Biochimica et Biophysica Acta, 1061 (1991) 309–312

 0005-2736/91/$03.50
ADONIS 0005273691000943

BBAMEM 70670

BBA Report

Water transport in human red cells: effects of 'non-inhibitory' sulfhydryl reagents

Gheorghe Benga [1,2], Octavian Popescu [1], Victoria Borza [1], Adriana Hodârnău [1], Victor Ioan Pop [1] and John Wrigglesworth [3]

[1] *Department of Cell Biology, Faculty of Medicine, Medical and Pharmaceutical Institute,* [2] *Institute of Hygiene and Public Health, Cluj-Napoca (Romania) and* [3] *Department of Biochemistry, King's College University of London, London (U.K.)*

(Received 23 October 1990)

Key words: Water diffusion; Red blood cell; Sulfhydryl reagent; NMR; (Human)

The water diffusional permeability of human red blood cells following exposure to various sulfhydryl group (SH) reagents have been studied using a nuclear magnetic resonance technique. Exposure of red blood cells up to 12 mM *N*-ethylmaleimide (NEM) or 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNE) alone does not affect water diffusion. In contrast, when DTNB treatment follows a preincubation of the cells with NEM, a small (18% at 37°C) but significant inhibition of water permeability occurs. The NEM and DTNB treatment of the cells caused no change of the cell shape and volume or of the cell water volume. Consequently, the inhibition observed after NEM and DTNB treatment has a real significance.

Because of its ready availability and simple structure, the red blood cell has been a favourite object for investigating water permeability (see Refs. 1–3 for recent reviews). Studies on the chemical modification of the plasma membrane have shown that mercury-containing sulfhydryl reagents (SH reagents) substantially inhibit water exchange through the red cell membrane [4–8]. This has lead to the suggestion that mercury-containing SH reagents act by closing the aqueous channels of membrane protein concerned with water exchange [9]. Studies using ^{203}Hg-labelled *p*-chloromercuribenzene sulfonate (PCMBS) have allowed for the identification of some of these membrane proteins involved in human red blood cells [10,11].

Among other SH reagents, NEM does not seem to have any significant inhibitory effect, whilst for DTNB there are several conflicting reports. Inhibitory values of 60% [5] and 30% [12] have been reported for the inhibition of osmotic water permeability by DTNB. This contrasts with the negative findings of Chien and Macey [13]. In isotopic measurements of water diffusional permeability, Brahm [7] also could not detect any inhibitory effect by DTNB.

However, all previous investigations involved measurements performed on a small number of subjects and/or at one single temperature. The aim of our work was to perform NMR measurements on the effects of NEM and DTNB on water diffusional permeability on the effects of NEM and DTNB on water diffusional permeability in erythrocytes obtained from a large group of subjects. Taking advantage of the doping NMR method (see Ref. 14 for full discussion), we have estimated not only the inhibition of water permeability induced by these compounds, but also their effects on the activation energy of water diffusion in human red cells.

The NMR measurements of the water diffusion exchange time (T_e) were performed by the spin-echo method [15], essentially as previously described [14–17]. The membrane permeability for water diffusion P, is related to $1/T_e$, the cell water volume (V) and the cell surface area (A), by:

$$P = \frac{V}{A} \cdot \frac{1}{T_e} \quad (1)$$

The inhibition of water diffusion across human red

Abbreviations: SH, sulfhydryl; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); PCMBS, *p*-chloromercuribenzene sulfonate.

Correspondence: G. Benga, Department of Cell Biology, Faculty of Medicine, Medical and Pharmaceutical Institute, 6 Pasteur Street, 3400 Cluj-Napoca, Romania.

