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Interactions Between Anion Exchange and Other Membrane Proteins in Rabbit Kidney Medullary Collecting Duct Cells

Agnes Janoshazi, Julian L. Seifter, and A.K. Solomon

Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115, and Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115

Summary. In separated outer medullary collecting duct (MCD) cells, the time course of binding of the fluorescent stilbene anion exchange inhibitor, DBDS (4,4′-dibenzamido-2,2′-stilbene disulfonate), to the MCD cell analog of band 3, the red blood cell (rbc) anion exchange protein, can be measured by the stopped-flow method and the reaction time constant, τ_{DBDS}, can be used to report on the conformational state of the band 3 analog. In order to validate the method we have now shown that the $ID_{50,DBDS,MCD}$ (0.5 ± 0.1 μM) for the H_2-DIDS (4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate) inhibition of τ_{DBDS} is in agreement with the $ID_{50,Cl^-,MCD}$ (0.94 ± 0.07 μM) for H_2-DIDS inhibition of MCD cell Cl^- flux, thus relating τ_{DBDS} directly to anion exchange. The specific cardiac glycoside cation transport inhibitor, ouabain, not only modulates DBDS binding kinetics, but also increases the time constant for Cl^- exchange by a factor of two, from τ_{Cl^-} = 0.30 ± 0.02 sec to 0.56 ± 0.06 sec (30 mM $NaHCO_3$). The $ID_{50,DBDS,MCD}$ for the ouabain effect on DBDS binding kinetics is 0.003 ± 0.001 μM, so that binding is about an order of magnitude tighter than that for inhibition of rbc K^+ flux ($K_{I,K^+,rbc}$ = 0.017 μM). These experiments indicate that the Na^+,K^+-ATPase, required to maintain cation gradients across the MCD cell membrane, is close enough to the band 3 analog that conformational information can be exchanged. Cytochalasin E (CE), which binds to the spectrin/actin complex in rbc and other cells, modulates DBDS binding kinetics with a physiological $ID_{50,DBDS,MCD}$ (0.076 ± 0.005 μM); 2 μM CE also more than doubles the Cl^- exchange time constant from 0.20 ± 0.04 sec to 0.50 ± 0.08 sec (30 mM $NaHCO_3$). These experiments indicate that conformational information can also be exchanged between the MCD cell band 3 analog and the MCD cell cytoskeleton.

Key Words kidney · medullary collecting duct · anion exchange protein · band 3 · cytoskeleton · stilbene anion exchange inhibitors · DBDS · Na^+,K^+-ATPase · ouabain

Introduction

In the medullary collecting duct of the kidney, Cl^-/HCO_3^- exchange, on the basolateral face of the cell, is essential to the urinary acidification process. Stone et al. (1983) showed that the specific stilbene anion exchange inhibitor, SITS (4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene), inhibits bicarbonate transport in this tubule segment, and Zeidel, Silva and Seifter (1986*c*) found that the Cl^-/HCO_3^- exchange in separated medullary collecting duct (MCD) cells was inhibited by another stilbene anion exchange inhibitor, DIDS (4,4′-diisothiocyano-2,2′-disulfonic stilbene), with an $ID_{50,Cl^-,MCD}$ of 0.5 μM, similar to the $ID_{50,Cl^-,rbc}$ of 0.04–1.2 μM for DIDS inhibition of red cell anion exchange (Cabantchik, Knauf & Rothstein, 1978; Knauf, 1979). Schuster, Bonsib and Jennings (1986) and Drenckhahn et al. (1985) have shown that there is a protein on the basolateral face of MCD cells that reacts immunochemically with antibodies to both the membrane bound and cytoplasmic fragments of human red cell band 3. In the human kidney, Wagner et al. (1987) have studied the membrane-spanning domain of the analog of band 3 in the intercalated cells of the collecting duct and report no immunological differences from the analogous domain of human red cell band 3.

In a previous study on the anion exchange protein in separated rabbit kidney outer MCD cells, Janoshazi et al. (1988) have used a fluorescent stilbene anion exchange inhibitor, DBDS (4,4′-dibenzamido-2,2′-disulfonic stilbene), to characterize the physical chemical properties of the anion exchange protein. We have found that the binding and the kinetics of stilbene reactions with the MCD cell anion exchange protein closely resemble those of human red cell band 3 and have concluded that the molecular structure is highly conserved.

DBDS binding to MCD cells requires at least two steps: a rapid bimolecular association, too fast for us to resolve; and a subsequent conformational change, whose kinetics can be determined from the characteristic reaction time, τ_{DBDS}, obtained by the stopped-flow rapid reaction method. The dependence of τ_{DBDS} on DBDS concentration can then be

used to determine both the equilibrium constant of the bimolecular association and the forward velocity of the conformational change; these constants can be used, in turn, to report on the conformational state of band 3. Lukacovic et al. (1984) previously used τ_{DBDS} to report on conformational changes in red cell band 3 caused by interactions with the mercurial sulfhydryl reagent, pCMBS (*p*-chloromercuribenzenesulfonate). Janoshazi and Solomon (1989) have shown that another stilbene anion exchange inhibitor, H_2-DIDS (4,4′-diisothiocyano-2,2′-dihydrostilbene disulfonate), decreases the rate constant for 3 μM DBDS binding ($\tau^{-1}_{DBDS,rbc}$) to red cell band 3 from 6.0 sec^{-1} to 0 sec^{-1} and the inhibition $ID_{50,DBDS,rbc} = 0.3 \pm 0.1$ μM. Since this ID_{50} is very close to the $ID_{50,Cl^-,rbc}$ of 0.47 ± 0.04 μM, that we have determined for H_2-DIDS inhibition of red cell Cl^- exchange, we concluded that changes in τ_{DBDS} provide an accurate reflection of changes in the DBDS binding site on band 3. Therefore, Janoshazi and Solomon (1989) used τ_{DBDS} to report on red cell band 3 conformation changes in studies on interactions between band 3 and other integral membrane proteins. We have found that a similar close relationship exists between H_2-DIDS modulation of τ_{DBDS} and τ_{Cl^-} in MCD cells and have concluded that changes in τ_{DBDS} also provide a satisfactory report of changes in the conformation of the band 3 analog in MCD cells.

We have therefore used τ_{DBDS} to study the relationship between the anion transport protein and other membrane components. The cardiac glycoside cation transport inhibitor, ouabain, stimulates Cl^- flux in MCD cells, which indicates that the anion transport protein in MCD cells is close enough to the Na^+,K^+-ATPase that configurational information can be passed between them. We have also found that cytochalasin E, which binds to a specific site on the spectrin/actin/band 4.1 complex of the red cell cytoskeleton, causes τ_{DBDS} in MCD cells to increase by more than a factor of two. Since Drenckhahn et al. (1985) have shown that antibodies to red cell spectrin react immunochemically with MCD cells, these experiments with cytochalasin E suggest that the MCD cell anion transport protein is closely linked to the MCD cytoskeleton.

