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Modulation of Water and Urea Transport in Human Red Cells: Effects of pH and Phloretin

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Summary. It has previously been shown by Macey and Farmer (*Biochim. Biophys. Acta* **211**:104–106, 1970) that phloretin inhibits urea transport across the human red cell membrane yet has no effect on water transport. Jennings and Solomon (*J. Gen. Physiol.* **67**:381–397, 1976) have shown that there are separate lipid and protein binding sites for phloretin on the red cell membrane. We have now found that urea transport is inhibited by phloretin binding to the lipids with a K_I of 25 ± 8 μM in reasonable agreement with the K_D of 54 ± 5 μM for lipid binding. These experiments show that lipid/protein interactions can alter the conformational state of the urea transport protein. Phloretin binding to the protein site also modulates red cell urea transport, but the modulation is opposed by the specific stilbene anion transport inhibitor, DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonate), suggesting a linkage between the urea transport protein and band 3. Neither the lipid nor the protein phloretin binding site has any significant effect on water transport. Water transport is, however, inhibited by up to 30% in a pH-dependent manner by DIDS binding, which suggests that the DIDS/band 3 complex can modulate water transport.

Key Words red cell · permeability · water · urea · phloretin · pH

Introduction

When Macey and Farmer (1970) showed that 0.5 mM phloretin inhibited urea transport across the human red cell membrane by almost 80% but had no effect on water permeability, they concluded that water and urea were transported through different channels. Subsequently, Owen and Solomon (1972) generally confirmed these results but also found that the effect of phloretin is linearly dependent upon the ether/water partition coefficient of the solute, inhibiting permeation for hydrophilic solutes, and stimulating permeation for lipophilic solutes. They suggested that this bimodal effect might be ascribed to two separate sites of phloretin action, one on the protein and the other on the lipids.

Jennings and Solomon (1976) found two phloretin binding sites in the red cell membrane, a high affinity binding to the proteins (K_D = 1.5 ± 1 μM, pH 6.0) and a lower affinity binding to the membrane lipids (K_D = 54 ± 5 μM, pH 6.0). They suggested that the absence of any significant phloretin effect on water transport could result from the summation of two opposing processes, inhibition of a protein-mediated water transport channel coupled with stimulation of water transport through the lipids. Wieth et al. (1974) found that phloretin inhibits anion exchange with a K_I of 2-5 μM, consistent with inhibition at the protein binding site and Forman et al. (1982) reported that phloretin competitively inhibits binding of the stilbene anion exchange inhibitor, DBDS (4,4′-dibenzamido-2,2′-disulfonic stilbene), with K_I = 1.6 μM, presumably acting at the DBDS binding site (*see also* Fröhlich, Bain & Weimer, 1986; Fröhlich & Gunn, 1987).

We have used concentration and pH dependence to determine which sites are involved in the phloretin effects on urea and water transport. Effects at pH 9.0 and concentrations <1.5 μM were attributed to binding of phloretin to the anion transport inhibition site while effects at concentrations >5 μM and at pH 6.0 were attributed to lipids, as discussed below. We also blocked the anion inhibition site by covalent treatment with DIDS (4,4′-diisothiocyano-2,2′-disulfonic stilbene) and compared the results ± DIDS. Though there are small phloretin effects on osmotic water fluxes at pH 7.4, there are no appreciable phloretin effects on water transport at either pH 6.0 or 9.0. Phloretin binding at the lipid site almost completely inhibits urea permeability. Phloretin binding to the protein site modulates urea permeability, and these effects are generally opposed by DIDS. We also found that DIDS alone, in the absence of phloretin, inhibits water transport in a pH-dependent fashion, water transport inhibition rising from 11 ± 3% at pH 7.4 up to a maximum of 30% at pH 10.

Materials and Methods

Materials

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), Tris (hydroxymethylaminomethane), MES (2[N-morpholino] ethanesulfonic acid), CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), CHES (2-[N-cyclohexylamino]ethanesulfonic acid), and NEM (N-ethylmaleimide) were obtained from Sigma Chemical Co. (St. Louis, MO). Phloretin was supplied by K & K Labs (ICN Biomedicals, Plainview, NY) and DIDS by Molecular Probes (Junction City, OR). All other chemicals were of reagent grade and obtained from Fisher Scientific (Medford, MA). Outdated blood was kindly donated by the Children's Hospital (Boston, MA).

