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EFFECT OF THIOUREA ON PCMBS INHIBITION OF OSMOTIC WATER TRANSPORT IN HUMAN RED CELLS

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The organomercurial reagent *p*-chloromercuribenzene sulfonate (PCMBS) is an inhibitor of osmotic water permeability in the human red cell membrane. We have found that thiourea, when added along with PCMBS to a red cell suspension, interferes with this inhibition and at high enough concentrations prevents the inhibition from developing altogether. For a 2 mM PCMBS concentration $K_i = 3 \pm 1$ mM. When thiourea is added at a later time, the PCMBS inhibition, which normally takes about 20 min to develop fully, is halted and remains fixed at the value attained by that time. Thiourea also inhibits the reversal of PCMBS inhibition by a 10 mM concentration of cysteine, the half-time for reversal increasing by more than an order of magnitude when [thiourea] = 50 mM. Possible implications for the nature of the water and urea transport pathways across the red cell membrane are discussed.

Introduction

The sulfhydryl reagent *p*-chloromercuribenzene sulfonate (PCMBS) inhibits water transport across the human red cell membrane, reducing the osmotic permeability coefficient, L_p, to a value consistent with the assumption that pathways allowing bulk water flow have been closed, and that only diffusion pathways remain [1,2]. This inhibition is presumably caused either by direct steric hindrance in a protein-associated hydrophilic pathway or by conformational changes which effectively close the pathway to bulk water flow. At maximal PCMBS concentration of about 2.0 mM, water permeability under an osmotic pressure gradient is reduced [2] by about 80% and water diffusion is reduced by 54% [3]. The effect of PCMBS on the permeability of other solutes is quite specific. PCMBS inhibits urea permeation to about 10% of its unperturbed value [1], whereas it has no effect on the permeability of lipophilic solutes and a very much smaller effect on alcohols such as ethylene glycol and glycerol than on amides. Phloretin, another inhibitor of solute diffusion into red cells, affects both alcohols and amides, but has no effect on water transport. The specificity of the effects of these different inhibitors led Macey and Farmer [1] and later Brahm and Wieth [4] to suggest that urea entered the cell through a specialized transport system rather than through an aqueous channel. These authors [4] and subsequently Brahm [5] have shown that urea inhibits the transport of its

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Abbreviations: PCMBS, *p*-chloromercuribenzene sulfonate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate.

sulfur-containing analog, thiourea, across the cell membrane and, conversely, that thiourea inhibits urea flux, possibly competitively. This observation indicates that these two molecules are transported by the same system, but does not bear directly on the question as to whether urea and thiourea are transported by the water channel. Solomon and Chasan [6] have shown that thiourea also inhibits other hydrophilic amides, but has little or no effect on alcohol or lipophilic solute transport, indicating that the inhibition is specific to amides. The existence of a shared pathway for water and small hydrophilic molecules is a matter of considerable debate and it was for this reason that we have looked for evidence of thiourea interaction with PCMBS inhibition of water transport.

Methods and Materials

Osmotic permeability was measured using the stop-flow light-scattering instrument of Terwilliger and Solomon [7] and the techniques of Levin et al. [8]. Blood was freshly drawn on the day of each experiment by venipuncture of a healthy human donor and placed in a heparinized flask (20 USP U/ml blood). The blood was immediately centrifuged and the supernatant fluid and buffy coat were removed by aspiration. The cells were then washed three times with a standard buffer of the following composition (in mM): NaCl, 125; KCl, 4.4; $NaHCO_3$, 24.9; $CaCl_2$, 1.2; $MgCl_2$, 0.5; Na_2HPO_4, 5.9, pH 7.4, total osmolality 300 ± 5 mOsm. Cells suspended in this buffer at 2% hematocrit were mixed with an equal volume of solution made hypertonic by addition of sodium chloride to the buffer. The response of the cells to a 250 mOsm gradient was the basis of all L_p measurements. Thiourea and PCMBS were added to the blood suspension as required and pH was readjusted to 7.4, if necessary, by addition of 1 N H_3PO_4 or 1 N NaOH. Experiments were carried out at room temperature (22–25 °C). Osmolalities of all solutions were measured using a Fiske model OS osmometer (Fiske Associates Inc. Uxbridge, MA). Cysteine, DTNB and PCMBS were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and obtained from Fisher Scientific Co., (Fairlawn, NJ).

