Biochimica et Biophysica Acta, 1065 (1991) 99–102

ADONIS 00052736002237

BBAMEM 70680

BBA Report

In vivo tritium contamination effects on water exchange across the erythrocyte membranes: a nuclear magnetic resonance study

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(Received 8 November 1990)
(Revised manuscript received 11 March 1991)

Key words: Tritium effect; Water exchange; NMR; (Rat erythrocyte membrane)

The effects produced by chronic in vivo tritium contamination on the process of water diffusion across the rat erythrocyte membranes were investigated using an NMR relaxation method. As the level of tritium intake increased, an inhibition of the water permeability was observed, reaching values of 40% for corresponding absorbed doses of about 100 mGy. The activation energy of the transport process was also significantly increased, suggesting that the mechanism of the water diffusion across the membrane had changed.

The transport of water across the biological membrane is of considerable importance for many physiological processes involved in the cell adaptation to various external factors.

The relative simplicity and availability of the erythrocytes have made them a primary target for the investigation of water transport processes across the cell membrane. Many studies have focused on the changes induced in the water transport across the erythrocyte membranes following chemical manipulations of the membranes [1–4] with the aim of locating the water transport pathway and on this basis to exploring further the membrane defects in pathological subjects.

Our interest was to explore the effects induced by ionizing irradiation on the erythrocyte water transport process and if possible to gain some insight into the underlying membrane structural changes. The effects of ionizing radiation on biological membranes have already been examined from other points of view: changes in membrane Na-K permeability [5], the content of sulphydril and disulphides groups [6], cell surface charge [7–9], receptors function [10,11] and fatty acids peroxidation [12,13]. All the experiments (at least to our knowledge) performed in order to reveal the effects of ionizing radiation on the erythrocytes were in vitro experiments at high absorbed doses $5–10^3$ Gy).

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In this report we present an experimental investigation of the effects produced by chronic in vivo low doses of tritium contamination on the water diffusional transport across the rat erythrocyte membrane.

In order to monitor the changes of membrane permeability, we used the method of NMR relaxation [14–16]. This method has been shown to be a valuable tool of investigation in both the physiology and pathology of the erythrocyte membrane [17,18].

The biological material for this experiment was collected from five groups of 20 Wistar male rats each. Four of them were chronically contaminated during 90 days by feeding them with tritiated water. The supplied tritium concentration conformed to that recommended by the International Commission of Radiological Protection as the maximum admissible concentration (MAC) for occupational exposures [19] as follows: $1.11 \cdot 10^3$ Bq/ml (= 1 MAC) for group A; $3.7 \cdot 10^3$ Bq/ml (= 3 MAC) for group B; $11.1 \cdot 10^3$ Bq/ml (= 10 MAC) for group C; $37 \cdot 10^3$ Bq/ml (= 30 MAC) for group D. The total exposure corresponds to a range of absorbed doses between 3.7 and 110 mGy (for calculations see Ref. 20). The fifth rat group (M) received tap water and was used as control. Rat blood was obtained by tail venipuncture in heparinized tubes and was used within 6 h. Representative blood samples were prepared by mixing equal aliquots of blood from 6 or 7 animals of each group.

A Coulter counter method (PS-4 Picoscaler-Medicor Budapest) was used to determine the cell volume and its

dimension (accuracy 97%) as well as the number of cells per unit volume (relative error better than 2%) for each sample.

The samples for NMR measurements were prepared by gently mixing an aliquot of 1 ml of the representative whole blood sample with an appropriate volume of $MnCl_2$ solution in order to obtain a final concentration of 17 mM $MnCl_2$.

The NMR measurements were carried out with a 90 MHz Bruker pulsed spectrometer. The time evolution of the transverse magnetization of water protons in the sample was monitored by the standard Carr-Purcell sequence ($\pi/2$ pulse of 8 μs, interpulses spacing of 100 μs). The sampled NMR signals were transferred to a Nicolet BNC-12 computer for data processing.

The sample temperature was measured directly by a thermocouple inserted in a capillary tube (accuracy within 0.3K). Our variable temperature set-up was modified in order to minimize the temperature equilibration which was reduced to about 5 min per temperature point.

The presence of Mn^{2+} paramagnetic ion drastically shortens the extracellular water transverse relaxation time (T_{2b}), leaving the intracellular water relaxation time (T_{2a}) almost unchanged. Thus extracellular water is labeled and differentiated on the NMR scale from intracellular water which in turn allows the kinetics of the water transport process across the membrane to be followed [15,16].