Methods

Blood outdated by no more than 3 days was washed three times with a buffer of the following composition, in mM: NaCl, 140; KCl, 4; $MgCl_2$, 0.5; HEPES, 30; pH 7.4, 300 ± 2 mOsm. Cells were resuspended to 25% hematocrit in this buffer with 12 mM NEM (±10 μM DIDS) for a 1-hr incubation at 37°C. Following incubation at pH 7.4, cells were washed an additional three times (with 25 volumes each time) with DIDS- and NEM-free buffers of identical osmolality and ionic content, at varying pH's from 6 to 10. HEPES was replaced with either 30 mM MES (pH 6, pK_a 6.1), Tris (pH 8.0, pK_a 8.3), CHES (pH 9.0, pK_a 9.3) or CAPS (pH 10.0, pK_a 10.4). Osmolalities were measured on a Fiske Model OS osmometer (Uxbridge, MA).

Following the third wash, samples for hematocrit (in triplicate) and hemoglobin determination (in quadruplicate) were taken. The measured cell volume at pH 7.4 (non-DIDS control), as determined by the hematocrit and the OD at 540 nm, was set equal to 100×10^{-12} cm³. All other cell volumes were then referenced to this value. It was assumed that cell surface area remained constant at 1.35×10^{-6} cm² when solving for the hydraulic conductivity, L_p (cm³ dyne^{-1} sec^{-1}).

Osmotic permeability was measured using the stopped-flow light-scattering apparatus of Terwilliger and Solomon (1981). Cells were resuspended a fourth time in the varying pH buffers to 2% hematocrit and were mixed with an equal volume of solution made hyperosmolal by the addition of NaCl to each buffer. The response of the cells to a 250-275 mOsm gradient after mixing was the basis for all determinations of L_p. After flow was stopped, scattered light intensity was sampled at 4-msec intervals over a period of 4 sec. This process was repeated 10 times for each experimental data point. Control sets of data were obtained in the same manner, except that the cell suspensions were mixed with solutions which were isosmolal with the cell suspension and at the same pH. All measurements were at 22–24°C.

In the course of our experiments at alkaline pH's we observed that DIDS treatment, by itself, causes a significant change in the volume of the red cell at the higher pH's, even though it only amounts to 1–3% at pH 7.4. We therefore routinely measured the initial and final red cell volumes which we entered into the computer program to determine L_p and the solute permeability coefficient, ω (mol dyne^{-1} sec^{-1}), for virtually all the experiments in the DIDS/pH series.

Urea permeability was determined by the minimum method of Sha'afi et al. (1970) and was based on the response of the cells to a 350–400 mOsm urea gradient, after mixing. The corrected values for cell volumes at each pH (±DIDS) was used to correct the fractional cell solids for each sample.

Phloretin was added to the 2% cell suspensions from either a 4 mM or a 100 mM stock solution in ethanol. Ethanol concentration was kept constant at 0.025% (vol/vol) by addition of pure ethanol to all controls and phloretin samples, as required, except for the experiments with 200 μM phloretin, in which the ethanol concentration was 0.05% and separate controls were run.

These experiments were carried out with NEM-treated cells. NEM reacts with five of the six sulfhydryl groups on band 3 and reaction of the remaining sulfhydryl group with the mercurial sulfhydryl reagent, *p*CMBS (*p*-chloromercuribenzene sulfonate), appears to control urea transport; we have determined the kinetics and the binding constant of this *p*CMBS/band 3 reaction in an extensive study (Toon & Solomon, 1986). There is another sulfhydryl group in NEM-treated red cells which appears to control water permeability, and we also characterized the binding constant and kinetics of this second reaction in the same paper. Though we have shown that NEM treatment does not affect the hydraulic conductivity of the cells (Toon et al., 1985), it affects the conformation of membrane transport proteins in other ways, as shown by Lauf, Adragna and Garay (1984). By using NEM-treated red cells we have the advantage of using a system whose permeability to urea and water we have studied in great detail, with the concomitant disadvantage of having made an alteration which could affect the affinities of the phloretin binding sites on the red cell membrane.

