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Relation between red cell anion exchange and urea transport

Michael R Toon and A K Solomon *

Biophysical Laboratory, Department of Physiology and Biophysics, Harvard Medical School, Boston, MA 02115 (U S A)

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The new distilbene compound, DCMBT (4,4′-dichloromercuric-2,2,2′,2′-bistilbene tetrasulfonic acid) synthesized by Yoon et al. (Biochim. Biophys. Acta 778 (1984) 385–389) was used to study the relation between urea transport and anion exchange in human red cells. DCMBT, which combines properties of both the specific stilbene anion exchange inhibitor, DIDS, and the water and urea transport inhibitor, pCMBS, had previously been shown to inhibit anion transport almost completely and water transport partially. We now report that DCMBT also inhibits urea transport almost completely and that covalent DIDS treatment reverses the inhibition. These observations provide support for the view that a single protein or protein complex modulates the transport of water and urea and the exchange of anions through a common channel.

We have previously reported the synthesis of a new distilbene compound, DCMBT (4,4′-dichloromercuric-2,2,2′,2′-bistilbene tetrasulfonic acid) which combines properties of the stilbene red cell anion exchange inhibitors and those of the water transport inhibitor, pCMBS (*p*-chloromercuribenzene sulfonate) (Yoon et al [1]). We have shown that DCMBT inhibits anion transport almost completely with a K_i of 15 μM and inhibits osmotic water flux by about 15–20% with a K_i of about 8 μM. When DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) is covalently bound to the red cells, DCMBT inhibition of water transport is significantly reduced, which provides very strong evidence that anion transport and water transport are mediated by the same protein or protein complex. We now report the results of analogous studies showing that DCMBT inhibits urea transport almost completely and that covalent DIDS binding reverses the inhibition. These results provide evidence that urea transport and anion exchange are modulated by the same protein or protein complex.

Our previous experiments with DCMBT were carried out in *N*-ethylmaleimide-treated cells because Solomon et al [2] have previously shown that the sulfhydryl group with which pCMBS interacts to inhibit water transport does not react with *N*-ethylmaleimide. Furthermore, *N*-ethylmaleimide reacts with 80% of the membrane sulfhydryl groups (Haest et al [3]), significantly reducing the number of sulfhydryls with which DCMBT can react.

As Fig. 1 shows, DCMBT inhibits urea permeability in *N*-ethylmaleimide-treated cells by 82% with a K_i of 5.4 ± 0.9 μM in one experiment, typical of three (average $K_i = 9 \pm 5$ μM, max inhibition = 96 ± 13%). In *N*-ethylmaleimide-treated cells, the K_i for pCMBS inhibition of urea transport is 2.7 ± 0.4 μM (after 1 h incubation). DCMBT also inhibits methyl urea permeability by

* To whom correspondence should be addressed.

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pCMBS, *p*-chloromercuribenzenesulfonic acid; DCMBT, 4,4′-dichloromercuric-2,2 2′,2′-bistilbene tetrasulfonic acid

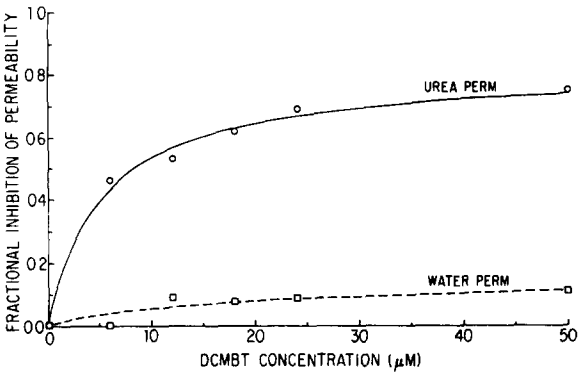

Fig 1 Inhibition of osmotic water (□) and urea (○) permeability by DCMBT in one of three experiments Washed red cells from recently outdated bank blood were treated at 25% hematocrit with 12 mM *N*-ethylmaleimide in the following buffer (in mM) NaCl 125 KCl, 4 4 $NaHCO_3$, 24 9 $MgCl_2$, 0 5 Na_2HPO_4, 5 9 pH 7 4 Measurement of osmotic water flux was by the method of Terwilliger and Solomon [4] and that of urea by the method of Sha'afi et al [5] Both permeability measurements were performed after 20 min incubation at 25°C with DCMBT with cells at 2% hematocrit in the same buffer Curves have been drawn by non-linear least squares to fit a single-site binding equation For urea inhibition, $K_i = 5\,4 \pm 0\,9$ μM and maximal fractional inhibition = $0\,82 \pm 0\,03$