Under paramagnetic doping conditions, due to the exchange process, the time evolution of the transverse magnetization is the sum of the two time exponentials with two apparent relaxation times T_{2A} and T_{2B}. Under the doping conditions used in this work (17 mM $MnCl_2$), the extracellular water relaxation time (T_{2b}) is sufficiently small and the long apparent relaxation time (T_{2A}) deduced from the slowest decaying region is well approximated by [15]

$$(T_{2A})^{-1} = (\tau_a)^{-1} + (T_{2a})^{-1}$$

where τ_a is the mean life time of a water molecule in the intracellular compartment (loosely defined as the exchange time through the membrane). Independent measurements of T_{2A} and T_a allow the calculation of τ_a. Ideally, T_a can be obtained from packed cell samples in the absence of any residual plasma. Actually, T_{2a} was measured in aliquots of 1 mM packed cells obtained after the centrifugation of whole blood samples at 1000 $\times g$ for 60 min leaving a 3% extracellular trapped plasma volume which was found to have negligible effects on T_{2a} [21].

The transverse relaxation times T_{2a} and T_{2A} were obtained experimentally for temperatures between 20 and 40 °C. This allows the calculation of τ_a within the same temperature range. Since the typical time for a full temperature range measurement of one sample was about 40 min, one should check the stability of T_{2A} against possible effects of the cellular intake of Mn^{2+} ions. We checked this at 37 °C, measuring T_{2A} immediately after Mn^{2+} addition and again 1 h later. No differences were found, which is in agreement with similar results on human whole blood [22].

The Arrhenius plots $\ln \tau_a = f(1000/T)$ remained linear over the entire range of temperatures allowing the calculation of the activation energies (E_a) of the diffusion process from

$$\tau_a = \tau_0 \exp(E/kT)$$

Tables I and II and Fig. 1 present some of these measured and derived values. Summing up, we may point out the following features:

(a) Within experimental error, the intracellular water relaxation times (T_{2a}) remain invariant with respect to the internally absorbed dose. This means that intracellular interactions between water molecules and cellu-

TABLE I

Experimental results for T_{2a}, τ_a and permeability inhibition

Relative errors for T_{2a} * and τ_a + are within 10%.

^3H concn. in drinking water 10^3 (Bq/ml)	Experimental parameters				
	25 °C			37 °C	
	T_{2a} (ms)	τ_a (ms)	inhibition (%)	T_{2a} (ms)	τ_a (ms)
M (control)	101	6.2	0	122	4.7
A (1.1)	96	7.8	21 ± 7	121	5.4
B (3.7)	101	7.5	19 ± 5	126	4.4
C (11.1)	98	9.9	38 ± 9	119	5.8
D (37)	95	10.4	41 ± 8	121	5.7

* Error estimates for $T_{2a}(T_{2A})$ are standard deviation (S.D.) of the least-square fit and include statistical error as well as systematic errors due to base line in the CPMG sequence [16].

\+ S.D. of τ_a and inhibition were calculated using the error propagation method.

TABLE II

*Activation energies E_a * and τ_0 values as a function of the tritium intake level*

^3H concn. in drinking water 10^3 (Bq/ml)	E_a (kJ/mol)	τ_0 (s)
M (control)	21.6 ± 4.2	$1 \cdot 10^{-6}$
A (1.1)	23.3 ± 4.2	$6 \cdot 10^{-7}$
B (3.7)	33.3 ± 6.5	$1 \cdot 10^{-8}$
C (11.1)	35.8 ± 6.5	$5 \cdot 10^{-9}$
D (37)	42.4 ± 7.2	$4.4 \cdot 10^{-10}$

* Estimates of the E_a errors are standard deviations calculated using the error propagation method using an absolute temperature accuracy within 0.5 K and a τ_a relative standard deviation of 10%.

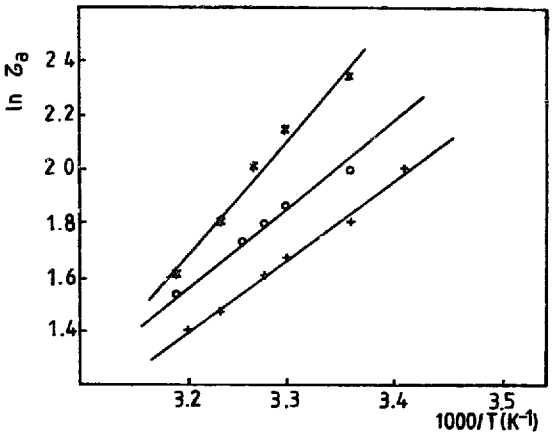

Fig. 1. Arrhenius plots $\ln(\tau_a) = f(1000/T)$. +——+, group M (control); o——o, group A (1 MAC); *——*, group C (10 MAC).

lar macromolecules are not influenced by internal irradiation at the levels used in this paper.

(b) The water exchange times τ_a show a clear dependence on the level of tritium contamination. This is better revealed at lower temperatures. The change of τ_a is a consequence of changes in both E_a and τ_0.

(c) Following tritium intake, a significant increase of the activation energy of the diffusional water transport from $E_a = 20$ kJ/mol for the control group up to $E_a = 40$ kJ/mol for the highest tritium contamination group is observed.

