Biochimica et Biophysica Acta, 772 (1984) 313–320
Elsevier

BBA 72085

SITE OF RED CELL CATION LEAK INDUCED BY MERCURIAL SULFHYDRYL REAGENTS

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(Received October 4th, 1983)
(Revised manuscript received January 30th, 1984)

Key words: Cation transport; Water transport; Band 3; Membrane reconstitution; Mercurial sulfhydryl reagent; (Erythrocyte membrane)

It has been suggested that the human red cell anion transport protein, band 3, is the site not only of the cation leak induced in human red cells by treatment with the sulfhydryl reagent pCMBS (*p*-chloromercuribenzene sulfonate) but is also the site for the inhibition of water flux induced by the same reagent. Our experiments indicate that *N*-ethylmaleimide, a sulfhydryl reagent that does not inhibit water transport, also does not induce a cation leak. We have found that the profile of inhibition of water transport by mercurial sulfhydryl reagents is closely mirrored by the effect of these same reagents on the induction of the cation leak. In order to determine whether these effects are caused by band 3 we have reconstituted phosphatidylcholine vesicles containing only purified band 3. Control experiments indicate that these band 3 vesicles do not contain ($Na^+ + K^+$)-ATPase as measured by ATP dephosphorylation. pCMBS treatment caused a significant increase in the cation leak in this preparation, consistent with the view that the pCMBS-induced cation leak in whole red cells is mediated by band 3.

Introduction

The sulfhydryl reagent pCMBS (*p*-chloromercuribenzene sulfonate) induces a cation leak in human red cells (Knauf and Rothstein [1]) and also inhibits water transport (Macey and Farmer [2]). It has been suggested by Brown et al. [3] that band 3, the anion transport protein, contains the site responsible for inhibition of water flux, based on experimental results obtained with DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), another sulfhydryl reagent. We have suggested that band 3 is also the site of the cation leak and that the same SH group is responsible for both water transport inhibition and the induced cation leak [4]. The experiments described in the present paper provide evidence for a cation leak that is to be attributed to a SH group in band 3.

Materials and Methods

Materials. *p*-Chloromercuribenzene sulfonate (pCMBS), *p*-aminophenylmercuric acetate (pAPMA), *p*-chloromercuribenzoic acid (pCMB), *N*-ethylmaleimide, L-cysteine hydrochloride (hydrate) and β-mercaptoethanol were obtained from Sigma Chemical Co (St. Louis, MO). 4,4′-Diisothiocyano-2,2′-disulfonic stilbene (DIDS) was obtained from the U.S. Biochemical Corp. (Cleveland, OH). L-α-Phosphatidylcholine was obtained from Sigma or from Avanti Polar Lipids (Birmingham, AL). Sephadex G-25 and G-50 was purchased from Pharmacia (Piscataway, NJ).

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pAPMA, *p*-aminophenylmercuric acetate; pCMB, *p*-chloromercuribenzoic acid; pCMBS, *p*-chloromercuriphenylsulfonic acid.

DEAE-cellulose, Bio-Gel A-15M, and Bio-Beads SM-2 were obtained from Bio-Rad (Richmond, CA). Rubidium (^{86}RbCl, spec. act. 2–8 mCi/mg) was purchased from New England Nuclear (Boston, MA). All other chemicals were of reagent grade.

Analytical procedures. Protein was determined by the method of Lowry et al. [5] with bovine serum albumin as a standard. For samples containing Triton X-100, 3% SDS was added to the alkaline copper reagent to prevent precipitate formation. Protein associated with vesicles was determined by dissolving vesicles in 10% sodium deoxycholate and 0.01 M NaOH and carrying out a Lowry analysis with control vesicles (no protein) as a blank. Gel electrophoresis was performed according to the method of Fairbanks et al. [6] except that 7.5% polyacrylamide gels were used instead of 5.6%. Gels were run on all band 3 preparations used for reconstitution and were found to contain at least 95% pure band 3.