Materials and Methods

Materials

DBDS, synthesized by the method of Kotaki, Naoi and Yagi (1971), was kindly provided through the courtesy of Dr. James A. Dix. H_2-DIDS and SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium) were obtained from Molecular Probes (Eugene, OR). DIDS was obtained from Pierce Chemical (Rockford, IL). Ouabain and digitoxigenin were obtained from Sigma (St. Louis, MO). All other chemicals, obtained from Fisher (Fairlawn, NJ) were reagent grade.

Preparation of MCD Cells

Highly purified suspensions of outer MCD cells were prepared from New Zealand white rabbits as described by Zeidel, Silva and Seifter (1986*b*) and Zeidel et al. (1986*c*). The inner stripe of the outer medulla was excised, digested with protease, and centrifuged on continuous (5–46%) Ficoll gradients at 2300 × *g* for 45 min. A homogeneous population of cells with morphologic and functional characteristics of outer MCD cells were located in the upper two fractions. These cells have a high glycolytic capacity, are rich in carbonic anhydrase, and use H^+-ATPase and Cl^-/HCO_3^- exchange to maintain intracellular pH (Zeidel et al., 1986*b*,*c*). Suspensions were either freshly prepared or had been stored at −20°C until use. The suspension was washed 2–3 times with PBS buffer of the following composition, in mM: NaCl, 150; Na_2HPO_4, pH 7.4, 300 ± 5 mOsm and kept at room temperature for about an hour prior to the experiments. To reverse any aggregation of the frozen MCD cells, the first wash contained 10 mM EDTA, which was omitted in the subsequent washes. The cells were vortexed for 5 min to disperse the suspension. Since the properties of these cells varied greatly from preparation to preparation, the results of our experiments are always expressed relative to a control in the same preparation.

For the protein determinations, the MCD cell suspension was sonicated for 5 min in a Branson bath sonicator (Model 12, Shelton, CN) and the protein content measured by the Bradford (1976) method.

Kinetics of DBDS Binding to MCD Cells

For the binding kinetics experiments, the MCD cell solution in PBS was mixed with an equal volume of 1–8 μM DBDS, in the same buffer, to provide an MCD cell concentration of 0.01–0.1 mg protein ml^{-1} in the stopped-flow apparatus (Model SFA-11, Hi-Tech Scientific, Salisbury, Wiltshire, England). This apparatus consisted of two hand-driven syringes, which rapidly drove 0.2 ml of solution through a mixing chamber into an observation cuvette in the light path of our single-beam spectrofluorimeter. The path length from the mixing chamber to the observation port, together with the time required to replace the solution in the observation chamber, gave an effective dead time of 40 msec, to which must be added an additional 10 msec for delays in the trigger circuit. DBDS binding was measured by fluorescence enhancement, excited at 350 nm (resolution 4 nm), path-length 10 mm, and monitored at 427 nm (resolution 8 nm, path-length 2 mm). The spectrofluorimeter output was interfaced on-line to our computer, whose data acquisition board (Lab Master, Scientific Solutions, Solon, OH) provided a 30 kHz conversion. 1000 data points, spaced at 4 msec, were collected for each run and 5–10 runs were averaged for each data point. The data were usually fitted by nonlinear least squares to a single exponential, except that, occasionally, there was a small, but measurable, slow component of unknown cause in the DBDS binding kinetics. When this second component was detectable, the time constant was 3–5 times higher than the fast component and the data were fitted to two exponentials, which were then deconvoluted so that the fast time constant, which is the one we use, could be determined accurately.

Fluorescence Method of Measuring Cl^- Exchange

Fresh MCD cells were loaded with the water-soluble dye, SPQ, which accurately reports intracellular Cl^- concentration (Illsley & Verkman, 1987). They were loaded with SPQ by incubation with 10 mM SPQ for 0.5–1 hr at 37°C in PBS buffer + 5 mM D-glucose, pH 7.4. Extracellular SPQ was removed by three washes in PBS, pH 7.4 and very low centrifugation speeds (<5000 × *g*). To minimize SPQ diffusion out of the cells, they were kept at 4°C in PBS (without glucose) until used. The stopped-flow experiments were carried out as described for DBDS binding. The SPQ-loaded MCD cells were mixed with equal volumes of buffer containing in mM: 50 (or 60) $NaHCO_3$; 110 (or 92) Na gluconate, bubbled with 5% CO_2, pH 7.4–7.9. The decrease in cell Cl^- caused a rapid increase in SPQ fluorescence (excited at 350 nm, resolution 12 nm; measured at 427 nm, resolution 8 nm), which was recorded at a data acquisition rate of 4 msec/point. The data from a large number of runs was averaged and corrected for the slow dye leakage that persists in MCD cells, which was measured independently for each set of measurements. The corrected rates of the Cl^- flux were fitted to a single exponential by nonlinear least squares, convoluted with a function describing Stern-Volmer quenching (K = 118.0 M^{-1}), by the method given by Illsley and Verkman (1987). Since the properties of these cells varied greatly from preparation to preparation, the results of our experiments are always expressed relative to a control in the same preparation.

Results

Theory

We have modeled the first steps of the DBDS binding reaction as a bimolecular association, too fast for us to measure, followed by a conformational change, according to Eq. (1), following the model of Verkman, Dix and Solomon (1983) in the red cell,

$$\text{DBDS} + \text{band 3} \overset{K_1}{\longleftrightarrow} \text{DBDS-band 3} \overset{k_2}{\underset{k_{-2}}{\longleftrightarrow}} \text{DBDS-band 3}^* \quad (1)$$

in which * denotes the DBDS/band 3 complex following its conformation change and K_1 is the dissociation constant for the bimolecular reaction. The kinetics of the reaction are given by Czerlinski (1966) in an equation, which can be simplified to:

$$\tau_{DBDS} = 1/k_2 + K_1/(k_2[\text{DBDS}]_{total}) \quad (2)$$

provided $k_2 \gg k_{-2}$ and $[\text{DBDS}]_{total} \gg [\text{protein}]_{total}$. The DBDS concentration in our suspensions (after mixing) was 0.5–4 μM, which is large compared to the number of DBDS sites (1.4 nmol DBDS sites/mg total cell protein) in suspensions, which contained from 0.01–0.1 mg protein ml^{-1}. In our previous study on DBDS binding to MCD cells (Janoshazi et al., 1988), it was reported that $k_{-2} = 0.5 \pm 0.3$ sec^{-1}, much smaller than the value for $k_2 = 9.9 \pm 0.8$ sec^{-1} in this study. A linear fit of τ_{DBDS} to $[\text{DBDS}]^{-1}$ gives k_2 = 1/intercept and K_1 = slope/intercept and, as will be shown in Fig. 5, the points fall on a straight line.