Results and Discussion

Phloretin Effects at pH 7.4

We initially studied the effects of phloretin at pH 7.4 and found, in two dose-response experiments on NEM-treated cells, that phloretin inhibition of urea permeability fitted the single-site inhibition curve in Fig. 1 (top) with $K_I = 18 \pm 1$ μM and maximum fractional inhibition, 0.96 ± 0.02. We compared the results in Fig. 1 with those in non-NEM-treated cells in the same experiment and found no change in the inhibition of urea permeability, $K_I = 25 \pm 4$ μM, maximum fractional inhibition, 1.07 ± 0.06. These results are in general agreement with those of Brahm (1983) who found the maximal fractional inhibition by phloretin to range between 98.3 and 99.4 at 500 μM phloretin. Brahm also found that incubation of red cells with 1 mM NEM caused a 10% increase in urea permeability, which is in reasonable agreement with our findings.

Macey and Farmer (1970) reported that phloretin had no effect on osmotic water permeability in normal red cells, and Brahm (1982) has ob-

tained the same result for diffusional permeability. We find that phloretin causes a small fractional increase in the hydraulic conductivity in normal cells (Fig. 1, bottom), amounting to 0.23 ± 0.03 with the stimulation constant, K_S = 39 ± 12 μM, which is in agreement with the increase in hydraulic conductivity of 30 ± 10% reported by Owen and Solomon (1972) at 250 μM phloretin.

In general, NEM has no effect on water permeability, as Brahm (1982) showed for diffusional permeability, and we found (Toon et al., 1985) for hydraulic conductivity. We were surprised, therefore, to learn that NEM treatment alters the effect of phloretin and turns the small *stimulation* we have observed in normal cells at pH 7.4 into a fractional *inhibition* of 0.18 ± 0.02 with a K_I of 53 ± 12 μM as shown in Fig. 1 (middle) for NEM-treated red cells. The effect, though small, is reproducible since the fractional inhibition in the other experiment in this series was 0.25 ± 0.03 (K_I = 51 ± 15 μM).

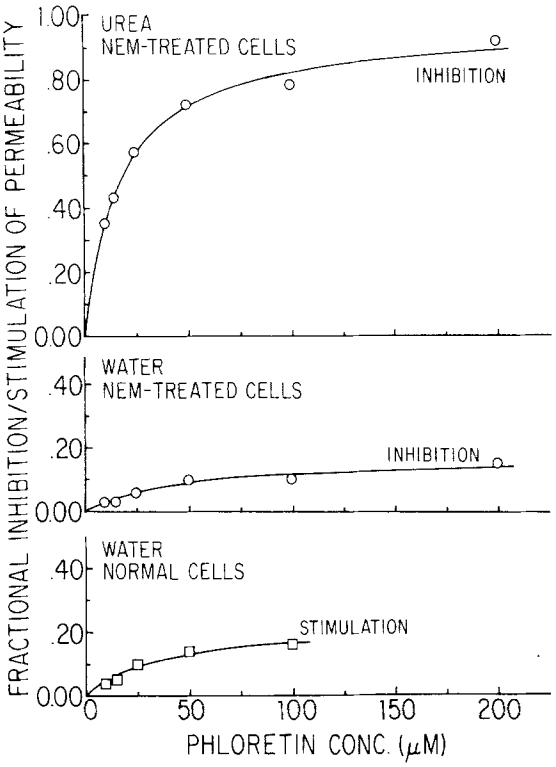

Fig. 1. Modulation of urea and water fluxes by phloretin at pH 7.4. *Top:* Phloretin inhibition of urea flux in NEM-treated red cells has been fitted by nonlinear least squares to a single-site binding curve with K_I = 18 ± 1 μM and maximum fractional inhibition of 0.96 ± 0.02. In a duplicate experiment, K_I = 21 ± 3 μM and maximum fractional inhibition = 1.04 ± 0.04. Data >0 and <10 μM is not shown in any of the experiments in Fig. 1. In NEM-treated cells, without phloretin, ω_{urea} = 20.6 × 10^{-15} mol dyne^{-1} sec (control cells, 17.6 × 10^{-15} mol dyne^{-1} sec^{-1}). *Middle:* The inhibition of osmotic water flux in NEM-treated red cells has been fitted to a single-site binding curve with K_I = 53 ± 12 μM and maximum fractional inhibition, 0.18 ± 0.02. In NEM-treated cells without phloretin, L_p = 1.45 × 10^{-11} cm^{-3} dyne^{-1} sec^{-1} (control, 1.53 × 10^{-11} cm^3 dyne^{-1} sec^{-1}). *Bottom:* In normal red cells, phloretin *stimulates* osmotic water fluxes and the data have been fitted to a single-site binding curve with K_S = 39 ± 12 μM and maximum fractional stimulation = 0.23 ± 0.03

