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Effect of pCMBS on anion transport in human red cell membranes

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The kinetics of binding of the mercurial sulfhydryl reagent, pCMBS (*p*-chloromercuribenzene sulfonate), to the extracellular site(s) at which pCMBS inhibits water and urea transport across the human red cell membrane, have previously been characterized. To determine whether pCMBS binding alters Cl^- transport, we measured Cl^-/NO_3^- exchange by fluorescence enhancement, using the dye SPQ (6-methoxy-*N*-(3-sulfopropyl)quinolinium). An essentially instantaneous extracellular phase of pCMBS inhibition is followed by a much slower intracellular phase, correlated with pCMBS permeation. We attribute the instantaneous phase to competitive inhibition of Cl^- binding to band 3 by the pCMBS anion. The ID_{50} of 2.0 ± 0.1 mM agrees with other organic sulfonates, but is very much greater than that of pCMBS inhibition of urea and water transport, showing that pCMBS reaction with water and urea transport inhibition sites has no effect on anion exchange. The intracellular inhibition by 1 mM pCMBS (1 h) is apparently non-competitive with $K_i = 5.5 \pm 6.3$ mM, presumably an allosteric effect of pCMBS binding to an intracellular band 3-related sulfhydryl group. After *N*-ethylmaleimide (NEM) treatment to block these band 3 sulfhydryl groups, there is apparent non-competitive inhibition with $K_i = 2.1 \pm 1.2$ mM, which suggests that pCMBS reacts with one of the NEM-insensitive sulfhydryl groups on a protein that links band 3 to the cytoskeleton, perhaps ankyrin or bands 4.1 and 4.2.

Introduction

It has been generally accepted that the sulfhydryl reagent, pCMBS (*p*-chloromercuribenzene sulfonate), has no effect on red cell anion flux though, as Knauf and Rothstein [1] first showed, it causes a massive cation leak. Knauf and Rothstein and subsequently Rothstein et al. [2] found no direct pCMBS effect on SO_4^{2-} efflux at 37°C, but did not specify the pCMBS concentration. Deuticke [3] reported that 10 μM pCMBS did not inhibit SO_4^{2-} exchange and Passow and Schnell [4], Passow [5] and Deuticke et al. [6] made similar statements in reference to unpublished experiments.

We have previously characterized the binding of pCMBS to the separate sites responsible for inhibition of water and urea transport (Toon and Solomon [7]), and Ojcius and Solomon [8] have used gel electrophoresis to show that these binding sites are located on band 3. Both Knauf and Rothstein [9] and Rao [10] have shown that anion exchange inhibitors slow the permeation of pCMBS, which is itself an anion, into the red cell. Consequently, it is to be expected that pCMBS would have an effect on anion exchange, either by competition at the transport site or as a result of pCMBS modification of band 3. Since our evidence [8] showed that pCMBS inhibits water and urea transport by binding to band 3, and since the binding sites can be differentiated by their kinetics [7], we were anxious to determine whether either or both of these sites were related to anion transport. The availability of the SPQ (6-methoxy-*N*-(3-sulfopropyl)quinolinium) method [11] to measure Cl^- flux by stopped-flow fluorescence enhancement makes it possible to determine the pCMBS effect on Cl^- exchange at room temperature, notwithstanding the rapid exchange kinetics.

Materials and Methods

Materials

pCMBS and NEM (*N*-ethylmaleimide) were obtained from Sigma Chemical Co. (St. Louis, MO). SPQ and DIDS (4,4'-diisothiocyano-2,2'-disulfonic stilbene) were obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of reagent grade and

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were obtained from Sigma or Fisher (Pittsburgh, PA). Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

Methods

After washing three times with PBS buffer (150 mM NaCl, 5 mM Na_2HPO_4 (pH 7.4)), red cells 1:20 (v/v) were hemolysed in 5 mM Na_2HPO_4 (pH 8.0 at 4°C) for 5 min. One-step ghosts were obtained by centrifuging the hemolysate at $12000 \times g$, for 10 min. The pink pellet was mixed at 1:5 (v/v) with PBS and appropriate amounts of 50 mM SPQ and 2 M NaCl, bringing the final concentrations to 5 mM SPQ and 150 mM NaCl (pH 7.4). NEM-treatment was carried out by adding NEM to the ghost suspension at this time. Both pCMBS and NEM were freshly prepared each time. One-step ghosts were resealed by incubation in PBS at 37°C for 1 h, then washed in PBS and centrifuged ($12000 \times g$ for 10 min) three times. Cl^-/NO_3^- exchange kinetics were measured at 20–24°C in a stopped-flow apparatus (Model SFA-11, Hi-Tech, Salisbury, Wiltshire, UK) whose cuvette was inserted in the light path of a Photon Technology Alphascan Spectrofluorimeter (Princeton, NJ). The time course of SPQ fluorescence (excitation 350 nm, emission 430 nm, slit widths 4 nm) was recorded at a data acquisition rate of 5–10 ms/point. To measure Cl^-/NO_3^- exchange, approximately 2% (v/v) one-step ghosts in PBS were mixed with equal volumes of 30–150 mM $NaNO_3$, 5 mM Na_2HPO_4, and an appropriate amount of sodium gluconate, which was used for adjustment of [Na] = 150 mM and 295 ± 5 mosM (pH 7.4). Osmolalities of all solutions were measured using a Fiske OS osmometer (Fiske Associates, Uxbridge, MA). Hematocrits were determined in quadruplicate by centrifugation at $5000 \times g$ for 30 min in microhematocrit tubes (Fisher, Pittsburgh, PA).