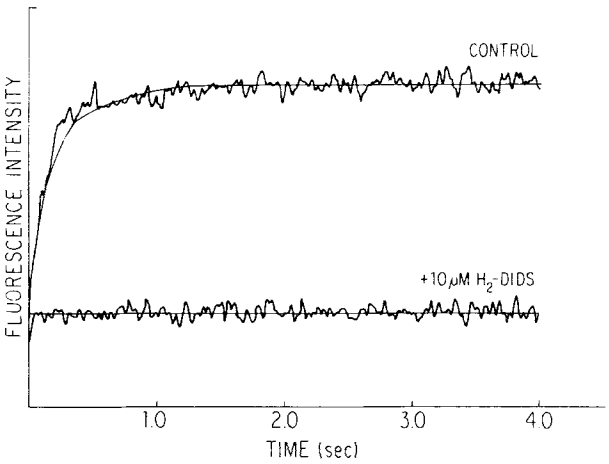

Fig. 1. Effect of 10 μM H_2-DIDs on DBDS time course. Frozen MCD cells (0.1 mg protein/ml) were incubated for 1–3 min with 10 μM H_2-DIDS at 20–23°C in PBS, pH 7.4. They were mixed with an equal volume of 8 μM DBDS (in PBS) in the stopped-flow apparatus. The control curve was fitted to two exponentials by nonlinear least squares with $\tau_{fast} = 0.14 \pm 0.01$ sec and $\tau_{slow} = 0.42 \pm 0.08$ sec. Treatment with 10 μM H_2-DIDS caused both exponentials to disappear

Effect of H_2-DIDS on τ_{DBDS}

Since H_2-DIDS, which is a specific high affinity inhibitor of red cell Cl^- exchange, with $ID_{50,Cl^-,rbc} = 0.3$ μM (Knauf, 1979), is analogous to DIDS, which inhibits MCD cell Cl^-/HCO_3^- exchange in MCD cells (Zeidel et al., 1986*b*), we expected H_2-DIDS also to inhibit the DBDS reaction with MCD cells. These experiments were made in pooled MCD cells, which had been preserved by freezing in order to obtain a homogeneous population of cells large enough for our measurements. Although these pooled cells are unsealed and no longer maintain their normal Cl^- permeability barrier, they retain the ability to bind DBDS, which may be presumed to bind to the extracellular face of MCD cells, as it has been shown to do in the red cell (Verkman et al., 1983). As previously pointed out by Janoshazi et al. (1988), and as discussed in Materials and Methods, the time course of DBDS fluorescence enhancement in MCD cells (Fig. 1) consists of a fast exponential, which characterizes the conformational change, as well as an occasional slow exponential, whose role we do not understand. Figure 1

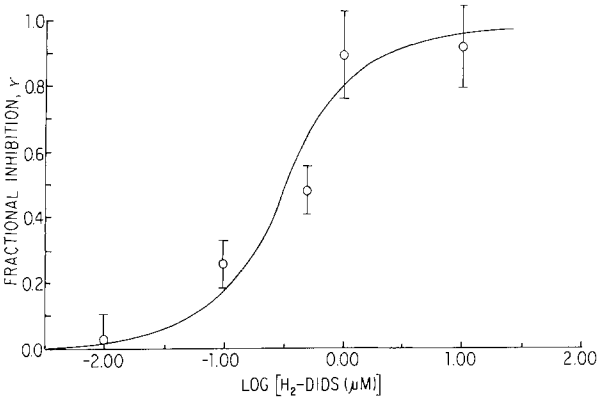

Fig. 2. Dose-response curve for H_2-DIDS effect on DBDS binding kinetics. Frozen MCD cells were incubated in PBS (4 μM DBDS, final concentration) with varying concentrations of H_2-DIDS and measured as in Fig. 1. The time courses were fitted to a double exponential by nonlinear least squares and the time constant of the fast component is used. $ID_{50,DBDS,MCD}$ =0.5 ± 0.1 μM. ν was set at 1.0 for $\tau^{-1} = 0$ sec^{-1} and ν =0 was set for τ^{-1} = 8.0 sec^{-1}. Average of two experiments in each of which an experimental point is the average of five measurements

also shows that H_2-DIDS reduces the amplitude of both exponentials to zero, consistent with the conclusion that the fluorescent enhancement reflects DBDS binding to MCD cell band 3. However, it might still be possible for the MCD cells to have additional nonspecific DBDS adsorption sites that could be saturated with H_2-DIDS.

Janoshazi et al. (1988) have shown that the physical chemical characteristics of the DBDS binding site in the MCD cell anion exchange protein are essentially the same as those for red cell band 3. If the fluorescence enhancement following DBDS binding to the MCD cell is to be attributed to interactions with the Cl^- exchange site, the $ID_{50,DBDS,MCD}$ for H_2-DIDS inhibition of DBDS fluorescence enhancement in MCD cells should be closely similar to that for H_2-DIDS inhibition of red cell Cl^- exchange. We determined the $ID_{50,DBDS,MCD}$ by plotting the time constant of the fast fluorescence enhancement process as a function of [H_2-DIDS] and found, as shown in Fig. 2, that $ID_{50,DBDS,MCD}$ = 0.5 ± 0.1 μM, in excellent agreement with the ID_{50} of 0.3 μM for inhibition of Cl^- flux in red cells. The data have been fit to a simple dose-response curve and the data are consistent with a single reacting species, which indicates that there are no nonspecific DBDS binding sites which affect our measurements of τ_{DBDS}.

Studies of Cl^- Exchange and Its Inhibition with H_2-DIDS

The fluorimetric method of measuring cell Cl^- with the dye, SPQ, makes it possible to study Cl^-/HCO_3^- exchange in the stopped-flow apparatus at 20–23°C. These experiments have to be carried out on freshly separated MCD cells, which have retained their Cl^- permeability barrier. Figure 3 shows that these cells are characterized by a Cl^-/HCO_3^- exchange with $\tau_{Cl^-,MCD}$ = 0.13 ± 0.02 sec, for exchange in 25 mM HCO_3^-. The observation that the flux is inhibited by 10 μM H_2-DIDS indicates that this flux is to be attributed to the anion exchange protein.

We next characterized the H_2-DIDS inhibition by obtaining the dose-response curve shown in Fig. 4, for which the $ID_{50,Cl^-,MCD}$ = 0.94 ± 0.07 μM. The agreement between the $ID_{50,Cl^-,MCD}$ in these direct experiments on Cl^- flux and $ID_{50,DBDS,MCD}$ = 0.5 ± 0.1 μM is very good[1], particularly because these Cl^- flux experiments used freshly prepared MCD cells with intact anion permeability barriers, while those on τ_{DBDS} used pooled frozen cells. These results provide very strong support for the conclusion that the time course of DBDS fluorescence enhancement represents reactions with a single MCD membrane component, the anion exchange protein.