Assignment of Phloretin Binding Sites to Membrane Proteins and Lipids

In order to determine the location of the binding sites, Jennings and Solomon (1976) made a Scatchard plot of phloretin binding to the separated red cell lipids at pH 6.0, where over 90% of the phloretin is uncharged HPhl, since phloretin is a weak acid with a pK_a of 7.3. They found only a single class of low affinity binding sites with K_D = 44 ± 3 μM and 5.2 ± 0.2 × 10^7 sites/equivalent cell, not significantly different from the low affinity sites in red cell ghosts with K_D = 54 ± 5 μM and 5.5 ± 0.5 × 10^7 sites/cell. The high affinity site was assigned to the proteins since there was no high affinity site in the lipids. Verkman and Solomon (1980) showed that HPhl binds to phosphatidylcholine vesicles 10^2 times more tightly than the charged form, (Phl^-), consistent with binding of HPhl to red cell lipids.

As stated in the Introduction, phloretin inhibits anion transport with a K_I of 2-5 μM (Wieth et al., 1974), consistent with the Jennings and Solomon (1976) high affinity site, of which there are 2.5 ± 1.5 × 10^6 sites/cell. In order to determine whether the anion transport inhibition was due to the charged form of phloretin, which predominates at pH 9.0, Forman et al. (1982) studied the pH dependence of the competitive inhibition of DBDS binding by phloretin. They computed the concentrations of Phl^- and HPhl and found a linear decrease of DBDS binding with increased [Phl^-], whereas there is no correlation with [HPhl], thus showing that Phl^- competes with DBDS binding to band 3 at the high affinity site.

Modulation of Urea Transport by Phloretin Binding to the Lipid Site

Figure 2 (bottom) shows that phloretin binding at pH 6.0 and at concentrations >5 μM, that is to the lipid site, inhibits urea permeability in NEM-treated red cells by 84% (50 μM point, experiment 6-1). At 25 μM phloretin, permeability was reduced by 53%

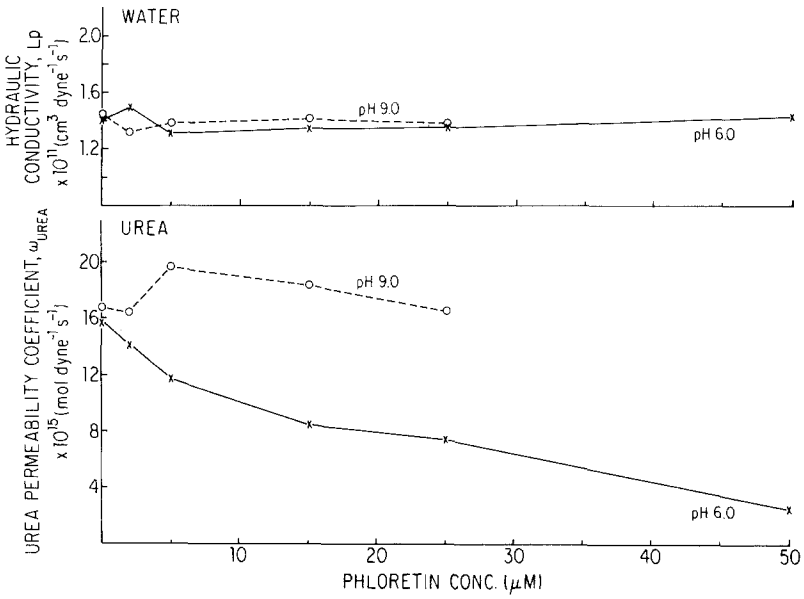

Fig. 2. Dependence of red cell water and urea permeability on phloretin concentration in the concentration range for *lipid* binding. There are three experiments at each pH in these series, 9-1 . . . 3 and 6-1 . . . 3 and each experiment covers the entire concentration range from 0.25 to 25 μM (except for 6-1 which went out to 50 μM). These data come from experiments 9-1 and 6-1 and include all the points except those from 0.25 to 1.5 μM phloretin. Similar results were obtained in the other four experiments