$(Na^+ + K^+)$-ATPase activity was assayed by incubating, for 30 min at 37°C, 0.5 ml of a solution containing 0.4–0.9 mg ghost or purified band 3 protein/ml, 140 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 30 mM imidazole (pH 7.25), $\pm 5 \cdot 10^{-4}$ M ouabain, and 6 mM ATP. The reaction was stopped at 30 min by the addition of 0.7 ml of a solution containing 0.5 M H_2SO_4, 0.5% ammonium molybdate, and 2% SDS. After vortexing, 0.02 ml of a solution containing 1.2% sodium metabisulfite, 1.2% sodium sulfite, and 0.2% 1-amino-2-naphthol-4-sulfonic acid was added. The color was allowed to develop for 30 min before the absorbance at 650 nm was determined. Since this assay determines inorganic phosphate, all ghost samples were washed, or protein extracts extensively dialysed against 10 mM Tris-HCl (pH 7.5), prior to assay. Protein determinations by the Lowry method were confirmed by duplicate assay using the Bradford technique (Bio-Rad), as Tris tends to interfere with the Lowry assay.

Intact cell experiments. Fresh heparinized (10 U/ml) adult human blood was used for both whole cell experiments and the preparation of red cell ghosts for the band 3 reconstitution studies. In whole cell experiments, cells were centrifuged, plasma and buffy coat aspirated, then resuspended in 5 vol. of a buffer of the following composition (in mM); NaCl, 142; KCl, 4.4; Na_2HPO_4 + NaH_2PO_4, 5; $CaCl_2$, 1.2; $MgCl_2$, 0.5; glucose, 15; pH 7.4, 23°C. Cells were washed four times in this buffer, and then resuspended to 50% hematocrit for treatment with 12 mM *N*-ethylmaleimide for 30 min at 37°C. Red cells were then centrifuged and resuspended in the above buffer containing mercurial reagents and $^{86}Rb^+$. The cumulative K^+ flux was computed from the $^{86}Rb^+$ uptake (considered to be a tracer for K^+) and the net K^+ efflux measured analytically, using the technique of Poznansky and Solomon [7]. Cation concentrations were determined with an Instrumentation Lab Model 143 flame photometer (Watertown, MA).

Experiments on water permeability were carried out on red cells that had been treated with either the anion transport inhibitor, DIDS, or the sulfhydryl reagent, *N*-ethylmaleimide. For the DIDS treatment, red cells were incubated with 100 μM DIDS for 30 min in the dark at 37°C; this treatment inhibited > 98% of the anion transport. The *N*-ethylmaleimide treatment consisted of incubation with 12 mM *N*-ethylmaleimide for 1 h at 37°C, the concentration used by Rao [8] to cause *N*-ethylmaleimide interaction with all five intracellular band 3 SH groups. Water permeability was measured at 23°C by the stopped-flow method as described by Terwilliger and Solomon [9].

Purification of band 3. Band 3 was prepared and reconstituted into lipid vesicles according to the method of Lukacovic et al. [10]. Red cell ghosts were washed with isotonic saline and solubilized overnight at 4°C in 0.5% Triton X-100. This extract was applied to a DEAE-cellulose column and bands 3, 4.2, and glycophorin were eluted with high salt concentration. This high salt fraction was applied to a (*p*-(chloromercuri)benzamido) ethyl-agarose 4B gel (synthesized according to Lukacovic et al.) which removed glycophorin and band 4.2. Pure band 3 was eluted with 0.1 mM cysteine after a low salt wash. 15 mM β-mercaptoethanol was added immediately after elution to prevent protein aggregation. Protein was then concentrated in an Amicon Model 52 stirred cell using a YM 10 ultrafiltration membrane (Amicon Corp., Lexington, MA) before Triton removal.

Calculations on the possible copurification of the $(Na^+ + K^+)$-ATPase along with purified band

3 are based on the following data: band 3 is 0.25 of ghost protein; there are 200 copies of $(Na^+ + K^+)$-ATPase and $1.1 \cdot 10^6$ copies of band 3 in a single red cell ghost, P_i produced by $(Na^+ + K^+)$-ATPase in the ghosts from which band 3 was prepared (four experiments) is 225 nmol P_i/mg protein per 30 min. Prior to reconstitution, we measured the $(Na^+ + K^+)$-ATPase of a purified band 3 suspension which contained 0.306 mg band 3. The lowest standard used in the assay was 5 nM P_i. The $(Na^+ + K^+)$-ATPase activity of band 3 was too low to detect.