The MCD cell $\tau_{Cl^-,MCD}$ of 0.13 sec (Fig. 3) is of the same order of magnitude as $\tau_{Cl^-,rbc} \approx$ 0.1–0.7 sec (depending upon HCO_3^- concentration; Janoshazi & Solomon, 1989). The time constant for Cl^- efflux from red cell ghosts into 20–25 mM HCO_3^- is ≈ 1 sec (Illsley & Verkman, 1987; Janoshazi & Solomon, 1989). These time constants for anion exchange are much faster than the rate of change of cellular pH, measured by Zeidel et al. (1986*c*) using an intracellular pH dye, which is also inhibited by DIDS with $ID_{50,pH,MCD}$ = 0.5 μM. The DIDS inhibition shows that the pH changes depend upon Cl^-/HCO_3^- exchange, but Zeidel et al.'s time constant of 1.5–4.5 min is very much slower than ours. The apparent discrepancy between these two time constants, both clearly dependent on anion exchange across the membrane, may be ascribed to a time delay be-

[1] We determine ID_{50} in our experiments, rather than K_I, which is the correct parameter to use in relating inhibition processes in different experiments. As discussed in footnote 1 of the preceding paper (Janoshazi & Solomon, 1989), these two measures of inhibition are not interchangeable. The stilbene inhibitors and Cl^- are all mutually competitive inhibitors, so comparison among ID_{50}'s needs the correction terms given in that footnote. Using these corrections, $(ID_{50})_{DBDS\ expt}$ should be 1.2 times greater than $(ID_{50})_{SPQ\ expt}$. Our value of $ID_{50,DBDS,MCD}$ = 0.5 ± 0.1 μM, as compared to $ID_{50,Cl^-,MCD}$ = 0.94 ± 0.07 μM, which is reasonable agreement in view of the uncertainties.

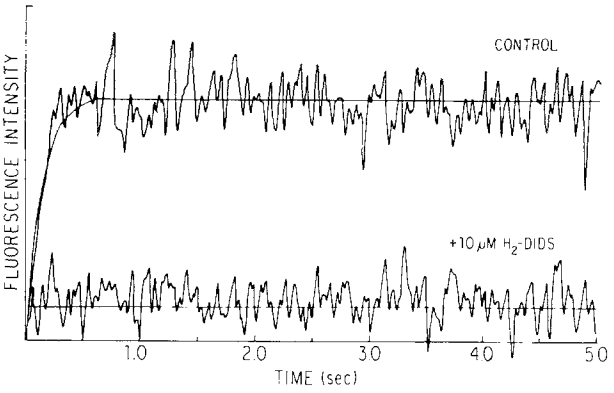

Fig. 3. Inhibition of Cl^- exchange by H_2-DIDS. Fresh MCD cells (0.1–0.2 mg protein/ml) were incubated with 10 mM SPQ for 30 min at 37°C in PBS, washed twice in PBS, pH 7.4, resuspended in PBS, pH 7.4, and incubated as in Fig. 1. They were mixed with an equal volume of 50 mM $NaHCO_3$ and 110 mM Na gluconate in the stopped-flow apparatus. Data from one experiment, typical of three. $\tau_{Cl^-} = 0.13 \pm 0.02$ sec. Inhibition with 10 μM H_2-DIDS was measured after incubation for 1–2 min at 20–23°C

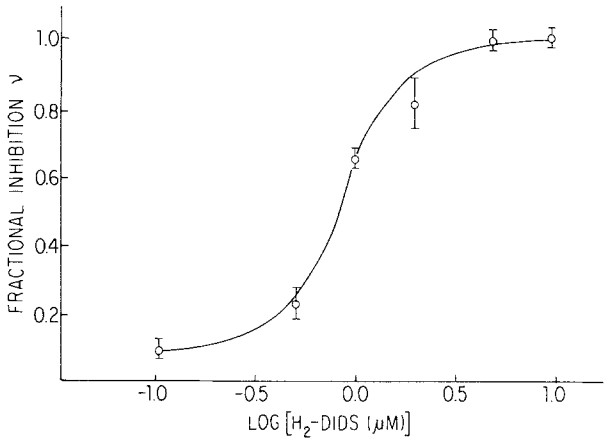

Fig. 4. Dose-response curve for H_2-DIDS inhibition of Cl^- exchange. Fresh MCD cells (0.1–0.2 mg protein/ml) were incubated with 10 mM SPQ for 30 min at 37°C in PBS, washed twice in PBS, pH 7.4, resuspended in PBS, pH 7.4, and incubated as in Fig. 1. They were mixed with an equal volume of 50 mM $NaHCO_3$ and 110 mM Na gluconate in the stopped-flow apparatus. Each experimental point was obtained on a separate MCD cell preparation and τ_{Cl^-} was determined from a single exponential fitted by least squares and normalized by dividing by $\tau_{Cl^-,control}$ (the τ_{Cl^-} in the absence of H_2-DIDS). ν was set at 1 for normalized $\tau_{Cl^-} = 0$ and set at 0 for normalized $\tau_{Cl^-} = 1$. $ID_{50,Cl^-,MCD} = 0.94 \pm 0.07$ μM

tween the time when the ions cross the membrane, which we measure directly, and the subsequent change in pH, which Zeidel et al.'s (1986*c*) measurements probe. Such a delay could arise if there were an additional diffusion barrier, or compartment, within the cell, which the anion had to traverse before reaching the alkalinization site, as if, for example, the organelles or some other compartment within the cell offered an impediment to Cl^- and/or HCO_3^- diffusion. If so, the intracellular Cl^- or HCO_3^- would not all be contained in a single homogeneous compartment and the system would be characterized by more than a single time constant.

Effect of Ouabain on DBDS Binding Kinetics and Cl^- Exchange in MCD Cells

In view of the resemblance of the MCD cell anion exchange protein to red cell band 3, and our finding (Janoshazi & Solomon, 1989) that ouabain modulates both DBDS binding kinetics and Cl^- exchange in red cells, we were anxious to see if the likeness to MCD cells extended to the ouabain interaction. Zeidel et al. (1986*a*) found that ouabain inhibited O_2 consumption in separated rabbit outer MCD cells by 8.7 ± 2.7% ($n = 5$), a figure which suggests that there is a small, but real, population of Na^+,K^+-ATPase in these cells, consistent with the necessity of maintaining normal K^+ and Na^+ gradients. Shaver and Stirling (1978) studied the uptake of ^3H-ouabain in slices of rabbit renal medulla and found a large uptake (≈4 × 10^6 ouabain sites/cell) in the basolateral face of the thick ascending limbs, with "little or no" uptake in the collecting duct, an observation which is consistent with Zeidel et al.'s (1986*a*) measurement of a small component of ouabain-sensitive O_2 consumption. Although Zeidel et al.'s data does not allow us to specify the face of the membrane, which contains the Na^+,K^+-ATPase, Shaver and Stirling (1978) have summarized the evidence showing a basolateral location for this transport protein in a number of epithelia. We conclude that the evidence in the rabbit outer MCD cells is consistent with a ratio of Na^+,K^+-ATPase to band 3, as great as, or greater than, the ratio of ≈400 Na^+,K^+-ATPase molecules to 10^6 copies of band 3 found in the human red cell membrane[2].

[2] The disproportion between the number of copies of the Na^+,K^+-ATPase and the anion exchange protein in MCD cells means that a large amplification factor is necessary for ouabain to have any effect on DBDS binding kinetics or Cl^- flux. We have considered this problem in detail in our previous red cell paper (Janoshazi & Solomon, 1989). In experiments in which the total amount of ouabain present in the system was small compared to the total amount of band 3, we showed that one molecule of ouabain could modulate the Cl^- flux of at least 30 anion exchange proteins and the DBDS binding kinetics of at least 200 copies of the protein.