Fig. 1 shows the time course of fluorescence intensity of a suspension of SPQ-loaded one-step ghosts during mixing with NO_3^- solution. The data were fitted by nonlinear least squares to a single exponential, $A = B_0 - B_1 \exp(-t/\tau)$. The initial rate of Cl^-/NO_3^- exchange was calculated from the initial rate of fluorescence change, $[dA/dt]_{t=0}$, which is equal to B_1/τ. We checked the Stern-Volmer relationship for the SPQ solution at room temperature, and obtained $A_0/A = 1.036 \pm 0.117[Cl]$, in which A and A_0 are the fluorescence intensities in the presence and absence of Cl^-. The value of $0.117 \pm 0.002\ mM^{-1}$ is the quench constant, K, which agrees with the value of $0.118\ mM^{-1}$ given by Illsley and Verkman [11]. Differentiating the Stern-Volmer equation,

$$\{d[Cl]/dt\}_{t=0} = \{[1.036 + K[Cl]]/KA\}[dA/dt]_{t=0} \quad (1)$$

To determine *intra*cellular A at $t = 0$, we must correct

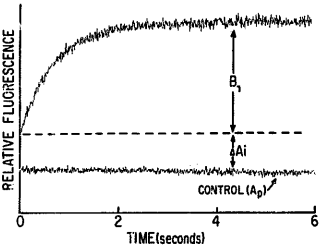

Fig. 1. Time course of Cl^-/NO_3^- exchange in one-step ghosts. Ghosts containing 5 mM SPQ in PBS were mixed with $NaNO_3$. B_1 is the fluorescence amplitude of the exponential time course. A_p is the fluorescence of SPQ-loaded ghosts when mixed with PBS (control) and ΔA_i is the fluorescence increment immediately after mixing ghosts with NO_3^- solution.

for SPQ leakage during the incubation of the SPQ-loaded sample (± pCMBS) at room temperature for periods up to 1 h. This *extra*cellular SPQ, after mixture with the extracellular Cl^- in the stopped-flow apparatus at $t = 0$, causes a baseline increment, ΔA_i, in the fluorescence intensity, as shown in Fig. 1. In Fig. 1, the control, A_p is the total fluorescence of the SPQ-loaded sample during mixing with PBS, which is almost constant over the entire 6 s time scale. Inserting the values of K, [Cl] and dA/dt at $t = 0$ in Eqn. 1, we obtain

$$\{d[Cl]/dt\}_{t=0} = \{158.9/[A_p - Q\,\Delta A_i]\}B_1/\tau \quad (2)$$

in which Q is the correction factor which converts the leakage, ΔA_i, to equivalent units of [Cl]; that is, Q corrects for the difference in the quenching factor between the mixed solution and PBS at $t = 0$. We have determined Q independently for several solutions. For example, $Q = 0.88 \pm 0.06$ in the absence of, and 0.95 ± 0.03 in the presence of, 0.5 mM pCMBS (final concentration).

We carried out the set of control experiments, whose Hill plots are given in Fig. 2, to show that incubation of pink ghosts for 5 min with 1 mM pCMBS does not modify the Hill coefficient (control, $n = 0.99 \pm 0.05$; + pCMBS, $n = 1.02 \pm 0.04$). Similar results were obtained after incubation with either 1 mM NEM for 1 h at 37°C, or with 1 mM pCMBS for 1 h at room temperature. In another control, we measured DIDS inhibition of Cl^-/NO_3^- exchange. DIDS was added to the SPQ-loaded pink ghost suspension 10 min before Cl^-/NO_3^- exchange measurements at room temperature, which means that the DIDS reaction was probably reversible. We obtained an $ID_{50,Cl}$ of 0.27 ± 0.03 μM in the presence of 45 mM NO_3^- solution, in very

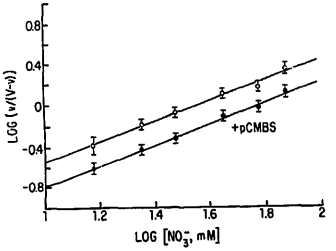

Fig. 2. Hill plot of the effect of pCMBS on Cl^-/NO_3^- exchange as a function of NO_3^- concentration. SPQ-loaded ghosts were incubated for 5 min at room temperature with 1 mM pCMBS. V is the maximal velocity estimated from the respective Lineweaver-Burk plots; v is the initial velocity of anion exchange. For the control: $V = 250 \pm 12$ mM s^{-1}, slope $= 0.99 \pm 0.05$, intercept $= -1.5 \pm 0.08$; +pCMBS: $V = 261 \pm 15$ mM s^{-1}, slope $= 1.02 \pm 0.04$, intercept $= -1.8 \pm 0.06$. One experiment typical of five. (Control, ○; +pCMBS, ●).

good agreement with the value of 0.2 μM for DIDS inhibition of red cell anion exchange (Knauf [12]). These experiments confirm that Cl^-/NO_3^- exchange in our system was mediated by band 3. It should be pointed out that both Cl^-/NO_3^- exchange and its pCMBS inhibition differ significantly in different bloods so that controls were always carried out with the same blood.

