation of stable bonds with membrane protein SH-groups may affect the functional integrity of, e.g., the red cell membrane channel proteins. Indeed, the K^+-selective Gárdos channels of the erythrocyte membrane [24] have been recently shown [10] to be activated by $HgCl_2$. Hg^{2+} is considered to increase cell membrane K^+ conductance by interaction with the channel protein SH groups (see, e.g., [25]). Hg^{2+} is known to readily enter cells [26], so that its effect could be exerted also from the intracellular side of the cell membrane. Upon activation of K^+-selective Gárdos channels, K^+ leaves the cells, this is osmotically obliged with water efflux and decrease of cell volume. However, as pointed out in [10], the Hg^{2+}-induced activity of the erythrocyte Ca^{2+}-dependent Gárdos channels could have been also due to an increased cytosolic Ca^{2+} level as a result of a Hg^{2+}-activated membrane Ca^{2+}-pump function. The sensitivity of erythrocytes to cellular loss of K^+ and the role of cation channels in triggering their "apoptosis-like" death [27] is similar to cellular K^+-loss in apoptotic death of nucleated cells: in both cases cells deal with sustained decrease of cell volume [28]. Our observation of erythrocyte volume decrease induced by treating the ~20% hematocrit samples with 1 mM $HgCl_2$ is in line with such behavior. However, the fact that the volume decrease could be in part reversed by the reducing action of added mercaptoethanol to the suspension of treated cells, suggests that Hg^{2+} is involved also in weaker interactions with membrane components. Our data further indicate that treatment of 20–30% suspensions of human erythrocytes with 1 mM $HgCl_2$ does not affect their membrane diffusional water permeability.

It should be also mentioned that a scanning electron microscopy and fluorescence spectroscopy study revealed that $HgCl_2$ disrupts the structure of the human erythrocyte membrane and of model phospholipid bilayers [11]. In particular, 1 mM $HgCl_2$ is found to induce gross alteration of the erythrocyte morphology from discoid to both stomatocytic and echinocytic forms. According to the bilayer couple hypothesis [29] stomatocytes are formed when a foreign molecular species (mercurial in our case) is inserted into the inner erythrocyte phospholipid monolayer, whereas echinocytes are formed when it is located into the outer leaflet of the lipid bilayer. The study on interactions of bivalent Hg(II) with model lipidic membranes containing the amino phospholipid species phosphatidylserine (PS) and phosphatidylethanolamine (PE) [30] has suggested that, in cell membranes, in addition to the membrane protein sulfhydryl groups, the binding sites for Hg(II) are the primary amino groups of PS and PE. Thus, as lipid membranes are readily permeable to Hg(II) [26] and the prevailing fraction (~80%) of the red cell membrane species PS and PE is located in the inner lipid monolayer, the interaction of Hg(II) with these lipid species is expected to favour stomatocytic forms. On the other hand, the interaction of $HgCl_2$ with phosphatidylcholine (PC), that represents the phospholipid class of the outer monolayer of the human erythrocyte membrane, contributes to the formation of echinocytes [11].

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Appendix A

The volume ratio v^*/v of the intracellular water of the erythrocyte in its $HgCl_2$-treated and control states is obtained from the V_{in}/V_{out} data of Fig. 3B as follows. For the control erythrocyte suspension of cytocrit percentage h, knowing that the intracellular water volume v of the human erythrocyte is 70% of its total isotonic cell volume [21], the ratio V_{in}/V_{out} of the intra- to extracellular water fractions in the sample is given as

$$V_{in}/V_{out} = 0.7h/(1-h). \tag{A1}$$

This ratio is decreased to $V^*_{in}/V^*_{out} = (V_{in}-x)/(V_{out}+x)$ when a certain amount x of water is transferred from the intracellular space into the extracellular solution of the sample due to erythrocyte shrinkage. It should be stressed that because of vastly different time scales of τ^*_{exch} and V^*_{in}/V^*_{out} determination by the CPMG technique (performed on the 10 ms scale) and of the $HgCl_2$-induced red cell shrinkage (that takes the 1 h scale, see Fig. 3), the cell volume during each particular determination

of the $M(t)$ signal may be considered as constant. With the notation $\eta = V_{in}/V_{out}$ and $\eta^* = V_{in}^*/V_{out}^* = (V_{in}-x)/(V_{out}+x)$, the volume ratio V_{in}^*/V_{in} of intracellular water in the modified and control state of the sample is calculated as:

$$\frac{V_{in}^*}{V_{in}} = \frac{V_{in}-x}{V_{in}} = \frac{\eta^*(1+\eta)}{\eta(1+\eta^*)}. \quad \text{(A2)}$$

Recalling that $V_{in}^* = N_0 . v^*$ and $V_{in} = N_0 . v$, where N_0 is the number of erythrocytes in the sample, it follows from Eq. (A2) that

$$v^*/v = \frac{\eta^*(1+\eta)}{\eta(1+\eta^*)} \quad \text{(A3)}$$

Assuming that s is constant, by means of Eq. (13) therefore:

$$P_d^*/P_d = (v^*/v) \; . \; (\tau_{exch}/\tau_{exch}^*) = \frac{\eta^*(1+\eta)}{\eta(1+\eta^*)}\frac{\tau_{exch}}{\tau_{exch}^*}. \quad \text{(A4)}$$

With the data of Fig. 3, i.e., the control values $\tau_{exch} = 12.1$ ms and $\eta = V_{in}/V_{out} = 0.20$ at time $t = 0$, and the constant values $\tau_{exch}^* = 5.6$ ms and $\eta^* = V_{in}^*/V_{out}^* = 0.08$ at time $t \approx 120$ min of erythrocyte treatment with $HgCl_2$, one obtains from Eq. (A4) the following permeability ratio:

$$P_d^*/P_d \approx 0.96. \quad \text{(A5)}$$

This result therefore proves that treatment of 20–30% suspensions of human erythrocytes with 1 mM $HgCl_2$ does not affect their membrane diffusional water permeability P_d. This is in agreement with the observation [21] that there was no change in P_d when the cell volume was decreased either by increasing the tonicity of the extracellular medium (by increasing the extracellular NaCl concentration from hypotonic concentration of 100 mM to a hypertonic concentration of 250 mM) or by increasing the extracellular pH from 5.5 to 9.5.

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