TABLE I

Effect of various SH reagents on diffusional permeability of the human red blood cells

Human blood was obtained by venipuncture, into heparinized tubes, of healthy male and female donors. The red blood cells were isolated by centrifugation and washed three times in 150 mM NaCl, 5.5 mM glucose, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (Hepes), (pH 7.4) (medium S), and finally suspended in medium S supplemented with 0.5% bovine serum albumin at a cytocrit of 50%. Incubations were performed at a hematocrit of 10% in medium S (except for the incubation with NEM which were performed at a hematocrit of 25% in medium S). The cells were sedimented by centrifugation and aliquots were used for NMR measurements as previously described [14–17,20]. The results are expressed as mean ± S.D. The statistical significance of the parameters of samples treated with SH reagents and control samples were calculated using the paired Student's *t*-test. The differences were significant for samples incubated with NEM plus DTNB ($P < 0.01$) and for samples incubated with PCMBS ($P < 0.001$). The permeabilities and % inhibitions were calculated as described in the text.

Compound	Conditions of incubation		No. of determinations	Temperature of measurement	P ($cm \cdot s^{-1}$ ($\times 10^3$))	% Inhibition
	time (min)	temperature (°C)				
None	60	37	18	15	3.27 ± 0.30	
			21	20	4.05 ± 0.35	
			22	25	4.54 ± 0.36	
			12	30	5.30 ± 0.34	
			22	37	7.21 ± 0.60	
			14	42	8.07 ± 0.66	
12 mM NEM	60	37	9	15	3.05 ± 0.56	–
			22	20	4.02 ± 0.56	–
			8	25	4.47 ± 0.71	–
			8	30	5.48 ± 0.60	–
			20	37	6.93 ± 1.21	–
			8	42	8.64 ± 1.59	–
10 mM DTNB	60	37	7	15	3.15 ± 0.50	–
			11	20	3.81 ± 0.30	–
			7	25	4.36 ± 0.42	–
			7	30	5.57 ± 0.63	–
			12	37	7.08 ± 0.57	–
			7	42	8.66 ± 0.82	–
12 mM NEM followed by	60	37				
10 mM DTNB	60	37	7	15	2.60 ± 0.48	20.5 ± 6.0
			12	20	3.45 ± 0.35	14.8 ± 5.0
			7	25	3.82 ± 0.35	15.9 ± 3.9
			7	30	4.69 ± 0.22	11.5 ± 2.5
			12	37	5.93 ± 1.00	17.8 ± 2.3
			7	42	7.32 ± 1.11	10.4 ± 2.9

TABLE II

Effect of sulfhydryl group reagents on the activation energy of water diffusion in human red blood cells

The preparation of samples and the measurements were performed as described in the text and in the legend to Table I. Results are expressed as mean ± S.D. Statistical significance was calculated by the Student's paired *t*-test.

Compound	Concentration (mM)	Number of determinations	$E_{a,d}$		Statistical significance compared to control (P)
			kcal/mol	kJ/mol	
Control		42	6.14 ± 0.77	25.7 ± 3.2	> 0.1
Incubated for 60 min at 37 °C		20	6.44 ± 0.78	26.9 ± 3.3	
NEM	1	6	6.16 ± 0.87	25.7 ± 3.7	> 0.1
	2	4	6.19 ± 0.74	25.9 ± 3.1	> 0.1
	12	5	6.20 ± 0.71	25.9 ± 3.0	> 0.1
DTNB	10	4	6.39 ± 0.54	26.6 ± 2.3	> 0.1
NEM	12				
followed by DTNB	10	4	6.04 ± 0.60	25.3 ± 2.5	> 0.1
PCMBS	1	4	9.21 ± 2.20	39.0 ± 9.2	< 0.001

blood cell membranes was calculated according to the formula:

$$\% \text{ Inhibition} = \frac{P \text{ control} - P \text{ sample}}{P \text{ control}} \times 100 \quad (2)$$

As no major morphological changes induced by exposure to SH reagents could be detected by optical microscopy, the cell surface area was assumed to have a constant value of $1.35 \cdot 10^{-6}$ cm^2, that was considered by Dix and Solomon [19] to be the best estimate for the human red cells.