Results

Does thiourea react with PCMBS?

The measurements described below indicate that thiourea interferes with the process by which PCMBS inhibits osmotic water transport. Before discussing the implications of these results for the red cell membrane, we first consider evidence that we are in fact not simply observing the consequences of a chemical reaction between thiourea and PCMBS. There is an equilibrium between uncharged thiourea and its protonated form which strongly favors the uncharged form. Nonetheless a reaction between the sulfur in the thiourea and the mercury in the PCMBS is not impossible, even at pH 7.4. In order to determine whether such a reaction takes place, we used Ellman's reagent, DTNB, which develops a yellow color (absorption maximum, 412 nm) when it reacts with an SH group [9]. As the absorbance values in Table I show, addition of cysteine to DTNB produces the typical yellow color of Ellman's reagent. When 0.1 mM PCMBS was added, the absorbance fell to 0.04, indicating that cysteine binds more strongly to PCMBS than to DTNB. Addition of thiourea to the solution produced no change, even when the thiourea and PCMBS were allowed to incubate for 1 h before addition of cysteine and DTNB. These experiments show that the cysteine-PCMBS bond is much stronger than the putative bond between

TABLE I

USE OF DTNB TO PROBE POSSIBLE THIOUREA-PCMBS REACTION

Preincubation of thiourea and PCMBS for 30 min (60 min) produced an absorbance of 0.03 (0.04). Preincubation for 35 min, followed by addition of DTNB prior to the PCMBS addition produced an A of 0.09. Thiourea did not react with DTNB alone ($A = 0.02$). All measurements were made in phosphate-buffered saline (0.15 M NaCl, 5 mM Na_2HPO_4, pH 7.4) at room temperature.

Reagents	Absorbance (412 nm)	
	Control	+ Thiourea (0.1–1.0 mM)
Cysteine (0.1 mM) + DTNB (0.1 mM)	1.28	1.17
+ PCMBS (0.1 mM)	0.04	0.04

PCMBS and thiourea. A number of control experiments are summarized in the heading to Table I. They indicate that in free solution there is no evidence for any interference by thiourea with the PCMBS-cysteine reaction.

Although the SH groups in membrane proteins are located in cysteine residues, it is quite likely that the reactivity of the SH groups in the protein is different from that in free solution. The DTNB measurements do not rule out a thiourea-PCMBS complex which is much less stable than the PCMBS-cysteine complex but more stable than the PCMBS-membrane protein complex. To address this concern, a mercuric sulfhydryl reagent affinity gel was prepared with *p*-chloromercuribenzoate (PCMB) by the method of Lukacovic et al. [10], and hemoglobin, which contains 10 sulfhydryl groups, was absorbed to the column. When a 1 mM cysteine solution was subsequently passed through, more than 99% of the hemoglobin was eluted, whereas a 1 mM thiourea solution removed less than 1% of the hemoglobin, as measured by absorption at 412 nm. This measurement indicates that hemoglobin sulfhydryl groups form stable complexes with a reagent which is very similar in its properties to PCMBS, and that thiourea does not break the PCMB-sulfhydryl bond whereas cysteine does. It has been suggested by Brown et al. [11] that the anion-transport protein band 3 provides the aqueous channel for water transport, and Solomon et al. [12] have advanced supporting evidence. Therefore it was important to determine whether or not thiourea could remove PCMB from the sulfhydryl groups of band 3. Consequently we separated and purified band 3 according to the method of Lukacovic et al. [10] using the same PCMB-Sepharose 4B column. Whereas 1 mM thiourea eluted 5–10% of the band 3, 1 mM cysteine eluted 90–95%. This confirms our view that mercuric sulfhydryl reagents bind much more tightly to sulfhydryl groups on band 3 than thiourea does, but it does not exclude the possibility that thiourea can remove a fraction of the PCMBS from band 3, or from any other membrane protein which may be involved in osmotic water transport. For this purpose we relied on a functional test which will be described in the next section.