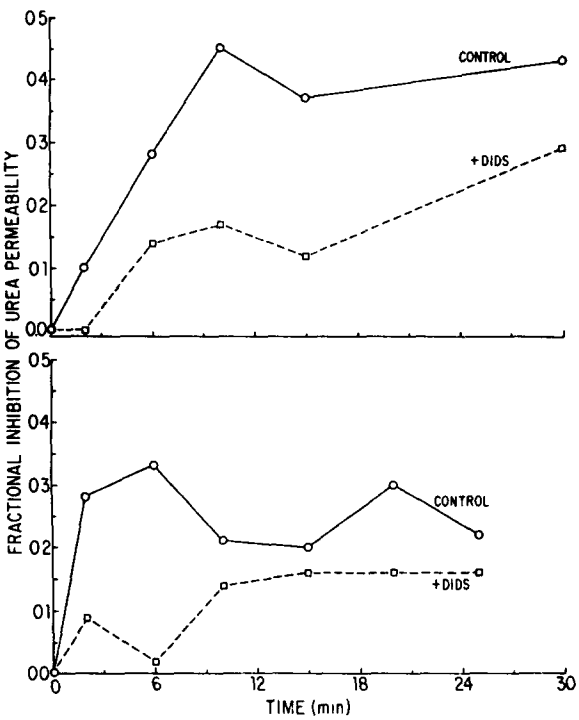

Fig 2 Effect of DIDS on the time-course of urea flux inhibition in two experiments Cells were treated as above with 12 mM *N*-ethylmaleimide ± 20 μM DIDS Cells were then washed three times with buffer and resuspended to 2% hematocrit for treatment with 6 μM DCMBT The lines were drawn to connect the points

$80 \pm 30\%$ (3 expts) with $K_i = 13 \pm 6$ μM These values of K_i for urea and methyl urea are similar to that of 8 μM for water Although DCMBT treatment always inhibited urea and methyl urea transport, the results were very variable as the large standard errors indicate Water inhibition in these experiments was also very variable and never exceeded 10%, lower than the figure of 15–20% previously reported

Fig 2 shows that covalent binding of DIDS slows the onset of 6 μM DCMBT inhibition of urea permeability in two experiments, typical of four The half-time for development of the urea inhibition in the control experiment is in the neighborhood of 2–5 min, similar to that previously shown for water inhibition As Fig 2 indicates, there is a tendency for the inhibition after DIDS treatment to approach control values at later times Notwithstanding this tendency, the DIDS effect is highly significant In 23 paired measurements in four experiments, DIDS treatment reduced the fractional inhibition of permeability by $0\,11 \pm 0\,02$ (S E, $P < 0\,001$, *t*-test) In the data shown in the bottom experiment in Fig 2, the difference in fractional inhibition caused by DIDS is significant at $P < 0\,02$, *t*-test in the top experiment, $P < 0\,01$ Control experiments in the absence of DCMBT showed that DIDS treatment had no inhibitory effect on either urea or water permeability (data not shown)

In our earlier experiments on the DCMBT effect on water transport, we pointed out that higher concentrations of DCMBT could override DIDS inhibition, indicating that DIDS does not totally block all the access routes by which DCMBT can reach the pCMBS site The present behavior fits this interpretation Although we have not explored the concentration dependence of the DCMBT/DIDS interaction, our impression is that higher concentrations of DCMBT will also override the DIDS protection of urea inhibition

The observation that DIDS slows the onset of DCMBT inhibition of urea permeability indicates that the DCMBT molecule which binds to the

DIDS site is also the molecule responsible for the urea transport inhibition Since DCMBT, fully extended, is 24 Å long, neither Hg atom can be more than 12 Å from the stilbene moiety, which places the pCMBS site within 12 Å of the stilbene binding site A similar argument places the pCMBS site for water inhibition within 12 Å of the stilbene site Although this argument is consistent with a single site that is responsible for both urea and water transport, as is the similarity in the values of K_i, this is not a unique interpretation If there are separate sulfhydryl sites for urea and water transport inhibition, each is within 12 Å of the stilbene site The previous DCMBT experiments showed that the transport of water and the exchange of anions are modulated by a single protein or protein complex The present experiments show that there is a similar linkage between the transport of urea and the exchange of anions

Our conclusions are entirely consistent with the results Toon et al [6] obtained with another sulfhydryl reagent, DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) Reithmeier [7] has shown that DTNB inhibits anion exchange by noncovalent binding to the anion transport inhibitor site in normal cells In *N*-ethylmaleimide-treated cells *, Toon et al [6] found that DTNB not only inhibited anion exchange but also inhibited osmotic water flux by $29 \pm 1\%$ with a K_i of $2\,5 \pm 0\,3$ mM, similar to the K_i for anion exchange inhibition of $1\,6 \pm 0\,3$ mM Covalent binding of DIDS suppressed the DTNB inhibition of water flux to $12 \pm 2\%$ These observations are in agreement with those reached by Yoon et al [1] on the basis of DCMBT inhibition of water and anion transport Toon et al [6] also reported that DTNB inhibits methyl urea transport by about 50% with a K_i of $0\,6 \pm 0\,2$ mM This inhibition is entirely suppressed by covalent DIDS binding which led them to conclude that band 3 was the principal constituent of the aqueous channel through which methyl urea passed

DCMBT is a very different molecule than DTNB and the only property shared by both inhibitors is their ability to act as sulfhydryl reagents Yet, as Reithmeier [7] showed, the DTNB action on anion transport is a result of noncovalent DTNB binding to the stilbene site and does not involve any sulfhydryl reaction Both these reagents are also able to inhibit water transport, urea and/or methyl urea transport and anion exchange Taken together, these several observations provide strong support for the existence of a common element, presumably band 3 either alone or complexed with other proteins, which modulates the transport of water and of urea and the exchange of anions through a common channel

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* In contrast to the results in *N*-ethylmaleimide-treated cells, experiments carried out on cells that had not been treated with *N*-ethylmaleimide showed no effect of 5 mM DTNB on water permeability, in agreement with the observations of Macey [8] and Brahm [9] as discussed by Toon et al [6] Neither Brahm nor Macey studied the effect of DTNB on water permeability in *N*-ethylmaleimide-treated cells

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