Reconstitution: Efflux experiments. Triton was removed by incubating 5 ml of the concentrated band 3 solution with 1.5 g Bio-Beads SM-2 for 4 h at 6°C. Prior to use, Bio-Beads were treated with methanol (Holloway [11]) and washed with deionized H_2O. Phosphatidylcholine (from egg yolk, stored in chloroform) was added to a round bottom flask and rotated under vacuum to obtain a thin layer coating the flask, The Triton-free protein solution in 2.0 ml of 36 mM Na_2HPO_4, 5 mM KCl (pH 7.5), containing 0.5 mCi $^{86}Rb^+$ was then added. The suspension formed by gentle swirling was then sonicated in a Bransonic B12 bath sonicator (Shelton, CN) for 3 min at 23°C and trapped radioactivity was separated from free radioactivity by gel filtration (Sephadex G-25) at 4°C. About 6 ml of vesicles were collected and 2-ml aliquots ± the sulfhydryl reagents were placed in 0.25 inch dialysis tubing which had been prepared for use by boiling in distilled water for 15 min. At the beginning of the experimental period, sulfhydryl reagents were also added to the transport buffer (36 mM Na_2HPO_4/5 mM KCl (pH 7.5)) in which the tubing was immersed. The protein concentration in these vesicles was 0.25 mg/20 mg lipid. $^{86}Rb^+$ efflux was measured at 23°C as described for sulfate efflux by Lukacovic et al. [10].

Reconstitution: Influx experiments. In order to obtain a more accurate measure of the kinetics, we devised the influx method, which uses a Sephadex column to separate the vesicles from the $^{86}Rb^+$ solution in which the uptake measurements were carried out, rather than measuring efflux from suspensions of vesicles contained in dialysis tubing.

To a 100 ml round bottom flask, 20 mg phosphatidylcholine (brought to 1 ml volume by addition of chloroform) was added and a lipid coat was formed by rotation under vacuum. To this lipid coat, 4 ml of Triton-free band 3 solution was added and a suspension was formed by gentle swirling. One ml fractions of this suspension (in 36 mM Na_2HPO_4 ± 5 mM KCl (pH 7.5)) were sonicated for 2 min at 23°C. The vesicles were stored overnight at 4°C and sonicated an additional 30 s the next day. The vesicles were then passed down a Sephadex G-50 column (45 × 1.5 cm) to remove β-mercaptoethanol (added to stabilize the protein after purification; see Lukacovic et al. [10]). Protein concentration at this point was 0.06–0.12 mg/ml.

Rb^+ influx was measured by incubating 0.1 ml $^{86}Rb^+$ in 36 mM Na_2HPO_4 + 5 mM KCl (pH 7.4) (spec. act. 10000–15000 cpm/nmol Rb^+) with 0.1 ml of vesicles, which had been pre-incubated with pCMBS (or buffer) for 30 min at 23°C. After a set time (usually 15 min) the vesicles were added to a 25 ml buret containing Sephadex G-50. Vesicles were eluted in 2 min at 4°C with the phosphate buffer (+5 mM KCl) and a clear separation from free radioactivity was obtained. The trapped $^{86}Rb^+$ was then counted in a Nuclear Chicago Gamma Counter (Des Plaines, IL). In order to determine the amount of $^{86}Rb^+$ which was bound to the vesicles but not transported, a run was carried out in which the vesicles and the radioactivity were mixed at 0°C and the gel filtration was performed immediately. Counts associated with the vesicles under these conditions were subtracted from all other incubation time points carried out at 20°C or above.

It was observed that high leak rates and low pCMBS stimulation of the leak were obtained when the chloroform used in lipid coat formation had been exposed to light, or when lipid stored in hexane was used.