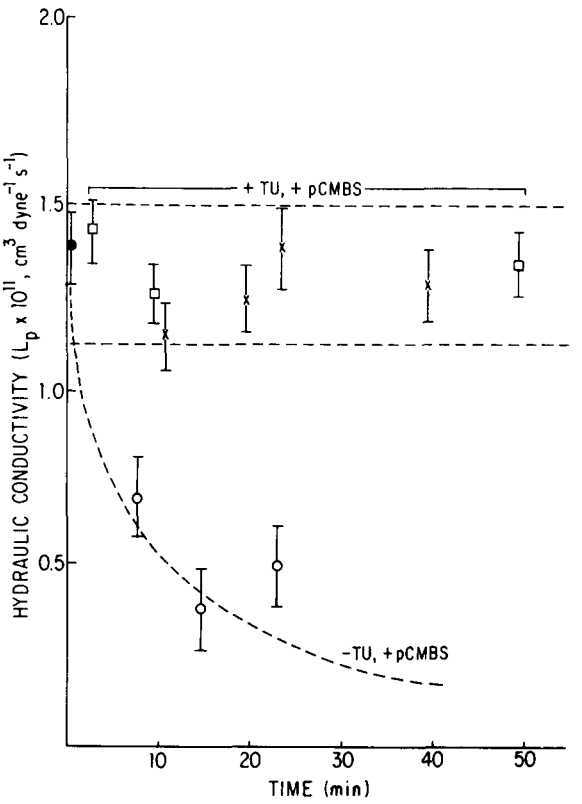

Fig. 1. Effect of thiourea (100 mM) and PCMBS (2 mM) on L_p. Thiourea was added at zero time (□) or after 5 min (×). ○, normal time course of PCMBS (2 mM) inhibition without thiourea. Dashed curve is an aid for the eye, not a fit. Control: −TU, −PCMBS (●).

Effect of thiourea on inhibition of water transport by PCMBS

The most striking effect of thiourea is the prevention of PCMBS-induced inhibition of osmotic water transport when 100 mM thiourea is added together with 2 mM PCMBS. Fig. 1 shows that L_p in cells to which 100 mM thiourea was added with 2 mM of PCMBS remains unchanged for at least 1 h although PCMBS normally produces its full inhibitory effect in 20 min, as shown by the dashed line in Fig. 1. In another control experiment, typical of three, L_p in the presence of 100 mM thiourea was $(1.15 \pm 0.01) \cdot 10^{-11}$ cm^3 · dyne^{-1} · s^{-1} as compared to a control value of $(1.25 \pm 0.15) \cdot 10^{-11}$ cm^3 · dyne^{-1} · s^{-1}, thus showing that thiourea alone has no effect on osmotic water transport. The dose-response curve in one experiment with 2 mM

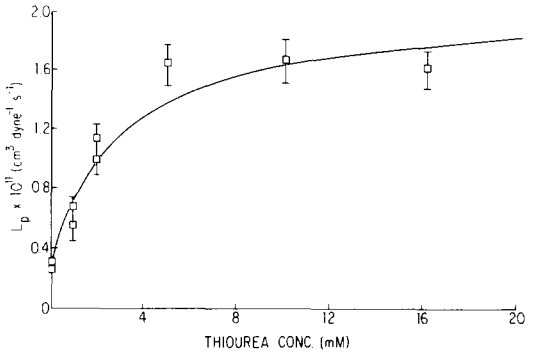

Fig. 2. Concentration dependence of thiourea effect on PCMBS inhibition of osmotic water transport. [PCMBS] = 2 mM.

PCMBS, typical of three, is shown in Fig. 2. The points have been fitted by non-linear least-squares to a single-site binding equation with a half concentration of 3 ± 1 mM thiourea. Thus half inhibition is reached when the thiourea concentration is of the same order of magnitude as the PCMBS concentration, rather than two orders of magnitude greater as used in the earlier experiments which first established the nature of the effect.

If the addition of thiourea is delayed relative to the PCMBS, L_p remains fixed at the approximate value attained by the time that the thiourea (100 mM) is added, as shown in Fig. 3. No reversal of inhibition is observed, indicating that once an osmotic water channel has been closed by PCMBS it cannot be opened by thiourea. Further experiments at lower concentrations show that there is no reversal by 10 mM thiourea, even after an hour's delay. Since thiourea is unable to reverse the PCMBS effect, a PCMBS-thiourea complex does not play a role in our experiments, confirming the results given above.