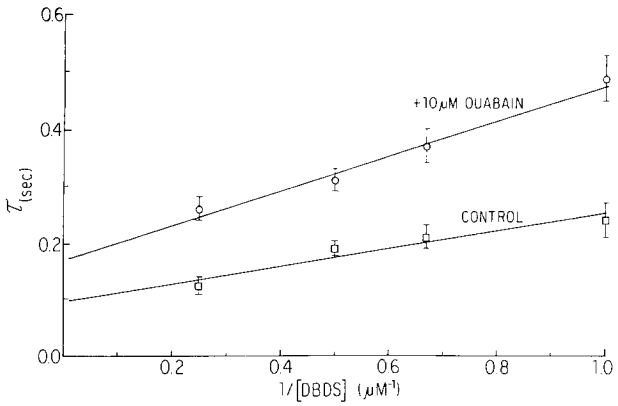

Fig. 5. Effect of ouabain on DBDS binding kinetics to MCD cells. Frozen MCD cells (0.14 mg protein/ml) were incubated in PBS with 10 μM ouabain for 10 min at 20–23°C, and then mixed in the stopped-flow apparatus with different concentrations of DBDS. The time course was fitted to a double exponential by nonlinear least squares and τ_{DBDS} was taken from the fast exponential. K_1 = 1.6 ± 0.3 μM (control); 1.8 ± 0.5 μM (+10 μM ouabain). k_2 = 9.9 ± 0.9 sec^{-1} (control); 5.7 ± 0.4 sec^{-1} (+ ouabain). Average of three experiments; in each of which, an experimental point is the average of five measurements

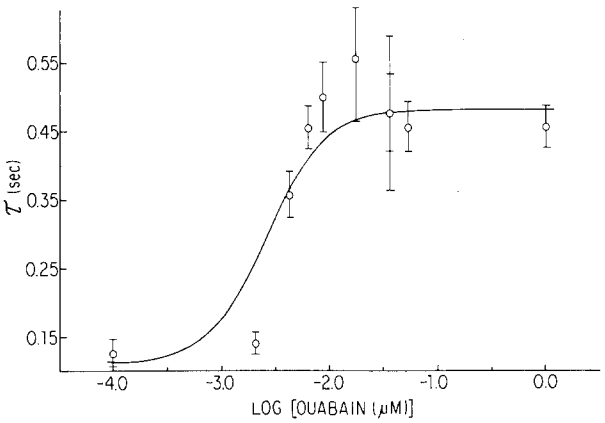

Fig. 6. Dose-response curve for ouabain effect on DBDS binding kinetics in frozen MCD cells. Frozen MCD cells (≈0.2 mg protein/ml) were incubated with different concentrations of ouabain for 1 hr at 37°C before mixing with 4 μM DBDS in PBS in the stopped-flow apparatus. The time course was fitted to a double exponential by nonlinear least squares and τ^{-1}_{DBDS} was taken from the fast exponential. $ID_{50,DBDS,MCD}$ = 0.003 ± 0.001 μM. Two experiments; each point is the average of 6–9 measurements

Figure 5 shows that 10 μM ouabain causes a significant change in the DBDS/band 3 binding kinetics; similar results have been obtained with 100 μM ouabain (*data not shown*). The forward rate constant of the conformational change, $k_{2,DBDS}$, is decreased by about 50%, from 9.9 ± 0.9 sec^{-1} to 5.7 ± 0.4 sec^{-1}, with very little change in K_1 (*see* Fig. 5 legend). We next carried out a set of experiments to see if this effect was related to the interaction of the cardiac glycoside with MCD cell Na^+,K^+-ATPase under physiological conditions. We first determined the dose-response curve for the ouabain effect on τ_{DBDS} and found that $ID_{50,DBDS,MCD}$ = 0.003 ± 0.001 μM, after 1 hr incubation at 37°C, as shown in Fig. 6. Ouabain binding to MCD cells is tighter by a factor of about five than human red blood cell binding, for which Solomon, Gill and Gold (1956) give $K_{I,K^+,rbc}$ = 0.017 μM for inhibition of human red cell K^+ flux, and also tighter than the $K_d \approx 0.02$ μM for ouabain binding to Na^+,K^+-ATPase isolated from the lamb kidney medulla (Wallick et al., 1980).

Solomon et al. (1956) found that the $K_{I,K^+,rbc}$ for inhibition of K^+ flux by another cardiac glycoside inhibitor, digitoxigenin, was 0.24 μM in a series of measurements, which showed that the $K_{I,K^+,rbc}$ depends upon the specific molecular structure of the glycoside. This dependence of binding affinity upon molecular structure provides a sensitive test that can be used to distinguish specific interactions with a characterized receptor from nonspecific binding. Having found that digitoxigenin also decreased the forward rate constant of the conformational change, we measured the dose response and found that $ID_{50,DBDS,MCD}$ = 0.09 ± 0.004 μM, after 10 min incubation at 20–23°C, as shown in Fig. 7. Thus digitoxigenin, like ouabain, binds more tightly to MCD cells than to red cells, and more than an order of magnitude less tightly than ouabain. The specificity of these high affinity binding constants, and their similarity to the red cell Na^+,K^+-ATPase binding constants, indicates that the binding site for the cardiac glycoside inhibitors is Na^+,K^+-ATPase.

An additional method of confirming that glycoside induced changes in τ_{DBDS} depend upon glycoside binding to a specific membrane site is provided by the time required for the effect to develop, since the effect of cardiac glycoside action on cation transport is very slow, while nonspecific absorption is generally rapid. For example, Chen and Verkman (1987) found that nonspecific binding of DBDS to brush-border membrane vesicles from the rat renal cortex was complete within <2 msec. We have found that the effect of both ouabain and digitoxigenin on τ_{DBDS} develops relatively slowly, as shown in Fig. 8. Appropriately for experiments on separated MCD cells[3], the 21.3 min $\tau_{ouabain}$ we measure

[3] Ouabain binding to its receptor is a bimolecular association with forward rate constant, k_{on}, and backward rate constant, k_{off}. As pointed out by Shaver and Stirling (1978) and Wallick et al. (1980), the rate constant, $\tau_{ouabain} = k_{on}[O] + k_{off}$, in which $[O]$ is ouabain concentration. Accurate comparisons between systems require determination of k_{on} and k_{off}. k_{on} depends, as Wallick et al. (1980) showed, on exact concentrations of Mg, K and ATP, which we do not know. We have also not determined k_{off} which, under the conditions of Shaver and Stirling (1978), is of the order of magnitude of $k_{on}[O]$ at 1 μM ouabain. The order-of-magnitude differences between our data and those of Shaver and Stirling (1978) and Wallick et al. (1980) are included for illustrative purposes; the differences are so great that exact kinetics are not important.