In view of the massive cation leak observed by Knauf and Rothstein [1], it was possible that incubation with 1 mM pCMBS for periods up to 1 h might produce a volume change that could affect our Cl^- flux measurements. Even though external [Na] = cell ([Na) + [K]), a differential between Na and K leak fluxes, or some other unspecified lesion might affect cell volume. Accordingly, we incubated red cells with 1 mM pCMBS for 2 h at room temperature and found that there was no pCMBS-induced volume change. In a 2-h period, the control hematocrit went from 0.151 ± 0.001 to 0.149 ± 0.002; after incubation with 1 mM pCMBS, the hematocrit went from 0.156 ± 0.002 to 0.157 ± 0.003. In view of the observations of Ralston and Crisp [13] and Clark and Ralston [14] of pCMBS-induced protein losses from well-washed white ghosts, we carried out more than ten additional control experiments with the pink ghosts used in our experiments. After treating pink ghosts with 1 mM pCMBS for 1 h at room temperature, we found less than 1% difference in fluorescence intensity between the pCMBS treated ghosts and controls, showing that pCMBS treatment also causes no change in volume in pink ghosts.

Results and Discussion

Competition of pCMBS for anion exchange site

There are several reasons to expect pCMBS to compete with Cl^- for the anion exchange binding site on band 3. In addition to the observations [9,10] that pCMBS permeation is inhibited by the stilbene inhibitors of band 3, there are the studies of Aubert and Motais [15] and Barzilay et al. [16] which show that organic sulfonates, such as benzene sulfonate, enter the red cell on the anion exchange carrier. In order to separate competition for the carrier from mercurial effects on membrane sulfhydryl groups, we began the exposure to pCMBS at the same time that we began the Cl^- flux measurement by including pCMBS in the NO_3^- solution in the other syringe in the stopped-flow apparatus, with no prior incubation. Thus the pink ghosts were not exposed to pCMBS until the instant of mixing and their effective exposure to pCMBS was limited to the 1–2 s required to complete the Cl^- measurement. Fig. 3 shows that pCMBS inhibits Cl^- flux under these conditions; the fractional inhibition has been fitted to a single-site binding curve with $ID_{50} = 2.0 \pm 0.1$ mM, which is equivalent * to $K_i = 0.87 \pm 0.03$ mM. Probably previous investigators did not find pCMBS effects on the anion transport system because they looked at lower concentrations in the range of the inhibitory concentrations for water and urea fluxes. When the data is plotted as a Hill plot in Fig. 4, the relationship is linear and the Hill coefficient

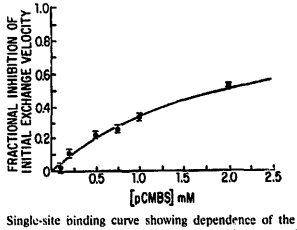

Fig. 3. Single-site binding curve showing dependence of the fractional inhibition of initial Cl^-/NO_3^- exchange velocity on pCMBS concentration under zero-time conditions. The pCMBS concentrations are after mixing; final $[NO_3^-] = 45$ mM. Maximal inhibition was fixed at 1; $ID_{50} = 2.0 \pm 0.1$ mM, as fitted by non-linear least squares.

* The equation relating ID_{50} to K_i is:

$$ID_{50} = (1 + ([NO_3^-]/K_m))K_i$$

in which we have used 45 mM for $[NO_3^-]$. The mean K_m for $NO_3^- = 35 \pm 3$ mM was determined from the controls in Figs. 7 and 8.

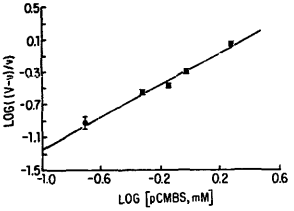

Fig. 4. Data from Fig. 3 shown in the form of a Hill plot. V and v are the Cl^-/NO_3^- exchange rates in the absence and presence of pCMBS, respectively. Slope, $n = 0.96 \pm 0.07$; intercept $= -0.286$.

is 1 (0.96 ± 0.07), showing that only a single site is involved in the zero-time pCMBS inhibition of Cl^- exchange.

Aubert and Motais [15] reported that the permeability of the organic sulfonates through the anion channel depends sensitively upon molecular configuration. For example, $ID_{50,Cl} = 47 \pm 15$ mM for benzene sulfonate while, for *p*-chlorobenzene sulfonate $ID_{50,Cl} = 5.5 \pm 2.2$ mM (Barzilay et al. [16]). Thus, our finding that pCMBS interacts with the anion exchange protein is consistent with the behavior of other organic sulfonates.

pCMBS binding to the anion transport protein site where it competes with Cl^- exchange does not involve reaction with a cysteine moiety. Thus, binding to this reversible transport site is different from the usual binding of mercurial sulfhydryl reagents which generally involves chemical reactions. Nonetheless, the transport site is a true reversible binding site whose occupancy by a molecule as large as pCMBS could affect the conformation of band 3. Verkman et al. [17] have observed that pCMBS binding to purified band 3 reconstituted into lipid vesicles leads to quenching of the fluorescence of the tryptophan residues in band 3 with $K_d = 0.44 \pm 0.06$ mM, with a time constant of the order of 1 min. This rapid reaction and the similarity of the binding constant to $K_i = 0.87 \pm 0.03$ mM for pCMBS inhibition of anion exchange suggests that the tryptophan fluorescence quenching observed by Verkman et al. [17] may be related to pCMBS binding to the band 3 transport site.