In contrast to our previous work, we have calculated the values of P from our own estimations of cell water volume. The cell volume was estimated from cell counts and measurements of the hematocrit. Cell water content was determined by drying a sample of red cells to constant weight and the calculation of the cell water as a fraction of cell volume was performed as described by Eilam and Stein [18].

We found no significant differences in the fractional volume of water between control human red blood cells: 0.730 ± 0.082 and the cells incubated with 12 mM NEM: 0.712 ± 0.072, 10 mM DTNB: 0.737 ± 0.108, or 12 mM NEM followed by 10 mM DTNB: 0.721 ± 0.115 (mean and standard error of the mean for five experiments). The conditions of incubation are mentioned in Table I.

The results of the NMR measurements of water diffusion were obtained on erythrocytes from a large number of subjects and cover measurements over the temperature range 15–42°C. As can be seen from the results in Table I, NEM or DTNB alone do not appear to significantly affect the red blood cell diffusional permeability. However, when the DTNB treatment is preceeded by a preincubation with NEM, a small but statistically significant ($P < 0.01$) inhibition of water diffusional permeability can be noticed (Table I). No increase in the activation energy of water diffusion occurs by treatment with the SH reagents used (Table II), exception the knonw effect of PCMBS [20]. Supposing that at pH 7.4 NEM and DTNB might not be at their maximum effectivity, the effects of incubation of red blood cells at pH 8.0 and 8.5 with 12 mM NEM or 10 mM DTNB or with both reagents have been investigated. There were no significant differences in the degree of inhibition of water diffusion induced by these reagents at pH ≥ 8.0 compared to their effects at pH 7.4.

It is for the first time that the effect of NEM and DTNB has been studied in detail by the NMR approach. The data reported here help to clarify the apparent conflicting reports concerning the effects of DTNB on red blood cell water permeability. It appears that DTNB inhibits this process only in cells previously exposed to an excess of NEM. The mean inhibition at 37°C was 18% and is thus in good agreement with the value of 12% previously reported by us [8] and with the 14% inhibition found by Toon et al. [21]. It is possible that the higher values of inhibition reported by Naccache and Sha'afi [5] and by Levitt and Mlekoday [12] reflect a particular behaviour of the blood cells in measurements of osmotic permeability.

The sites of action of DTNB have been studied by Toon et al. [21]. It appeared that one covalent binding site for this reagent does not inhibit water transport. There is an additional low-affinity (non-covalent) DTNB site that inhibits water transport in NEM treated cells. This inhibition can be partially reversed by the specific anion exchange transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate. Reithmeier [22] has also shown that the low-affinity DTNB site inhibits anion transport.

On the other hand, Dawson and Widdas [23] have studied in detail the inhibition of glucose permeability of human erythrocytes by NEM. They found that a relatively high concentration of NEM (around 12 mM) is required in order to induce a strong inhibition, while smaller concentrations will only partially inhibit glucose permeability.

In view of the above mentioned studies on the inhibitory effects of DTNB and NEM on anion and, respectively, on glucose transport, we may interpret our results as follows. While the inhibition of either anion or glucose transport alone will not affect the water transport of human red cells, a summation of both inhibitory effects couuld inducea small decrease in water permeability. This is most probably not an expression of a non-specific membrane damage. On one hand, Dawson and Widdas [23] have excluded a non-specific membrane effect after incubation of human red cells with relatively high concentrations of NEM (above 12 mM) by showing that the permeability of cells to malonamide is not affected. On the other hand, a non-specific membrane damage will result in a penetration of Mn^{2+} ions and hence a decrease in the T_c values obtained by NMR measurements [17], contrary to what we found after exposure of red cells to NEM and DTNB.

The present results indicate that in addition to the drastic inhibition induced by mercurials other SH-reagents (so far considered as 'non-inhibitory') may also have inhibitory effects on water permeability. Although much smaller than those of mercurials, these effects could be relevant for the decreased water permeability of red cells from individuals with Duchenne muscular dystrophy [24–26] where such small, but statistically significant inhibitions have been reported.