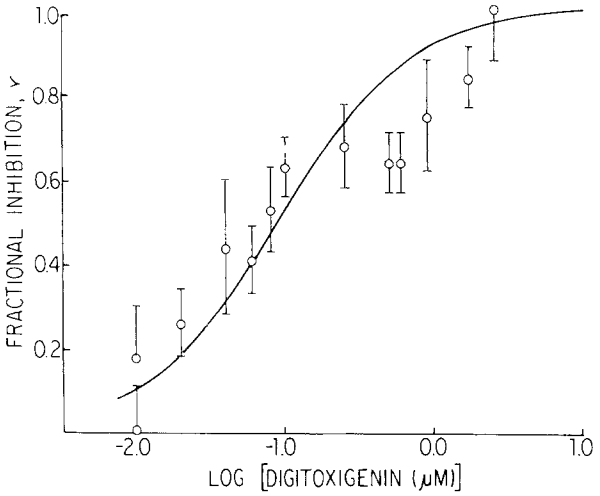

Fig. 7. Inhibition of τ_{DBDS}^{-1} by digitoxigenin. Frozen MCD cells in PBS (0.1 mg protein/ml) were incubated with different concentrations of digitoxigenin for 10 min at 20–23°C and mixed with 4 μM DBDS, as described in the legend to Fig. 6. To solubilize the digitoxigenin, C_2H_5OH (0.1%) was added to experimental and control suspensions. Since the alcohol concentration varied with the digitoxigenin concentration, each point was normalized to its own alcohol control. The data was fitted by nonlinear least squares to a single exponential. ν was set at 1.0 at normalized $\tau_{DBDS,dig}^{-1} = 0.31$ (maximum inhibition, 70%, 2.5 μM digitoxigenin) and at 0 for normalized $\tau_{DBDS,dig}^{-1} = 1.0$ (minimum inhibition, 0 digitoxigenin). $ID_{50,DBDS,MCD} = 0.09 \pm 0.004$ μM. Average of two experiments; each point is the average of 10 measurements

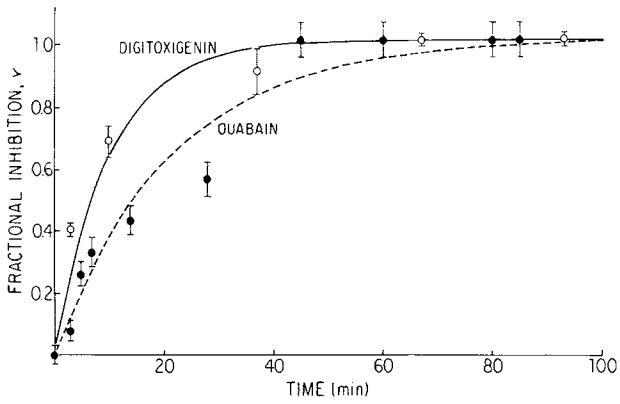

Fig. 8. Time dependence of digitoxigenin and ouabain effect on DBDS binding kinetics. Previously frozen MCD cells (0.1 mg protein/ml) in PBS were incubated with 0.1 μM digitoxigenin or 1.0 μM ouabain at 20–23°C and mixed with an equal volume of PBS buffer containing 4 μM DBDS. The fraction of the maximum effect (70% inhibition for digitoxigenin; 50% inhibition for ouabain) on τ_{DBDS} was measured as a function of time. The control was an independent experiment in which MCD cells were mixed with 4 μM DBDS without cardiac glycosides to give the minimum effect. The time course for the development of the glycoside effect was fitted to a double exponential by nonlinear least squares and $\tau_{glycoside}$ was calculated from the fast exponential. $\tau_{digitoxigenin} = 8 \pm 2$ min; $\tau_{ouabain} = 21.3 \pm 0.9$ min. Average data from two experiments; each point is the average of 5–10 measurements

for the development of the effect at 1 μM ouabain is very much faster than the τ of ≈350 min for the binding of 1 μM ^{3}H-ouabain to the thick ascending limb in rabbit renal medulla slices, as given by Shaver and Stirling (1978), and very much slower than $\tau \approx 0.3$–6 min for 1 μM ^{3}H-ouabain binding by isolated lamb kidney Na^+,K^+-ATPase given by Wallick et al. (1980). Since digitoxigenin lacks the sugar moiety, its time course is expected to be faster than that for ouabain. The time constant, $\tau = 8 \pm 2$ min at 0.1 μM is smaller than that for ouabain and would be even smaller at a comparable 1 μM digitoxigenin concentration. The molecular specificity of these time constants provides further support for the argument that the cardiac glycosides bind to the Na^+,K^+-ATPase and that our effects cannot be ascribed to nonspecific adsorption.

In order to confirm that conclusions reached on the basis of DBDS binding kinetics apply to anion exchange itself, we have determined the effect of ouabain directly on Cl^- exchange flux in fresh separated MCD cells. Figure 9 shows the results of one experiment, typical of three, in which 10 μM ouabain increases τ_{Cl^-} from 0.30 ± 0.02 sec to 0.56 ± 0.06 sec (average $\tau_{Cl^-,ouabain}/\tau_{Cl^-,control} = 1.9 \pm 0.3$). Furthermore, as Fig. 9 also shows, the Cl^- exchange disappears when the anion exchange protein has been inhibited by H_2-DIDS. In order to see

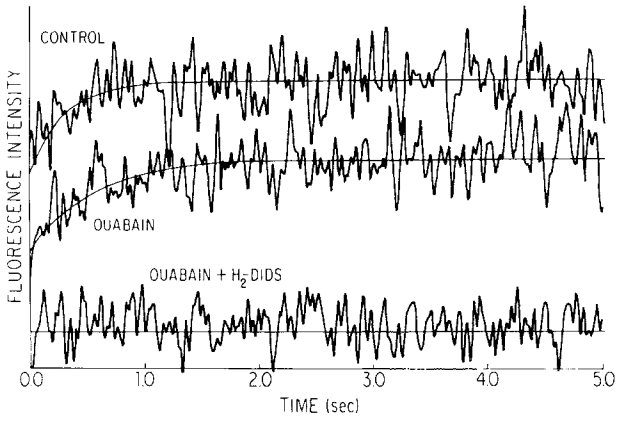

Fig. 9. Effect of ouabain on Cl^- flux. Fresh MCD cells (0.13 mg protein/ml) were incubated with 10 mM SPQ for 30 min at 37°C in PBS, washed twice in PBS, pH 7.4 and resuspended in PBS, pH 7.4. They were mixed with an equal volume of 60 mM $NaHCO_3$ and 92 mM Na gluconate in the stopped-flow apparatus. The base line shift is independent of the order of the experiments, which was randomized and cannot be attributed to SPQ leakage. Four runs were carried out with (and without) ouabain (10 μM ouabain incubated at 20–23°C, for 10 min). After correction for dye leakage, the time course was fitted to a single exponential by nonlinear least squares to give $\tau_{Cl^-,control} = 0.30 \pm 0.02$ sec; $\tau_{Cl^-,ouabain} = 0.56 \pm 0.06$ sec. Eight runs were averaged for the experiments with 15 μM H_2-DIDS, which was incubated with ouabain-treated cells for 3 min at 20–23°C. The H_2-DIDS curve, whose origin was the same as the preparation treated with ouabain alone, has been shifted vertically to increase visibility. One experiment, typical of three