Results and Discussion

Characteristics of SH groups on band 3

Rao [12] has shown that there are five SH groups on the intracellular portions of band 3, all of which are reactive to either *N*-ethylmaleimide or pCMBS; the pCMBS interaction is inhibited by *N*-ethylmaleimide and vice versa. At pH 7.4, pCMBS is a lipid insoluble anion; Rao [8] has

shown that covalent reaction of the specific anion transport inhibitor, 1-isothiocyano-4-benzene sulfonic acid with resealed ghosts increases the half-time for pCMBS entry into the ghosts from 40 min in the control to 190 min. This experiment shows that sulfonic acid anion transport inhibitors in the same class as DIDS effectively prevent interaction of pCMBS with the five intracellular SH groups. Brown et al. [3] observed that *N*-ethylmaleimide did not inhibit water transport and did not protect the SH group with which DTNB interacted. Since Rao [12] had shown that all five of the intracellular SH groups reacted with *N*-ethylmaleimide, the observation of Brown et al. [3] made it likely that none of these five SH groups was responsible for water transport inhibition. In two stopped-flow experiments we have found [4] that neither DIDS, nor *N*-ethylmaleimide has any effect on pCMBS inhibition of osmotic water permeability, which shows that none of the five intracellular SH groups is responsible for the pCMBS inhibition. A sixth SH group has been found on band 3 by Steck et al. [13] and by Ramjeesingh et al. [14], who have located this group in the 17 kDa membrane-bound fragment between the chymotrypsin and trypsin cuts in band 3.

Cation leak in whole red cells induced by mercurial sulfhydryl reagents

In order to establish whether this group, which we have denoted as the cryptic, 6th SH group, could be responsible for the cation leak, it was first necessary to show that the pCMBS-induced cation leak was still present in red cells that had been treated with *N*-ethylmaleimide under the conditions that Rao had used to block the five intracellular SH groups. Twelve experiments were carried out by measuring $^{86}Rb^+$ uptake in *N*-ethylmaleimide-treated red cells. The results of one of these experiments, typical of three in which the pCMBS dose response was measured, is shown in Fig. 1, from which it can be seen that the pCMBS-induced cation peak persists in *N*-ethylmaleimide-treated red cells. Although the pCMBS concentration for 50% stimulation of the induced cation leak can not be determined quantitatively from these experiments, it is consistent with the 0.1–0.2 mM pCMBS concentration half-point for inhibition of osmotic water transport (Chasan and Solomon, unpublished data; Levitt and Mlekoday [15]). Another experiment in the same series showed that the effect of 0.1 mM pCMBS could be completely suppressed by 5 mM β-mercaptoethanol, consistent with participation of an SH group. These experiments show that pCMBS can react with an *N*-ethylmaleimide-insensitive SH group to cause a large cation leak, similar to the pCMBS reaction with an *N*-ethylmaleimide-insensitive SH group which inhibits water transport, as discussed above.

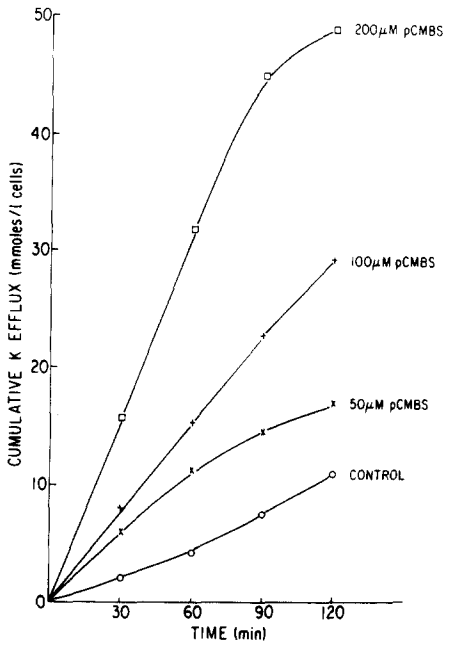

Fig. 1. Cation leak induced by pCMBS in *N*-ethylmaleimide-treated red cells. Uptake of $^{86}Rb^+$ in red cells previously treated with 12 mM *N*-ethylmaleimide for 30 min at 37°C. The curves were drawn by eye to connect the points. No control data is shown for normal cells without *N*-ethylmaleimide treatment, since these data are not relevant to the pCMBS effect we are studying. However, normal red cells maintain an essentially constant cell K^+ for many hours in our buffer [7].