in experiment 6-1 which agrees reasonably with the results of two other experiments (49 and 38% inhibition, experiments 6-2 and 6-3). The data in experiment 6-1 can be fit by nonlinear least squares to a single-site binding curve with $K_I = 25 \pm 8$ μM, in reasonable agreement with Jennings and Solomon's K_D of 54 ± 5 μM for phloretin binding to red cell ghost lipids at pH 6.0 (normal cells). Phloretin inhibits the flux completely, as shown by the maximum fractional inhibition of 1.2 ± 0.2. As would be expected, there is little phloretin effect at pH 9.0 where the ionized form predominates. Brahm (1983) has shown that the permeability coefficient for urea diffusion (i.e., the nonsaturable component) amounts to ≈0.3% of the permeability coefficient for the saturable process and has concluded that urea flux is mediated by a membrane protein. Our experiments show that the conformation of the urea transport protein is modulated by lipid/protein interactions, a conclusion which is independent of whether the urea transport goes through an aqueous pore or by a specialized transport protein. This is another example of the sensitivity of red cell membrane proteins to lipid perturbations, such as the conformational change in band 3 induced by lipid soluble anesthetics which we had previously observed (Forman et al., 1985).

Specificity of DIDS Binding

In the experiments that follow, DIDS was used as a probe that is specific to band 3, and it is necessary to consider just how specific the binding is. At a DIDS concentration of 10 μM, Lepke et al. (1976) found 72% of H_2-DIDS in band 3, about 10–13% in glycophorin, 5% in a shoulder on band 3, and the rest distributed along the gel. Subsequently, when Ship et al. (1977) reacted red cells with 10 μM DIDS, they found that 90% of the radioactivity was in band 3, 5% in a shoulder tentatively identified as glycophorin and the remaining 5% distributed along the gel.

In order to relate these peaks to the transport processes we are studying, it is necessary to show that there are enough copies of the urea and water transport protein(s) to be visible. The number of pores required to transport water across the red cell has been calculated[1] to be 1.3×10^5 (Solomon et al., 1983) which is in fair agreement with the 5.5×10^5 band 3 dimers (and in better agreement with 2.8×10^5 tetramers). For urea transport, the number of copies required ranges between 0.6×10^5 and 10^7 depending on whether the flux proceeds via a pore or a transport protein. 10^5–10^7 copies are sufficient to make a clearly visible peak on the gel. The only other DIDS-binding protein with the requisite number of copies (Haest, 1982) which was found by both Lepke et al. (1976) and Ship et al. (1977) is glycophorin (though the number of copies and the iden-

[1] The equivalent pore radius of the human red cell has recently been revised to 6.5 Å (Solomon, 1986). Incorporation of this value in the computations for the number of aqueous pores per red cell, reduces the number of pores to 1.3×10^5 from the value of 2.7×10^5 previously given (Solomon et al., 1983). A similar calculation reduces the number of channels required for urea permeation from 1.3×10^5, as computed by Toon et al. (1985), to 0.6×10^5 per cell.

tity of the small shoulder found by Lepke et al. is not clear). However, sulfhydryl reagents significantly inhibit both urea and water fluxes and glycophorin contains no sulfhydryl groups (Marchesi, 1979). Although this argument does not rigorously exclude the possible contribution of glycophorin, it makes it likely that, for our purposes and under our conditions, DIDS binding serves as a specific probe for band 3.