Sulfhydryl groups, pCMBS binding sites and band 3

Rothstein et al. [2] and Rao [10] report that there are approx. $24 \cdot 10^6$ pCMBS binding sites on a red cell membrane but that the number of extracellular groups is very much smaller, amounting to $(1–2) \cdot 10^6$/cell. Of these, $1.2 \cdot 10^6$ sites/cell are located on band 3 [8] and another $(0.15–0.3) \cdot 10^6$ sites/cell are located on band 4.5 (Deziel et al. [18]) so that these two proteins account for virtually all the extracellular pCMBS binding sites. In addition, the pCMBS site which inhibits the monocarboxylate carrier is located on the extracellular face (Deuticke [19]), but the number of these sites is much smaller than 10^6/cell. Rao [10] determined that the intracellular uptake of pCMBS, which was inhibitable by a stilbene inhibitor, followed a logarithmic time course, for which we have computed $\tau = 52$ min.

Lux et al. [20] have shown that there are five sulfhydryl groups on the human band 3 which they sequenced. According to their topological and hydropathic analysis, only a single cysteine, Cys-479, is in an extracellular locality where it can react with extracellular reagents. Two of the four intracellular sulfhydryls (Cys-201 and Cys-317) are located in the hydrophilic cytosolic domain which, it is well known (Grinstein et al. [21]), plays no role in anion exchange. The remaining two cysteines, Cys-843 and Cys-885, are contained in a 7–9 kDa fragment, located in the membrane domain near the cellular loop between crossings 12 and 13 and accessible only from inside the membrane (Rao [22], Ramjeesingh et al. [23]). Cys-843 and Cys-885 are close enough that they cross-link during the preparation of white ghosts and are no longer available to react with NEM in these ghosts (Rao [22], Ramjeesingh et al. [23]). Rao [22] showed that treatment of white ghosts with either 1 mM pCMBS at 0°C for 5–10 min, or 0.5 mM NEM for 1 h at 37°C, blocked all the *intra*cellular sulfhydryl groups of band 3. After NEM-treatment, these blocked sulfhydryl groups could not subsequently react with pCMBS, and vice versa. The effect of NEM-treatment was confirmed by the assay of Haest et al. [24] who measured the NEM non-reactive sulfhydryls in ghost membrane proteins and reported that there was only 1 (actually 1.2) such sulfhydryl per band 3 molecule, which is accounted for by the extracellular NEM non-reactive site, Cys-479.

Sha'afi and Feinstein [25] reported that pCMB (*p*-chloromercuribenzoate) inhibits red cell osmotic water permeability by 77%. Since inhibition could be reversed by cysteine, a sulfhydryl group was implicated, but one that didn't react with maleimides since pretreatment with NEM did not affect water transport, or its inhibition by pCMB. Solomon et al. [26] showed that the stilbene anion transport inhibitor, DIDS, which prevents pCMBS from crossing the cell membrane, has no effect on pCMBS inhibition of water flux. Though the experiments of Solomon et al. do not identify the specific protein involved in water transport inhibition, they do prove that the sulfhydryl group responsible for water flux inhibition must be one of those on the outer face of the membrane.

Polyacrylamide gel electrophoresis was used to localize the responsible sulfhydryl to a specific protein. Since NEM had no effect on mercurial sulfhydryl inhibition of red cell water flux, Sha'afi and Feinstein [25]

studied [^{14}C]pCMB binding to NEM-treated intact cells and showed that virtually all of the label was found in band 3. Ralston and Crisp [13] studied the distribution of [^{203}Hg]pCMBS in NEM-treated red cell ghosts and reported that bands 3 and 4.9 were the major proteins that bound the pCMBS; there were traces of binding on bands 4.1 and 4.2 while binding to the other components was negligible. Since bands 4.1, 4.2 and 4.9 are cytoplasmic, the ghosts were permeable to pCMBS; and since all the cytoplasmic sulfhydryls in band 3 react with NEM when the reaction is complete, the pCMBS binding to band 3 observed by Ralston and Crisp [13] in NEM-treated cells may be assigned to an extracellular site, in agreement with the observations of Sha'afi and Feinstein [25].

Benga et al. [27] made a detailed study of [^{203}Hg]pCMBS binding to NEM-treated red cells under conditions in which water transport was inhibited and found most of the radioactivity in bands 3, 4.2 and 4.5. The observation of pCMBS binding in band 3 confirms the findings of Sha'afi and Feinstein [25] and Ralston and Crisp [13]. The new question raised by the experiments of Benga et al. concerns the possible roles of band 4.5, the glucose transport protein, and band 4.2 in water transport. Since band 4.2 is an intracellular protein, it can not be responsible for transmembrane water permeation; furthermore, the observation of radioactivity in band 4.2 shows that the radioactivity in the observations of Benga et al. was not confined to the extracellular face. If band 4.5 is responsible for a primary role in water transport, it needs to have an extracellular NEM-non-reactive sulfhydryl group. Three of the five sulfhydryls in band 4.5 are accessible from outside faces, either cytoplasmic or extracellular, in addition to two nonreactive sulfhydryls that appear to be in the membrane lipid (Deziel et al. [18]). Since the extrafacial sulfhydryl, presumably Cys-429 (May et al. [28]), reacts with either pCMBS or NEM (Deziel et al. [18]), Cys-429 is probably not the sulfhydryl group responsible for water transport inhibition. Since Cys-429 is the only extracellular sulfhydryl group on band 4.5, band 4.5 is probably not the protein primarily responsible for water transport.