Sha'afi and Feinstein [16] have shown that other mercurial SH reagents such as *p*-chloromercuribenzoate (pCMB) and *p*-aminophenylmercuric acetate (pAPMA) also inhibit osmotic water transport whereas the nonmercurial SH reagent iodoacetamide is without effect. The results of one experiment typical of three in which these SH reagents have been applied to *N*-ethylmaleimide-treated red cells, are given in Table I. In contrast

TABLE I

EFFECT OF SULFHYDRYL REAGENTS ON CATION FLUXES IN *N*-ETHYLMALEIMIDE-TREATED RED CELLS

The experimental period was 2 h at 23°C. Cells were pretreated with *N*-ethylmaleimide as described in Methods and the sulfhydryl reagents were added with the $^{86}Rb^+$ at the beginning of the experiments. IAM, iodoacetamide.

	Hct		Net cation flux (efflux − influx) (mM/(l ave cell))		Total K^+ efflux (mM/(l ave cell))
	Initial	Final	Na^+	K^+	
N-Ethylmaleimide-treated red cells	0.384	0.377	7.6	8.1	10.0
+1 mM IAM	0.395	0.390	3.9	5.3	7.9
+0.25 mM pCMB	0.364	0.320	−11.5	27.0	31.2
+0.5 mM pAPMA	0.341	0.320	−29.2	45.9	45.8

to normal red cells which maintain an essentially constant cell K^+ for many hours in our buffer [7], *N*-ethylmaleimide-treated cells leak K^+. Since there is a net cation leak in these cells, but the water content remains almost constant, as the hematocrit shows, there must be a change in the Donnan potential difference across the membrane so that anions can enter to maintain the volume. As Table I shows there is very little change in the total K^+ efflux when 1 mM iodoacetamide is added to the *N*-ethylmaleimide-treated cells, whereas either of the mercurial sulfhydryl reagents increases the total K^+ efflux by a factor of three to five. Treatment with the mercurials also induces a net Na^+ leak down the Na^+ gradient, in agreement with the observation of Sutherland et al. [17] that pCMBS treatment induced roughly equivalent fluxes of Na^+ and K^+ across the cell membrane. The conclusion to be drawn from Table I is that a cation leak can be induced in *N*-ethylmaleimide-treated red cells by mercurial sulfhydryl reagents analogous to the inhibition of water flux produced by these same sulfhydryl reagents. Sha'afi and Feinstein [16] used 1 mM concentrations of the mercurials in their studies on water flux whereas we were limited to lower concentrations because *N*-ethylmaleimide-treated red cells hemolysed at higher mercurial concentrations. In order to complete the comparison between cation leak and water flux inhibition we have shown that the pCMB and pAPMA inhibition of water flux (Sha'afi and Feinstein [16]) also applies to *N*-ethylmaleimide-treated cells (Toon and Solomon, unpublished data). Thus, the selectivity characteristics of the reactions with sulfhydryl reagents are very similar for these two processes and are consistent with the view that they are both mediated by the same SH group.

Cation leak in reconstituted band 3 vesicles

Although these red cell experiments show that an *N*-ethylmaleimide-insensitive SH group is responsible for a large cation leak, they do not localize the SH group to band 3, since there are SH groups on other transmembrane proteins, such as the $(Na^+ + K^+)$-ATPase and band 4.5, which might be responsible for the effect. We therefore carried out experiments on separated band 3, incorporated into phosphatidylcholine vesicles. Band 3 was isolated by the method of Lukacovic et al. [10] which provides 95% pure band 3 containing no band 4.5. The authors found that the anion transport effectiveness of sulfate exchange in reconstituted band 3 vesicles was 24% of that in the intact red cell and subsequent control experiments in this laboratory have confirmed this finding.