Effect of Phloretin Interactions at the Protein Site on Urea Transport

In order to study the protein site, we have explored the effects of phloretin binding on water and urea flux at pH 9.0 in the concentration range 0 to 2 μM and have made comparisons with effects at pH 6.0. In this concentration range, the results at pH 6.0 include significant contributions of Phl^-, which is recruited to the protein binding site because the binding affinity is approximately equal to the phloretin concentration. The results in Fig. 3 (bottom) show unexpected and dramatic changes in the permeability of NEM-treated red cells at both pH's. Addition of 0.5 μM phloretin at pH 9.0 increases the permeability coefficient by 55% and 0.5 μM more brings it 10% lower than it was at the origin. In the presence of 10 μM covalently bound DIDS, this excursion is almost entirely suppressed. There are similar, but smaller, effects at pH 6.0. At first sight, these sharp maxima might be dismissed as experimental error but this is not the case. Our computer was not programmed to give the experimental errors in these experiments, but each point in Fig. 3 is the average of 10 runs under identical conditions, and we estimate the error in our measurements to be in the range of 5–10%. Outside of the 0–1.5 μM concentration range, the system is well behaved as is shown in the rest of the data from the same experiment in the bottom panel of Fig. 2 (pH 9.0; permeability scale smaller by a factor of two). The system behaves as if there were a resonance (or a series of resonances) of band width 0.1–0.25 μM, which is operative over the range of 0 to 1.5 μM phloretin. Each resonance peak appears to be opposed by DIDS, as an examination of the bottom panels in Fig. 3 shows. We do not understand the nature of these resonances, but they were present in all the experiments at pH 9.0, many at pH 6.0 and also in a series of six preliminary experiments at pH 7.4, including those shown in Fig. 1.

Phloretin has a large dipole moment and Andersen et al. (1976) have shown that absorption of the non-ionized form of phloretin at the surface of thin lipid membranes induces dipole potentials which dramatically increase the cation conductance and

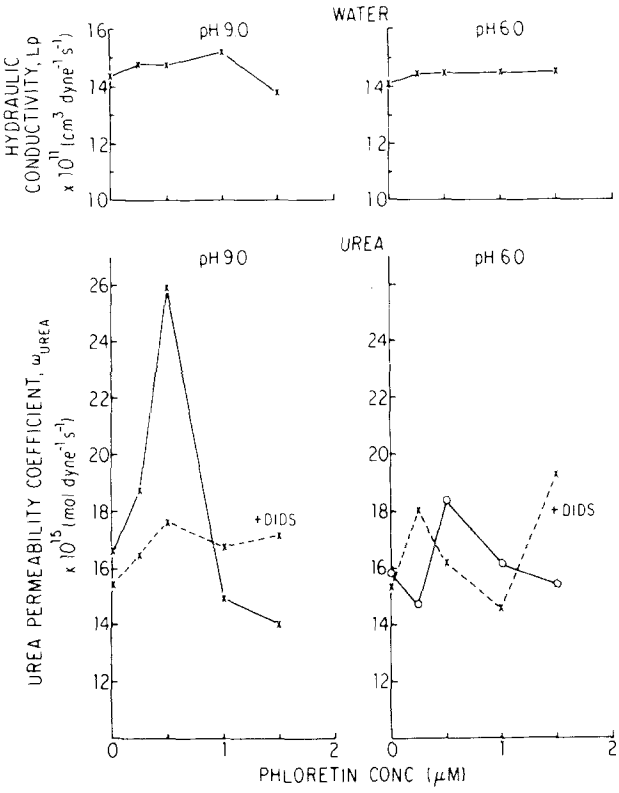

Fig. 3. Dependence of red cell water and urea permeability on phloretin concentration in the *protein* binding range. These data come from experiments 9-1 and 6-1 and include all the points between 0.25 and 1.5 μM phloretin

decrease the anion conductance of membranes treated with ion carriers, such as valinomycin. The effect becomes apparent at phloretin concentrations in the micromolar range, and it occurred to us that the phloretin dipole moment might be a factor in the excursions in urea permeability that we have observed in the micromolar range. However, there is no evidence for any sharp peaks or valleys in the concentration dependence of ion conductance; instead the changes in conductance are smooth functions of phloretin concentration over the range from 10^{-6} to 10^{-4} M both in Andersen et al.'s (1976) study and a subsequent one by Melnik et al. (1977). Fröhlich and Gunn (1987) have also concluded, on the basis of their studies of competition with Cl^- transport, that the phloretin effect on anion transport is not mediated by facial dipole potentials.