When Macey and Farmer [29] first showed that pCMBS inhibited red cell water transport, they also observed pCMBS inhibition of urea flux. The urea flux inhibition is much faster than water flux inhibition, as Macey [30] subsequently showed. Toon and Solomon [7] characterized the binding characteristics of both the water and urea transport inhibition sites and reported that the $K_{i,app}$ for pCMBS inhibition of urea flux is 0.09 ± 0.06 μM, three orders of magnitude tighter than $K_{i,app} = 160 \pm 30$ μM for water flux inhibition. The on-rate for pCMBS binding to the urea inhibition site is 310 ± 200 M^{-1} s^{-1}, two orders of magnitude faster than that for binding to the water inhibition site, 1.77 ± 0.03 M^{-1} s^{-1}. These differences in affinity and rate made it possible to label the urea and water inhibition sites separately with [^{203}Hg]pCMBS. Ojcius and Solomon [8] blocked all the NEM-reactive sites in red cells and also treated the cells with DIDS to block [^{203}Hg]pCMBS access to the cytoplasm. Under these conditions virtually all of the label was found in band 3, except for a small unexplained peak at approx. 46 kDa; binding to band 4.5 was negligible. Under conditions in which the urea and water inhibition sites were labelled separately, the time course of uptake to each site correlated very well ($r = 0.97$ for each site) with the time courses of pCMBS binding, calculated from the kinetic constants of Toon and Solomon [7]. Since Cys-479 is the only extracellular sulfhydryl group in band 3 available to accomodate both binding sites, Ojcius and Solomon suggested that the initial rapid binding to the high affinity fast urea inhibition site caused a conformational change in band 3, changing the binding characteristics of the partner in the band 3 dimer (tetramer) to those of the low affinity pCMBS water flux inhibition site. Taken together, the polyacrylamide gels of Sha'afi and Feinstein [25], of Ralston and Crisp [13], of Benga et al. [27] and of Ojcius and Solomon [8] clearly demonstrate that pCMBS binds to band 3 when water transport is inhibited and provide very strong evidence that the site for pCMBS inhibition of water transport is on band 3.

The early course of pCMBS effects on anion exchange

We wanted to determine whether pCMBS interaction with the sites that inhibit water and urea transport had any effect on Cl^-/NO_3^- exchange. In the course of these experiments, we measured the time course of 1

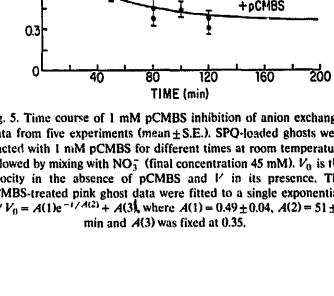

Fig. 5. Time course of 1 mM pCMBS inhibition of anion exchange. Data from five experiments (mean ± S.E.). SPQ-loaded ghosts were reacted with 1 mM pCMBS for different times at room temperature followed by mixing with NO_3^- (final concentration 45 mM). V_0 is the velocity in the absence of pCMBS and V in its presence. The pCMBS-treated pink ghost data were fitted to a single exponential, $V/V_0 = A(1)e^{-t/A(2)} + A(3)$, where $A(1) = 0.49 \pm 0.04$, $A(2) = 51 \pm 8$ min and $A(3)$ was fixed at 0.35.

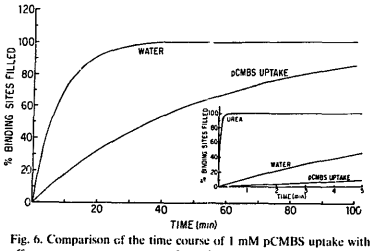

Fig. 6. Comparison of the time course of 1 mM pCMBS uptake with effects on transport processes. Data for pCMBS uptake taken from Rao [10] and for water and urea inhibition from Toon and Solomon [7]. For pCMBS uptake $\tau = 52 \pm 0.1$ min, for water, $\tau = 8.1 \pm 0.3$ min and for urea, $\tau = 0.05 \pm 0.04$ min.

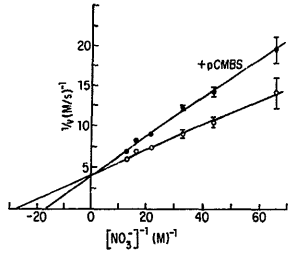

Fig. 7. Lineweaver-Burk plot of Cl^-/NO_3^- exchange inhibition by 1 mM pCMBS after 5 min reaction at room temperature. Inhibition is competitive, with $K_i = 1.5 \pm 0.3$ mM. For the control: K_m for NO_3^- = 37 mM, slope = 0.146 ± 0.005 s and intercept (with the y-axis) = 4.0 ± 0.2 (M/s)$^{-1}$; for the pCMBS-treated ghosts: slope = 0.235 ± 0.006 s and intercept = 3.8 ± 0.2 (M/s)$^{-1}$.

mM pCMBS inhibition of anion exchange over a two hour period, as Fig. 5 shows. After the initial drop at zero time, the flux continued to fall slowly with time until it was about 65% inhibited at 120 min. The curve was fitted to an exponential decay with $\tau = 51 \pm 8$ min, as shown by the solid line in Fig. 5. The control remained essentially constant over the first 80 min and then dropped slowly.