Cysteine rapidly reverses PCMBS water inhibition, indicating that cysteine can permeate to the relevant PCMBS site and react with the PCMBS. In order to study in more detail the mechanism by which thiourea has its effect we investigated its influence on the reversal process. Fig. 4 shows the time course of 10 mM cysteine reversal of fully developed PCMBS inhibition (2 mM, 30 min) in

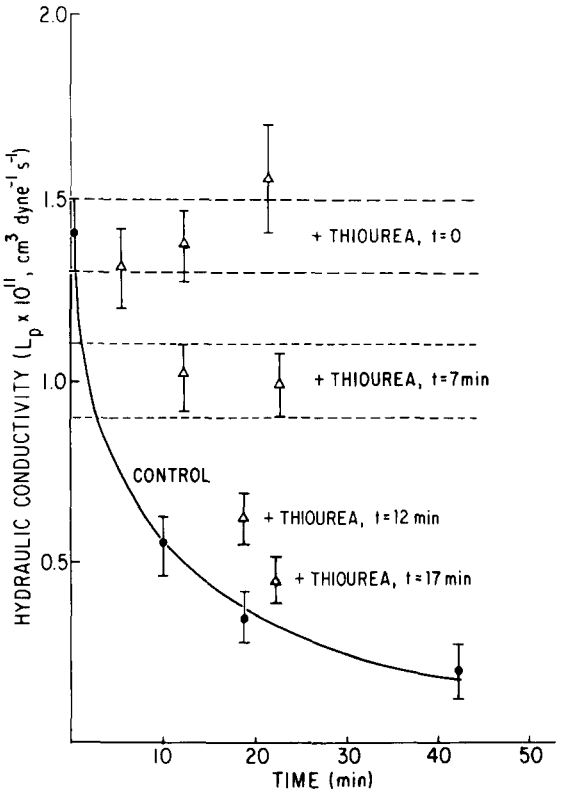

Fig. 3. Effect of delayed addition of thiourea (100 mM) to PCMBS treated blood. The solid points and curve show control PCMBS (2 mM) inhibition. The other points represent measurements made on PCMBS-treated blood to which thiourea was added at the indicated times after addition of PCMBS.

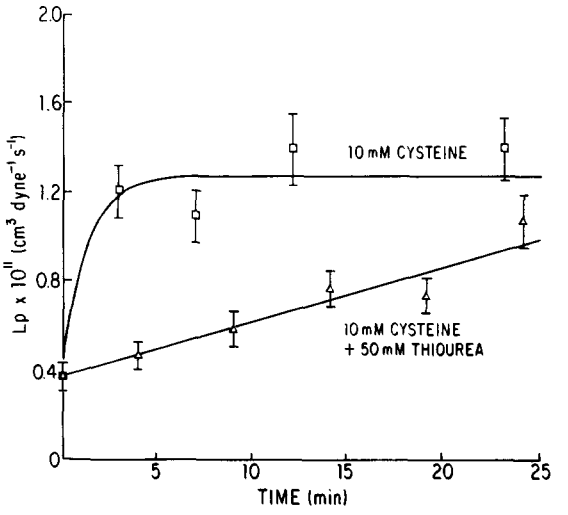

Fig. 4. Cysteine reversal of PCMBS inhibition of osmotic water permeability. □, cysteine (10 mM) added to blood 35 min after PCMBS (2 mM); △, cysteine (10 mM) and thiourea (50 mM) added to blood 35 min after PCMBS (2 mM).

one experiment, typical of three. The data have been fitted by non-linear least-squares to an exponential with a half-time of 1.3 ± 0.9 min. 50 mM thiourea has a dramatic effect on the cysteine reversal, slowing the process down so that the time for half reversal has been increased from 1.3 min to greater than 25 min.