What can be concluded in the absence of a physical mechanism to account for the sharp changes in urea permeability in the micromolar range of phloretin concentration? First, it seems safe to conclude that these are protein effects because Jennings and Solomon (1976) have shown that there is a high affinity protein binding site at

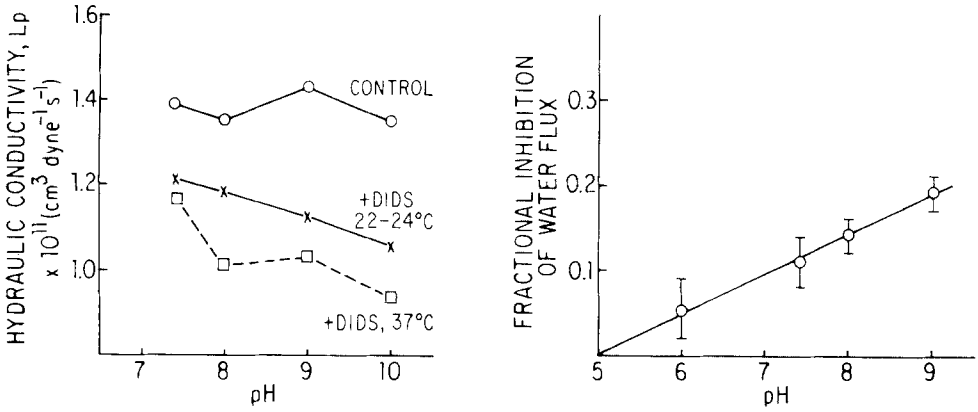

Fig. 4. DIDS inhibition of water permeability as a function of pH. In these experiments, the cells were initially treated with DIDS for 1 hr at 37°C at pH 7.4, as described under Methods, and then washed three times which removed all unreacted DIDS. This initial treatment was followed by a second incubation for 1 hr at the pH given in Fig. 4. The experimental conditions for Jennings and Passow's (1979) initial incubation (12 μM H_2-DIDS, pH 7.0–7.4, 20–60 min, 37°C, 10% hematocrit) were similar to ours (10 μM DIDS, pH 7.4, 60 min, 37°C, 25% hematocrit), and so we assume that their observations are applicable to our system. *Left:* The control data were obtained in the absence of DIDS at 22–24°C. In the two lower sets of data, the first incubation with 10 μM DIDS was carried out at 37°C as described under Methods and the second incubation was carried out at the temperature and pH shown in the Figure. *Right:* This shows the averaged data from all the pH series (with the second incubation at 22–24°C) which includes the following number of experiments: pH 6.0 (3); pH 7.4 (6); pH 8.0 (3); pH 9.0 (6). The line has been drawn by error-weighted least squares and has a slope of 0.046 ± 0.002 (pH unit)$^{-1}$ and a correlation coefficient of 0.997

this concentration at pH 6.0 and because of the determinations of Wieth et al. (1974), Forman et al. (1982), Gunn, Gilbert and Fröhlich (1987) and Fröhlich and Gunn (1987) of phloretin inhibition of anion transport and DBDS (or other stilbene anion exchange inhibitors) binding in this concentration range. Furthermore, the observation that the phloretin modulation of urea permeability is consistently opposed by 10 μM DIDS suggests that band 3 is involved in protein-mediated urea transport.

Comparison of the urea transport effects in Figs. 2 and 3 shows that there is a sharp difference between protein and lipid-mediated effects, consistent with Owen and Solomon's (1972) explanation for the bimodal effect of phloretin on hydrophilic and lipophilic solutes.

Phloretin Effects on Water Permeability

As the top of Fig. 3 shows, there is little or no effect on water permeability of NEM-treated red cells in the concentration range, 0–1.5 μM. The data in the top panel of Fig. 2 show that the lipid site exercises little effect on water permeability in NEM-treated red cells. The effects at pH 7.4 shown in Fig. 1 are small enough that they are often obscured at pH 6.0. All the data on osmotic water permeability in NEM-treated red cells argue persuasively against Jennings and Solomon's (1976) suggestion that phloretin exercises opposing effects on two routes of water permeation. Instead, our data support the findings in the literature that phloretin has little, if any effect on water permeability (Macey & Farmer, 1970; Owen & Solomon, 1972; Brahm, 1982) though it has significant and important effects on urea permeability, as Macey and Farmer (1970) first pointed out.