We thank Rodica Gabor and Marieta Dan for technical assistance. We also wish to thank the Ministry of Education and Teaching, the Academy of Medical Sciences (Romania) and The Wellcome Trust (U.K.) for financial support.

References

1 Benga, Gh. (1988) Prog. Biophys. Mol. Biol. 51, 193–245.
2 Macey, R.I. and Brahm, J. (1989) in Water Transport in Biological Membranes (Benga, Gh., ed.), pp. 25–40, CRC Press, Boca Raton.
3 Benga, Gh. (1989) Int. Rev. Cytol. 114, 273–316.
4 Macey, R.I. and Farmer, R.E.L. (1970) Biochim. Biophys. Acta 211, 104–106.
5 Naccache, P. and Sha'afi (1974) J. Cell Physiol. 83, 449–456.
6 Benga, Gh., Pop, V.I., Ionescu, M., Holmes, R.P. and Popescu, O. (1982) Cell Biol. Int. Rep. 6, 775–781.
7 Brahm, J. (1982) J. Gen. Physiol. 79, 791–819.
8 Benga, Gh., Pop, V.I., Popescu, O., Ionescu, M. and Mihele, V. (1983) J. Membr. Biol. 76, 129–137.
9 Macey, R.I. (1984) Am. J. Physiol. 246, 195–203.
10 Benga, Gh., Popescu, O., Pop, V.I. and Holmes, R.P. (1986) Biochemistry 25, 1535–1538.
11 Benga, Gh., Popescu, O., Borza, V., Mureşan, A., Mocsy, I., Brain, A. and Wrigglesworth, J.M. (1986) Eur. J. Cell Biol. 41, 252–262.
12 Levitt, D.G. and Mlekoday, H.J. (1983) J. Gen. Physiol. 81, 239–253.
13 Chien, D. and Macey, R.I. (1979) in Membrane Transport in Biology (Giebisch, G., Tosteson, D.C. and Ussing, H.H., eds.), p. 30, Springer-Verlag, Berlin.
14 Benga, Gh., Pop, V.I., Popescu, O. and Borza, V. (1990) J. Biochem. Biophys. Methods 21, 87–102.
15 Conlon, T. and Outhred, R. (1972) Biochim. Biophys. Acta 288, 354–361.
16 Benga, Gh., Borza, V., Popescu, O., Pop, V.I. and Mureşan, A. (1986) J. Membr. Biol. 89, 127–130.
17 Benga, Gh., Popescu, O., Borza, V., Pop, V.I. and Hodârnău, A. (1989) J. Membr. Biol. 108, 105–113.
18 Eilam, Y. and Stein, W.D. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), p. 318, Plenum Press, New York.
19 Dix, J.A. and Solomon, A.K. (1984) Biochim. Biophys. Acta 773, 219–230.
20 Benga, Gh., Pop, V.I., Popescu, O., Hodârnău, A., Borza, V. and Presecan, E. (1987) Biochim. Biophys. Acta 905, 339–348.
21 Toon, M.R., Dergi, P.L., Lukacovic, M.F. and Solomon, A.K. (1985) Biochim. Biophys. Acta 818, 159–170.
22 Reithmeier, R.A.F. (1983) Biochim. Biophys. Acta 732, 122–125.
23 Dawson, A.C. and Widdas, W.F. (1964) J. Physiol. 172, 107–122.
24 Serbu, A.M., Marian, A., Popescu, O., Pop, V.I., Borza, V., Benga, I. and Benga, Gh. (1986) Muscle Nerve 9, 243–247.
25 Benga, Gh., Popescu, O., Pop, V.I., Borza, V., Mureşan, A., Hodârnău, A., Benga, I. and Ionescu, I. (1988) in Biomembranes: Basic and Medical Research (Benga, Gh. and Tager, J.M., eds.), pp. 204–219, Springer-Verlag, Berlin.
26 Benga, Gh., Popescu, I., Pop, V.I., Borza, V., Hodârnău, A., Popescu, M., Serbu, A.M. and Benga, I. (1989) Muscle Nerve 12, 294–301.