Discussion

It has previously been shown by Naccache and Sha'afi [13] that PCMBS reacts very slowly with the site in the red cell membrane which controls water permeability. Over a 20 min period the osmotic permeability coefficient is reduced by 80% or more, presumably as a result of the interaction between the mercurial sulfhydryl reagent and a sulfhydryl group located in a membrane protein. The experiments that we have carried out lead to two phenomenological conclusions about this reaction. First, thiourea inhibits the PCMBS reaction by binding, within a period of three minutes or less, to a site characterized by $K_i = 3$ mM (for [PCMBS] = 2 mM). Second, thiourea markedly delays the effectiveness of cysteine in reversing the PCMBS inhibition.

In principle it is possible that this reversal is slowed simply because thiourea prevents PCMBS from leaving its binding site. However, the speed of cysteine-caused reversal of inhibition suggests that cysteine must reach the PCMBS-protein binding site in order to have an effect. Measurements made in this laboratory reinforce this conclusion (Toon, M.R. and Solomon, A.K., personal communication). Cells were exposed to 2 mM PCMBS for 30 min, then resuspended in buffer alone or in buffer containing either 5 mM of cysteine or an equal concentration of the larger and less permeant sulfhydryl reagent, glutathione. Suspension in buffer alone for 30 min failed to reverse the PCMBS inhibition, indicating that reversal by cysteine cannot occur simply through a scavenging reaction in the extracellular solution. The half-time for cysteine reversal was 1 min, compared to 20 min for glutathione. Similar results have been reported in an analogous situation, the PCMBS-induced cation leak in red cells. Cysteine reverses this process rapidly, whereas reversal by albumin is slow and incomplete, leading Rega et al. [14] to conclude that the PCMBS site is located in a cleft or channel that albumin cannot penetrate due to steric hindrance, whereas cysteine can. Similarly, Rothstein et al. [15] find that reversal of PCMBS binding to the red cell membrane is faster and more complete by cysteine than by albumin. In the present experiment, since thiourea is a smaller molecule than cysteine, it should be able to bind to a site in a channel which normally admits cysteine.

On the assumption that the water-transport channel is located in a protein, possibly band 3, the reaction involved in the inhibition of osmotic water transport may be written schematically as

$$\text{PCMBS} + \text{protein} \underset{\text{cysteine}}{\overset{\rightarrow}{\leftarrow}} \text{PCMBS} - \text{protein}^* \qquad (1)$$

using PCMBS-protein* to denote the form of the complex that inhibits water transport. Eqn. 1 is to be considered as a black-box description of the reaction which makes no assumptions about the cause of the delay in the water inhibition process. The effect of thiourea is to isolate the reaction product, PCMBS-protein*, from the reactants. In a membrane system, such an isolation could have either a physical or chemical cause. Physical occlusion of the relevant diffusion pathway could prevent either cysteine or PCMBS from getting to the PCMBS-protein* site. Alternatively, chemical interference could result from an interaction between thiourea and protein causing a conformation change which would, for example, internalize the site and prevent access. Whatever the cause, since thiourea does not affect the hydraulic conductivity, it does not directly occlude or close the aqueous channel. If the route to the PCMBS reaction site on the protein went by way of an ancillary channel, thiourea could bind to the protein and change the properties of the ancillary channel without affecting the hydraulic permeability of the major aqueous channel. Such an ancillary channel could be either a direct route from the outside of the cell or a side-road off the main aqueous channel. Changes in the ancillary channel would not affect the main osmotic water pathway. Thus Rao's [16] observation that PCMBS can enter the red cell slowly through the anion channel and that PCMBS permeation can be inhibited by anion-transport inhibitors is irrelevant to the argument.

The observation that thiourea, which is known to inhibit urea transport, also interacts with the water transport system suggests a relationship between urea and water transport. However, the K_i values for inhibition of urea transport reported by Mayrand and Levitt [17] are 12 mM on the inside of the cell and 35 mM on the outside, much larger than the K_i of 3 mM for the thiourea effect on the PCMBS system. These differences apparently indicate that separate thiourea binding sites are involved in the two processes. However, both urea and PCMBS can cause conformational changes in proteins and it is conceivable that such changes could affect the thiourea binding site and hence modify K_i. Thus, although the effect of thiourea on PCMBS inhibition of osmotic water transport is well established by the present measurements, our results cannot be used to determine whether or not there is a connection between the urea and water-transport processes in the red cell.

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