In order to discriminate between effects on the urea and water flux inhibition sites, we decided to pick times of exposure to pCMBS which favored one site or the other. Since high concentrations were required to produce the inhibition, it was not practicable to lower the concentration so that we could have more time to make the observation, particularly for the high affinity fast urea site. The predicted fractions of occupied sites, at 1 mM pCMBS, are shown in Fig. 6. If it were possible to make a measurement after 1–2 min incubation with pCMBS, we could assign the differential effect between zero and 2 min to the urea site. Practically, we are unable to complete the repetitive series of runs required to establish a time course that fast; the earliest practicable time is 5 min, by which time the urea site is completely filled and the water site is 46% filled. The data in Fig. 5 shows that there is essentially no increment in inhibition between $t = 0$ and 5 min, which means that pCMBS reaction with the urea transport inhibition site has no effect on anion transport.

The lack of supplementary inhibition in the first five minutes also means that the kinetics observed at that time are representative of those at zero time. As Fig. 7 shows, 5 min incubation with 1 mM pCMBS inhibits Cl^- flux competitively with $K_i = 1.5 \pm 0.3$ mM, as given in Table I, confirming the competitive nature of the zero time process. Our interpretation is supported by the calculation that the ID_{50} of 3.4 ± 0.7 mM computed * from this K_i is in reasonable agreement with the $ID_{50,Cl}$ of 2.0 ± 0.1 mM for the zero-time experiment in Fig. 3. These values are orders of magnitude greater than the $K_{i,app}$ for the pCMBS effect on urea flux, confirming the conclusion that pCMBS interaction with the urea flux inhibition site has no effect on Cl^- flux. Another tentative conclusion flows from the 5 min experiments. Since the water flux inhibition site is almost half filled by this time, the fact that we observe no additional Cl^- flux inhibition indicates that the water flux inhibition site also has no effect on Cl^- flux.

As previously pointed out (Solomon et al. [26]) the observation that pCMBS inhibition of water and urea sites does not affect Cl^- exchange does not mean that water and urea do not enter the red cell through an aqueous channel in band 3. It only means that the rate determining step for Cl^- exchange involving, as it

TABLE I

pCMBS inhibition constants

Conditions	K_i (mM)	Relative velocity	Number of expts.
No NEM treatment			
zero time, 1 mM pCMBS	0.87 ± 0.03	0.79 ± 0.02	1
5 min, 1 mM pCMBS	1.5 ± 0.3	0.80 ± 0.02	1
60 min, 1 mM pCMBS	5.5 ± 6.3	0.50 ± 0.06	4
NEM treatment			
60 min, 1 mM pCMBS, 1 mM NEM	2.1 ± 1.2	0.61 ± 0.04 [a]	2
60 min, 1 mM pCMBS, 12 mM NEM	1.4 ± 1.5	0.22 ± 0.02	2

[a] The controls differ with different bloods. The control for these experiments at 1 mM NEM was 0.88 ± 0.07.

* See footnote on p. 33.

does, a 1:1 obligatory anion exchange, is different from the rate determining step for urea and water flux. The present experiments, taken together with the earlier ones of Ojcius and Solomon [8], show that reaction of pCMBS with the external sulfhydryl site on band 3 has no effect on the rate-determining step for the anion exchange process.

Effects at longer times of incubation

Since the only extracellular cysteine on band 3, Cys-479, is occupied when urea and water influx is inhibited by pCMBS (Ojcius and Solomon [8]), there appear to be no additional extracellular band 3 sites to account for the increased Cl^- inhibition between 5 min and 120 min, shown in Fig. 5. The τ of 51 ± 8 min for this incremental Cl^- flux inhibition is in remarkably close agreement with the τ of 52 min computed from Rao's [10] measurement of pCMBS uptake by resealed pink ghosts. This close correspondence leads us to infer that the incremental inhibition of Cl^- flux is to be assigned to intracellular pCMBS sites on band 3. We have found that the multiple washings required to produce white ghosts cause changes in the transport properties of water and urea and in the kinetics of stilbene inhibitor binding to band 3 (Ojcius et al. [31], Janoshazi and Solomon [32]) and so have carried out the present experiments on pink one step ghosts produced by a more gentle treatment which does not remove all the cell hemoglobin, leaving perhaps 1–2% (Nash and Meiselman [33]). Generally, the characteristics of pink ghosts are closer to those of native red cells than those of white ghosts. It is possible, though we do not think it probable, that the cross-linking between Cys-843 and Cys-885 that Rao [22] observed in white ghosts does not take place in pink ghosts, which means that we cannot exclude the possibility that these cysteines are available for reaction with intracellular pCMBS in our experiments.