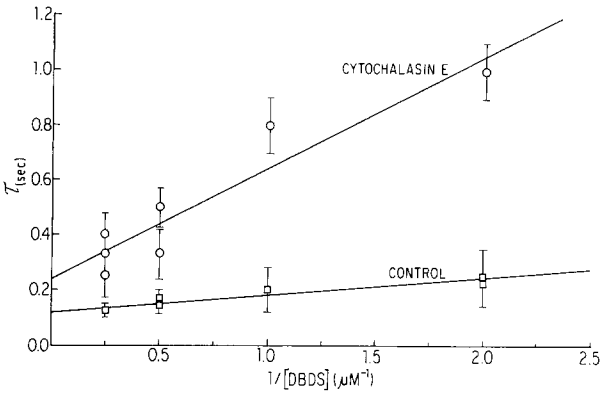

Fig. 10. Cytochalasin E effect on time course of DBDS binding to MCD cells. Frozen MCD cells (0.100–0.125 mg protein/ml) were incubated for 30–60 min ± 2 μM cytochalasin E at 20–23°C in PBS, pH 7.4. They were mixed in a stopped-flow apparatus with an equal volume of PBS to make an 0.5–4 μM DBDS solution. To solubilize the cytochalasin E, C_2H_5OH (0.1%) was added to experimental and control suspensions. $K_1 = 0.6 \pm 0.1$ μM (control); 1.7 ± 0.3 μM (+CE). $k_2 = 9.9 \pm 0.9$ sec^{-1} (control); 4 ± 1 sec^{-1} (+CE). Average of two experiments in each of which one experimental point is the average of 4–6 measurements

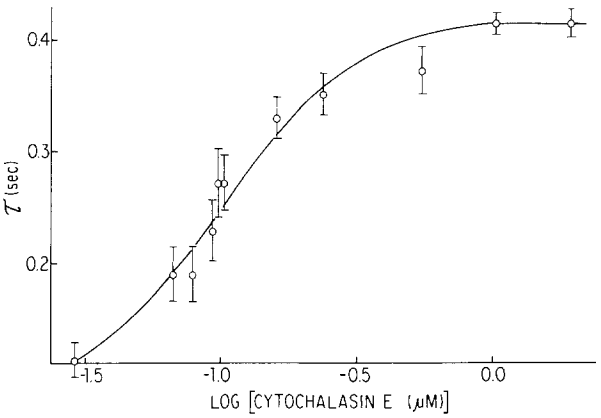

Fig. 11. Dose-response curve for cytochalasin E effect on DBDS binding to MCD cells. Frozen MCD cells (0.10 mg protein/ml) were incubated with various concentrations of CE for 30–50 min in PBS, pH 7.4 at 20–23°C. To solubilize the CE, C_2H_5OH (0.1%) was added to experimental and control suspensions. After incubation, the cells were mixed with 4 μM DBDS in PBS in the stopped-flow apparatus. The curves are double exponentials fitted to the data by nonlinear least squares and τ_{DBDS} was taken from the fast exponential. $ID_{50,DBDS,MCD} = 0.076 \pm 0.005$ μM in two experiments. One fresh MCD cell experiment (0.15 mg protein/ml) showed $ID_{50,DBDS,MCD} = 0.04 \pm 0.01$ μM

whether the ouabain effect depended upon the extracellular cationic environment, we carried out an additional set of three experiments in which the extracellular Na^+ was replaced with K^+. The ouabain effect was independent of this change, since 10 μM ouabain increased τ_{Cl^-} by a factor of 2.8 ± 1.2, not significantly different from the factor of 1.9 ± 0.4 observed with Na^+. The *increase* in τ_{Cl^-} produced by ouabain in MCD cells is similar to, but in the opposite direction from, results in the red cell, in which 10 μM ouabain *decreases* $\tau_{Cl^-,red\ cell}$ by a factor of about two (Janoshazi & Solomon, 1989). In these phenomenological experiments there is no *a priori* way to predict whether ouabain would be expected to increase or decrease τ_{Cl^-}; our experiments are designed simply to determine whether ouabain modulates Cl^- flux. Since Fig. 9 shows that the specific cardiac glycoside transport inhibitor, ouabain, does modulate anion exchange, we may conclude that the anion and cation transport proteins are close enough together that conformational information can be exchanged between them.

Cytochalasin Treatment of MCD Cells

Inside the human red cell there are cytochalasin binding sites both on the glucose transport protein and the cytoskeleton. Cytochalasin E (CE) binds to a spectrin/actin/band 4.1 complex in the cytoskeleton with a $K_d \approx 1$–0.01 μM (Lin & Lin, 1978; Lin, 1981). CE does not react with the glucose transport protein, but cytochalasin B (CB), which also binds to the CE site in the cytoskeleton, is a specific inhibitor of glucose transport with $ID_{50,gluc,rbc} = 0.3$–0.5 μM. Since CE can displace CB from the cytoskeletal site, the glucose transport inhibitory properties of CB are usually studied in the presence of CE (*see* Cushman & Wardzala, 1980). In the red cell, Janoshazi and Solomon (1989) have found that cytochalasin B (in the presence of CE) has a significant effect on τ_{DBDS} suggesting some form of linkage between band 3 and the glucose transport protein.

In MCD cells, we found that CE had a large effect on the kinetic and binding constants that describe DBDS binding to the anion transport protein, as shown in Fig. 10. CE (2 μM) causes K_1 to increase from a control value of 0.6 ± 0.1 μM to 1.7 ± 0.3 μM, decreasing the binding affinity by a factor of almost three. In addition, the rate constant, k_2, decreases from the control value of 9.9 ± 0.9 sec^{-1} to 4 ± 1 sec^{-1}. The dose-response curve in Fig. 11 shows that these effects were produced at physiological concentrations of CE, since the $ID_{50,DBDS,MCD}$ of 0.076 ± 0.005 μM is in the range for CE interaction with the red cell spectrin/actin/band 4.1 complex. These kinetics and binding affinity experiments, taken together, show that CE binding in the MCD cell causes a significant change in the conformation of the DBDS binding site on the MCD cell anion transport protein. Conformational information can be easily transported between the intra-

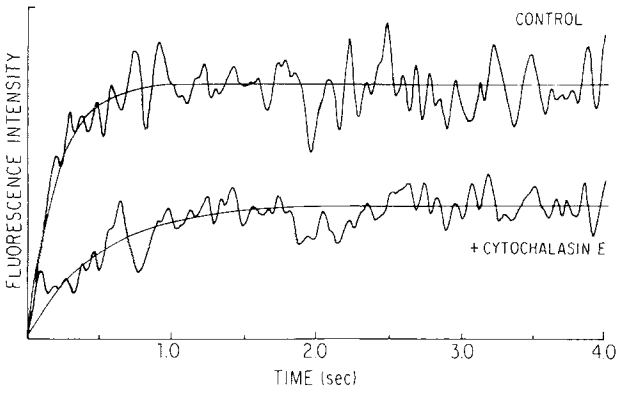

Fig. 12. Cytochalasin E effect on Cl^- flux of MCD cells. Fresh MCD cells (0.2 mg protein/ml) were incubated with 10 mM SPQ for 30 min at 37°C in PBS, pH 7.4, washed twice and resuspended in the same medium. They were then incubated with 2 μM cytochalasin E for 30 min, and mixed with an equal volume of 60 mM $NaHCO_3$ and 92 mM Na gluconate in the stopped-flow apparatus. The data were fitted with a single exponential by least squares. For the control, $\tau_{Cl^-} = 0.20 \pm 0.04$ sec; +CE, $\tau_{Cl^-} = 0.50 \pm 0.08$ sec

cellular and extracellular faces of the anion transport protein, as was shown in the red cell by Fossel and Solomon (1981) who reported that extracellular DIDS affected the conformation of enzymes in the intracellular glycolytic complex, which is bound to the cytoplasmic moiety of red cell band 3. Salhany, Cordes and Gaines (1980) also showed that DIDS binding modulated the reaction between red cell band 3 and intracellular hemoglobin (Hb).