(d) The NMR results may be directly related to the membrane permeability via [23]:

$$P_d = (V/A)(\tau_a)^{-1}$$

were V and A are the cell volume and surface, respectively. The relative change of the transmembrane diffusional water permeability (P_d) may be calculated from this relation as long as one knows the variation of the geometrical factor V/A induced by tritium contamination. The cell dimensions distribution as revealed by Coulter counter measurements was identical for irradiated and non-irradiated samples, with an average diameter value of 4 μm and a relative dispersion of 8%. Moreover, the observation of erythrocyte by light microscopy did not revealed any modification of the cell shape for any of the experimental groups. Therefore, one may assume that the radioactive contamination did not induced dimensional modifications of red blood cells. The permeability inhibition results were discussed on the assumption that the V/A was constant in all the samples investigated.

Under this condition the relative change of the water diffusion permeability (the inhibition) due to internal contamination can be expressed as

$$\text{inhibition} = 1 - \tau_{\text{control}}/\tau_{\text{contamination}}$$

As can be seen from Table I, the inhibition at 25°C begins to be observed even at the lowest level of contamination and increases as the level of the tritium intake increases reaching a value of 40% for group D ($37 \cdot 10^3$ Bq/ml).

The experimental results as pointed out above, indicated both an inhibition of the water transport and a change of the transport mechanism across the membrane (as reflected by the changes of E_a) induced by internal tritium contamination. Any further assumptions should rely on the knowledge of the transport mechanism for normal rat erythrocytes. The water exchange time values (τ_a) as well as the corresponding transport mechanism for rat erythrocytes have not been previously reported and we can not compare our control group results with similar ones from the literature, but we can refer to those reported on human erythrocytes.

For human erythrocytes, a water exchange time of $\tau_a = 13$–18 ms at 25°C was reported [21,22] with activation energies ranging between $E_a = 21$ and 25 kJ/mol [16,21,22].

Our control group data gave an exchange time which was shorter by a factor of 2 and no significant difference for activation energy, indicating that the water diffusion process is more rapid than for human erythrocytes, but the basic diffusion mechanisms are most probably the same. Therefore, we shall assume that the water diffusion transport for rat erythrocytes has the same molecular background as that for human erythrocytes, namely that the water transport across the red cell membrane involves membrane proteins (band 3 or 4.5) [2,3] and membrane lipids [24].

Although neither the extent nor the mechanisms of water transport by these two pathways have been clearly established, any change in the permeability should reflect changes within these two transport pathways.

At this point we should underline the strong similarities on the NMR scale between the inhibition and the activation energy changes induced by irradiation as found in this study and those produced by sulphydril oxidation of human erythrocyte membranes in the presence of *p*-chloromercurybenzensulfonate (PCMBS).

It has been shown that 2 mM PCMBS produce a 50% inhibition of water diffusion [1,25]. This inhibition was accompanied by an increase of the activation energy up to $E_a = 40$ kJ/mol [26] close to that obtained for lipid bilayers (45–55 kJ/mol) [27]. Therefore, under such blocking conditions the water transport was considered to proceed via lipid pathways. This behaviour has been explained assuming an interaction of PCMBS with the SH-group found in the hydrophobic zone belonging to one of the major transmembrane proteins, band 3 or 4.5 [2,3].

In view of similarity between the results mentioned above with our experimental results, one may assume

that the permeability inhibition produced by tritium intake is a consequence of SH-groups oxidation at the outer face of the membrane induced by the attack of water radiolysis radicals. Most probably due to SH oxidation, conformational modification of the proteins involved in water transport does occur, which may produce the observed changes. This hypothesis, based only on the similarity with effects induced by PCMBS is in good agreement with the results obtained by methods other than NMR on human erythrocyte irradiated in vitro at high doses, results which prove the additive effect of irradiation and PCMBS [5,6]. These studies as well as other [7,28] have established that high dose irradiation of suspended erythrocytes does produce SH oxidation and consequently conformational changes of the membrane proteins, modified protein–protein and protein–lipid interactions, which in turn, alter the membrane properties, including the ionic Na-K permeability. Our results seem to confirm that such effects exist and are also detectable for in vivo chronically irradiated erythrocytes, even at low absorbed doses.

We should mention that in vitro irradiation effects have also been reported on other types of cell (human and murine lymphocytes, human fibroblasts, human and murine platelets) [29]. These studies using various methods, have revealed micromorphological modifications, surface charges and surface receptor changes pointing out modifications of membrane protein induced by irradiation as also suggested by the results of this study.

Finally, we point out an interesting observation concerning the values of the water exchange times (τ_a), which at 37 °C are rather unmodified by the irradiation conditions. This is an overall result of two compensating effects: the increase of activation energy (E_a) with a simultaneous decrease of the preexponential factor (τ_0) as the irradiation dose increases. Although the transport mechanism is changed by irradiation effects (as reflected by E_a and τ_0), the net water permeability at physiological temperatures is apparently not affected by the internal irradiation.

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