After 1 h incubation, the inhibition of Cl^-/NO_3^- exchange appears to be non-competitive as the Lineweaver-Burk plot in Fig. 8 suggests (see legend). The K_i of 5.5 ± 6.3 mM (Table 1) is more than an order of magnitude greater than the figure of 0.16 mM for pCMBS inhibition of water flux, confirming the conclusion that the water inhibition site plays no role in Cl^- flux. The inhibitory effect of pCMBS shown in Fig. 8 includes both the zero time component and the 60 min component. The time course in Fig. 5 shows that the zero time inhibition amounts to 16% of the total Cl^- flux. By 60 min, the inhibition has risen by a further 34% to reach a total of 50% inhibition, so that the non-competitive process evident in Fig. 8 is the dominant process at 60 min.

As Segel [34] points out, irreversible binding leads to a Lineweaver-Burk plot similar to that for non-competitive inhibition. The experiments in Fig. 7 show that

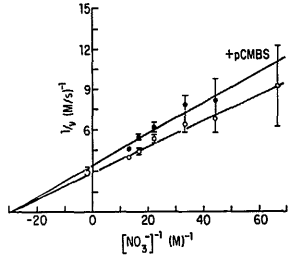

Fig. 8. Lineweaver-Burk plot of Cl^-/NO_3^- exchange inhibition by 1 mM pCMBS (1 h, room temperature). Inhibition is apparently non-competitive, with $K_i = 5.5 \pm 6.3$ mM. For the control: K_m for $NO_3^- = 33 \pm 4$ mM, slope = 0.095 ± 0.007 s and intercept = 2.86 ± 0.26 $(M/s)^{-1}$; for the pCMBS treated ghosts: $K_m = 34 \pm 7$ mM, slope = 0.115 ± 0.018 s and intercept = 3.38 ± 0.51 $(M/s)^{-1}$. The large error in K_i arises from the combinations required for the propagation of errors; the individual slopes and intercepts have much smaller errors. Though K_m is the same, as required for non-competitive pCMBS inhibition, the difference in the intercept, also required, only has $P < 0.1$ (*t*-test) and the apparent conclusion that pCMBS inhibition is non-competitive is not strictly significant.

binding to Cys-479 is reversible and in any case, the extracellular site is already filled long before 60 min. Thus, independent of whether the process in Fig. 8 is non-competitive inhibition or irreversible binding, pCMBS binding to an inhibitor site which is available from the cytosol leads to an allosteric change which modulates the rate-determining step for anion exchange, which is located in the 17 kDa membrane transport sequence in the transmembrane channel. We will denote the cysteine in the cytosol with which the entering pCMBS reacts as the site of the first-reactive-encounter. To qualify as the first-reactive-encounter site, the cysteine must not only react with pCMBS but also serve as an allosteric effector. The identity of the first-reactive-encounter site depends upon the detailed topology of the band 3/cytoskeleton interface. Since the kinetics are well defined and reproducible, the first-reactive-encounter site must be maintained in an ordered configuration. Suitable candidates include: Cys-201 and Cys-317 in the 43 kDa cytosolic domain (or possibly Cys-843 and Cys-885 in the loop between crossings 12 and 13) as well as cysteine groups in the linking proteins which are bound to band 3.

Sulfhydryl reactions in NEM-treated cells

After NEM-treatment, sulfhydryl groups that can react with pCMBS still remain in the cytoplasm. Though none of these appear to be on band 3, the sulfhydryl groups on proteins that interact with band 3 need to be enumerated since we have found cytosolic

pCMBS effects on anion exchange in NEM-treated red cells. Bands 4.1 and 4.2 associate with one another and the cytoplasmic domain of band 3, and bands 4.1 and 4.9 are associated with spectrin-actin complexes in the cytoskeleton (Bennett [35]). The presence of a major pCMBS binding site on band 4.9 reported by Ralston and Crisp [13] is confirmed by the finding of Benga et al. [27] that there is one (actually 0.84) sulfhydryl group on band 4.9. Benga et al. [27] also report sulfhydryl groups on bands 4.1 (actually 1.34) and band 4.2 (actually 1.29). These are in agreement with the report of Haest et al. [24] of NEM-nonreactive sulfhydryl groups on bands 4.1 (0.5 groups) and 4.2 (0.7 groups), and are also supported by the finding of Ralston and Crisp [13] that bands 4.1 and 4.2 are labelled by pCMBS in polyacrylamide gels.

Since we have presented evidence that conformational information can be exchanged between band 3 and band 4.5 (Janoshazi and Solomon [32]; Janoshazi et al. [36], the possible role of cytosolic NEM-nonreactive sulfhydryl groups on band 4.5 should be examined. Although Deziel et al. [18] found that all five of the cysteines on band 4.5 in a solubilized preparation reacted with high concentrations of NEM (15 mM, 37°C) within 20 min, the reaction with the three sulfhydryl groups available when band 4.5 was reconstituted into a bilayer took much longer. The initial phase of NEM reaction, which required about 20 min, is presumed to represent reaction with the extracellular Cys-429; the subsequent phase, which presumably represents reaction with the two available cytosolic cysteines, required two hrs for completion. Other investigators used less drastic NEM treatments. The pCMBS reactive sulfhydryl on band 4.5 reported by Benga et al. [27] in NEM-treated red cells may be presumed to be in the cytosol since, when access to the cytosol is prevented as in the experiments of Ojcius and Solomon [8], there is no pCMBS binding to band 4.5 in NEM-treated red cells. Although the finding of Haest et al. [24] of three (actually 3.6) NEM-nonreactive sulfhydryl groups in band 4.5 in ghosts appears to conform to the general conclusion that cytosolic cysteines are present after NEM treatment, the NEM concentrations used by Haest et al. [24] should have reacted with all the band 4.5 sulfhydryls and it is not clear why they did not.