DIDS Effects on Water Permeability

In the course of these studies we carried out a number of control experiments in which L_p was measured in NEM-treated red cells that had been treated with 10 μM DIDS at pH 7.4 followed by incubation at pH's from 6.0 to 10.0. Unexpectedly, we found that DIDS had a significant effect on L_p, particularly at the higher pH's, and we therefore began a systematic study. Brahm (1982) had shown that DIDS has no effect on the diffusional permeability of water across the normal red cell membrane. However, when we looked closely at the effect of DIDS on L_p at pH 7.4 in NEM-treated cells, we found that DIDS inhibits osmotic water flux significantly, lowering it by 11 ± 3% ($P < 0.001$, t test) in six experiments at pH 7.4, 22–24°C. As pH increases from 7.4 to 10 the inhibition of water increases steadily with pH, reaching a maximum of about 30% at pH 10.0 at 37°C, as shown in the left panel of Fig. 4.

The initial DIDS binding site under our condi-

tions of DIDS binding is located in the 17-kDa membrane-bound fragment of band 3 between the trypsin and the chymotrypsin cuts. As Jennings and Passow (1979) showed, there is a second H_2-DIDS binding site located in the 35-kDa fragment produced when chymotrypsin reacts at the outside of the membrane. The initial H_2-DIDS binding reaction causes a sizable fraction (10%–35%) of the second isothiocyano groups of H_2-DIDS to react also with the 35-kDa fragment, cross-linking the two fragments. When all the unbound H_2-DIDS has been washed off and there is a second incubation at pH 9.5, Jennings and Passow showed that the second isothiocyano moiety of the bifunctional reagent, H_2-DIDS, binds to the 35-kDa fragment, so that each DIDS molecule is tethered to band 3 by two isothiocyano bonds, one on the 17-kDa fragment, and one on the 35-kDa fragment. Jennings and Passow's experiments were carried out with H_2DIDS which has no double bond between the two aromatic rings and hence is more flexible than DIDS, a difference which may well have important conformational consequences. If DIDS can also cross-link to a second site as the pH is raised, the DIDS molecule will be more highly constrained to a specific locus. Either steric hindrance by the constrained DIDS molecule, or DIDS-induced conformational changes in band 3 could provide the physical barrier that impedes water flux across the membrane.

Mechanism of Urea and Water Transport Across the Cell Membrane

The experiments that have been reported provide further information about the mechanism of urea and water transport across the human red cell membrane, which can be best interpreted in terms of a specific model for these transport processes. There is now a great deal of evidence that there are interactions between water and urea transport (Chasan & Solomon, 1985) and that ligands which interact with band 3, such as phloretin and *p*CMBS, can modulate these fluxes (Toon & Solomon, 1986). Our observations of the antithetical effects of phloretin and DIDS on urea transport shown in Fig. 3 provide further evidence of a band 3/urea transport link.

It also is clear that transport of water and urea can be modulated by more than a single protein, as can be concluded from experiments in a number of different laboratories, including those of Macey (Macey & Farmer, 1970; Macey, 1984) Brahm (1983), Levitt (Mayrand & Levitt, 1983), Dix (Dix et al., 1985) and our own (Toon et al., 1985; Toon & Solomon, 1986). A model which accommodates all of these observations is a transport assembly, containing more than one protein, with an aqueous pore as a centerpiece and inhibitory sites on adjacent proteins which modulate the transport by allosteric means.

We had suggested (Solomon et al., 1983) that the aqueous channel runs through band 3 and also serves as the conduit for anion exchange. One important obstacle to this suggestion is the fact that DIDS, which completely inhibits anion exchange, had hitherto been found to have no effect on water fluxes. We had accommodated this problem by suggesting that the conformational change which follows DIDS binding (Macara, Kuo & Cantley, 1983; Verkman, Dix & Solomon, 1983) causes DIDS to swing out of the aqueous channel and be internalized in the protein. Our present observation that DIDS can inhibit water transport by 30% at high pH shows that there is a relationship between DIDS binding to the red cell membrane and water transport and is consistent with the presence of an aqueous channel in band 3.

Conclusions

In addition to the evidence relating to the aqueous channel, discussed above, the following conclusions may be drawn from the data reported in this paper:

1) It is possible to modulate urea transport under conditions which have little or no effect on water transport, as shown in Fig. 2. These experiments provide a detailed confirmation of Macey and Farmer's (1970) original observation.

2) Lipid/protein interactions can specifically modify the conformation of the protein which modulates urea transport.

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