The Cl^- flux experiments whose results are given in Fig. 12 show that CE not only deforms the DBDS binding site, but also modulates MCD cell Cl^- transport. CE (2 μM) causes the Cl^- flux to decrease by a factor of more than two, τ_{Cl^-} increasing from its control value of 0.20 ± 0.04 sec to 0.50 ± 0.08 sec, showing that the conformational change reported by the DBDS binding kinetics is associated with a profound change in the primary function of the MCD cell anion transport protein.

Although there is no evidence that a protein analogous to the red cell glucose transport protein is present on the basolateral face of the MCD cell, we probed the cell for possible effects of CB on DBDS binding kinetics in the presence of CE. Our inability to detect such a response indicates that either the response is too small for us to detect, or that the analogy between rbc and MCD cell membrane transport systems does not extend to the glucose transport protein.

Several of the major cytoskeletal elements in the red cell are also found in MCD cells. As already pointed out in the introduction, Drenckhahn et al. (1985) have obtained immunochemical evidence showing that ankyrin, the red cell cytoskeletal protein that binds band 3 to spectrin, is present in MCD cells. Their evidence shows that both the band 3 and the spectrin binding domains of ankyrin are co-localized with the membrane sites for band 3 on the basolateral face of MCD cells. Antibodies to brain spectrin are also bound on this face of the cell. These experiments show that components of the red cell spectrin/actin/band 3 complex are present in MCD cells. Furthermore, Drenckhahn and Merte (1987) have shown that the band 3 analog is not uniformly distributed along the basolateral membrane, but rather is localized to specific regions; they have suggested that this distribution may result from binding of band 3 through ankyrin to the cytoskeleton. Thus, it seems reasonable to suggest that the cytoskeletal components include the cytochalasin E binding site, all the more so because it is known that cytochalasins have profound effects on transporting epithelia (Ausiello, Hartwig & Brown, 1987). At the least, the CE experiments show that intracellular ligand binding can modulate the rate of anion exchange in MCD cells. If we can also assume that CE binds to the cytoskeleton in MCD cells, our experiments indicate that perturbations of the cytoskeleton are transmitted all the way across the membrane to the extracellular binding site for DBDS, as if pulling a string on the cytoskeleton could raise a flag at the the extracellular site for anion exchange inhibition.

Discussion

Discharge of the primary duties of both the rbc and the MCD cell are dependent on a rapid Cl^-/HCO_3^- exchange, so it is not surprising that the anion exchange proteins are similar, and probably functionally identical, in these two cells. In most other respects, the cells appear to differ widely since the MCD cells are nucleated and contain a number of organelles, including a small population of mitochondria, which enable the MCD cells to support about half of their energetic needs by respiration (Zeidel et al., 1986*b*). In contrast, the red cell has no nucleus and no organelles and meets its relatively small requirements for energy solely by glycolysis. The major function of the red cell is the carriage of O_2 and CO_2, which depend upon both the anion exchange protein and the very large intracellular [Hb]. The red cell cation transport maintains osmotic equilibrium by lowering the total intracellular cation content to balance the requirements imposed by the high [Hb]. The cation leak in the red cell is very slow, so that only a sluggish transport of Na^+ and K^+ is necessary to keep the cell in osmotic

balance. The relative velocity of the anion and cation transport processes is illustrated dramatically by the ratio of the 400 copies of the Na^+,K^+-ATPase to the 5×10^5 dimers of band 3.

Since the MCD cell is primarily concerned with proton transport, it has no need of the high concentrations of Na^+,K^+-ATPase found in the neighboring thick ascending limb cells in the outer renal medulla, which are devoted to transport of Na^+ as required for operation of the countercurrent mechanism and for regulation of extracellular volume. As Zeidel et al. (1986*b*) pointed out, there is a slow cation leak in MCD cells since uncharged NH_3 permeates the cell membrane much faster than NH_4^+. Thus, there is a requirement for at least some membrane-bound Na^+,K^+-ATPase. Similar in a way, to the red cell, MCD cells depend upon anaerobic glycolysis for a significant fraction of their metabolism, probably because of their location deep within the renal medulla where O_2 tension is low.

In view of the similarities of the physical chemical characteristics of the DBDS binding sites in the red cell and MCD cell, it is not surprising that we now find that ouabain binding modulates anion transport in the MCD cell, as in the red cell, and that cytochalasin E, which appears to interact with the anion exchange protein in the red cell, also does so in MCD cells. In the red cell, Fossel and Solomon (1978) have previously shown that the glycolytic enzymes are linked together in a megadalton complex, which interacts with band 3, and which may provide the locale for the segregated pool of ATP, which Proverbio and Hoffman (1977) have shown to be preferentially metabolized by the red cell Na^+,K^+-ATPase. Recently, Janoshazi and Solomon (1989) provided evidence suggesting that the Na^+,K^+-ATPase and the glucose transport protein are also linked to red cell band 3 to form a transport protein complex responsible for the transport of both anions and cations.

When our observations in the MCD cell are taken together with the immunochemical evidence that ankyrin, band 3 and spectrin are co-localized on the basolateral face of the MCD cell membrane, it becomes tempting to suggest that the entire multiprotein complex of band 3, Na^+,K^+-ATPase and cytoskeleton are present also as multi-protein complex in MCD cells. The likeness is further supported by the fact that a significant fraction of the energetic needs of MCD cells are supported by glycolysis, even though we have presented no evidence of a link between the glucose transport system in MCD cells and the MCD analog of band 3. Evidence is now accruing that several elements of such a complex are transferred to other cell types together with the anion exchange protein. In the turtle urinary bladder, Drenckhahn et al. (1987) have found that the anion exchange protein is co-localized with actin, isoforms of ankyrin and spectrin on the basolateral cell surface. In the superficial cortex of the human eye lens, immunochemical evidence for band 3 and ankyrin has been obtained by Allen et al. (1987) who report that these same cells have previously been found by others to contain spectrin, actin and band 4.1. Furthermore, Nelson and Veshnock (1987) showed that Na^+,K^+-ATPase, purified from the canine kidney outer medulla, binds specifically to ankyrin, purified from the human red cell, with an apparent K_d of 1–2.5 nM. The observation that so many elements of the multi-protein transport complex (anion exchange protein/cytoskeleton/Na^+,K^+-ATPase) are transferred together to these other types of cell provides support for the supposition that the functional interactions we have observed are important to the function of anion exchange not only in red cells and MCD cells, but also in a variety of other cells and tissues.

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