In the experiments reported below, we treated our pink ghosts with 1 mM NEM (1 h, 37°C), twice the NEM concentration used by Rao [22] to block all the NEM-sensitive band 3 sulfhydryl groups in white ghosts, so it is likely that all the intracellular NEM-sensitive sulfhydryl groups on band 3 had reacted with NEM under our conditions. These considerations lead to the following conclusions about the availability of cysteine residues. In pink ghosts reacted with 1 mM pCMBS for 1 h in the absence of NEM, there probably are few or no unreacted sulfhydryls in the band 3 membrane

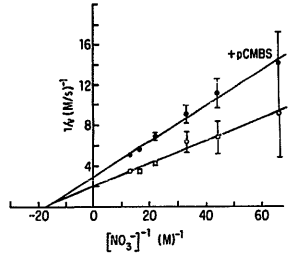

Fig. 9. Lineweaver-Burk plot of Cl^-/NO_3^- exchange inhibition of NEM-treated pink ghosts by 1 mM pCMBS for 1 h. Ghosts were pre-treated with 1 mM NEM for 1 h at 37°C, washed three times with PBS and then reacted with 1 mM pCMBS for 1 h at room temperature. $K_i = 2.1 \pm 1.2$ mM. For the control, K_m for $NO_3^- = 55$ mM, slope = 0.199 ± 0.009 s and intercept = 2.0 ± 0.3 $(M/s)^{-1}$; for the pCMBS treated ghosts slope = 0.173 ± 0.009 s and intercept = 2.97 ± 0.3 $(M/s)^{-1}$. One experiment typical of two.

domain, as we have discussed. In pink ghosts, treated additionally with 1 mM NEM there are almost certainly no unreacted sulfhydryls in the band 3 membrane domain. In both cases, the sulfhydryl groups are available on the linking proteins, ankyrin and bands 4.1 and 4.2.

pCMBS effects in NEM-treated cells

NEM is lipid soluble and readily permeates the ghost; since it is not an anion, there is no zero time inhibition by NEM competition for the anion exchange site. Nonetheless, 1 mM NEM by itself produces a small inhibition, reducing the Cl^- flux by a factor of 0.88 ± 0.07, in one experiment. In combination with 1 mM pCMBS, after 60 min incubation, the inhibition is apparently non-competitive with $K_i = 2.1 \pm 1.2$ mM, as shown in Fig. 9 and Table I; the relative anion flux falls by a factor of 0.61 ± 0.04 (two experiments). This decrease in relative velocity from 0.88 to 0.61 shows that NEM has changed the characteristics of the pCMBS binding site. This change is accompanied by a shift in K_m from 35 ± 3 mM in normal controls * to 55 mM in NEM-treated cells (Fig. 9, legend). We conclude that the Cl^- flux inhibition resulting from this combined treatment is to be attributed to an allosteric effect caused by pCMBS reaction with sulfhydryl sites probably on one or more of the linking proteins, or possibly band 4.5, but not on band 3. Thus, a perturbation on one protein in the band 3 transport complex leads to a modulation in the rate of anion transport, providing a further example of communication among the red cell membrane transport proteins.

* See footnote on p. 33.

Treatment with 12 mM NEM leads to more profound effects, inhibiting the flux by a factor of 0.45 ± 0.04 in one experiment (0.19 ± 0.01 in another). Further incubation with 0.5–1 mM pCMBS for 1 h reduces the Cl^- flux even further by a factor of 0.34 ± 0.03 in one experiment (0.09 ± 0.02 in the other). Inhibition is apparently non-competitive and $K_i = 1.4 \pm 1.5$ mM (Table 1, two expts). Since 1 mM NEM saturates all the NEM-reactive sulfhydryl groups in white ghost membranes [22], the additional inhibition caused by the higher NEM concentrations in pink ghosts may also reflect further interactions with other chemical groups, such as the α-amino groups of peptides and the imidazole group of histidine (Smyth [37]). Since these NEM and pCMBS treatments cause the Cl^- flux to fall to 10–25% of control, it is clear that disruption of so many sulfhydryl links and other bonds causes far-reaching damage to the membrane fabric.

The time course of the pCMBS inhibition of Cl^- flux is divided into two phases: the zero-time inhibition by competition for the anion transport site and the longer term effects which may clearly be ascribed to intracellular reactions. Assuming that pCMBS reacts with a sulfhydryl group and that the Cl^- inhibition is the result of a band 3 conformational change, it follows that interaction of pCMBS with the first-reactive-encounter site in the cytosol leads to an allosteric conformational change in band 3. After further treatment with NEM, the remaining candidate sulfhydryl groups responsible for the further Cl^- flux inhibition are those on the linking proteins, ankyrin and bands 4.1 or 4.2, which are not only not on band 3, but also are remote from the rate limiting region of band 3. Thus, we may regard band 3, together with its links, as part of a protein assembly which can react cooperatively and allosterically to control and modulate the anion flux mediated by band 3.

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