In order to determine whether any cation flux in the reconstituted vesicles is to be attributed to traces of $(Na^+ + K^+)$-ATPase not removed from band 3, we measured the $(Na^+ + K^+)$-ATPase activity in purified band 3 and found that the activity was too low to detect. We therefore used the concentration of the lowest standard in our assay of $(Na^+ + K^+)$-ATPase activity as a conservative estimate of the sensitivity of the detection method.

This leads to an upper limit of $3.3 \cdot 10^{-6}$ molecules of $(Na^+ + K^+)$-ATPase per molecule of band 3. The maximum $(Na^+ + K^+)$-ATPase contribution to the leak flux in reconstituted vesicles can be computed from this figure and the Rb^+ leak flux of 0.6 mol Rb^+/mol $(Na^+ + K^+)$-ATPase per s observed for porcine kidney $(Na^+ + K^+)$-ATPase reconstituted into azolectin vesicles (Karlish and Stein [18]). If the Karlish and Stein data are representative of the human red cell $(Na^+ + K^+)$-ATPase, the maximum $(Na^+ + K^+)$-ATPase contribution would be 0.2% of our observed baseline Rb^+ influx.

We have carried out more than 12 reconstitution experiments, in the course of which we have become increasingly successful at making reconstituted vesicles which are well sealed against K^+ leakage in baseline experiments in the absence of pCMBS. In four efflux experiments, we found that 0.2 mM pCMBS increased the cation leak by a factor of 1.3 ± 0.1 which is significant at a level of $p < 0.02$ (t-test). Data from a typical experiment is shown in Fig. 2. It can be seen that 0.2 mM *N*-ethylmaleimide caused no significant increase in K^+ flux above the baseline and that 5 mM β-mercaptoethanol reversed the pCMBS effect (two experiments). Although, as Fig. 2 shows, pCMBS does induce a cation leak in reconstituted band 3 vesicles, the effect is very much smaller than in whole red cells (Fig. 1).

We used the influx method in a second set of experiments to avoid the problems associated with measurement of efflux from suspensions of vesicles contained in dialysis tubing. In two experiments, with $KCl_{inside} = 0$ mM and $KCl_{outside} = 5$ mM, 0.5 mM pCMBS increased the Rb^+ influx by an average of 56% (59% and 52%); when KCl_{inside} was increased to 5 mM, the pCMBS stimulation increased to 174% (137% and 210%). In the absence of intravesicular KCl, we assume the $^{86}Rb^+$ uptake takes place by Na^+: K^+ exchange as in pCMBS-treated whole red cells. Since the addition of 0.5 to 1.0 mM pCMBS caused an increased cation leak under all conditions, these results are consistent with the view that the pCMBS induced cation leak in whole red cells is mediated by band 3.

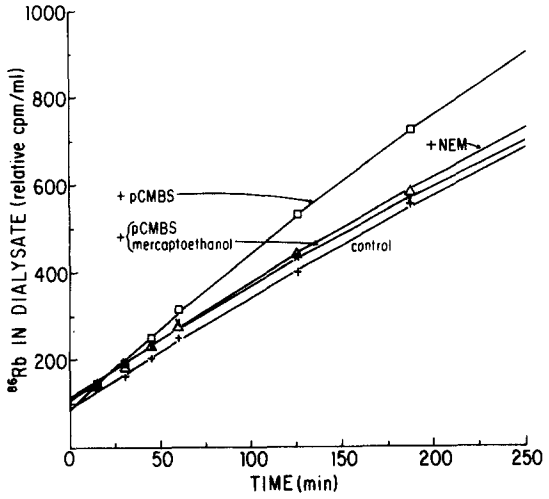

Fig. 2. Effect of pCMBS on cation efflux from reconstituted band 3 vesicles. Band 3 was prepared and reconstituted into lipid vesicles as described in the text. The curves are exponentials fitted to the data by least squares. The figure is typical of four experiments with 0.2 mM pCMBS, in two of which 0.2 mM *N*-ethylmaleimide and 5 mM β-mercaptoethanol were added.

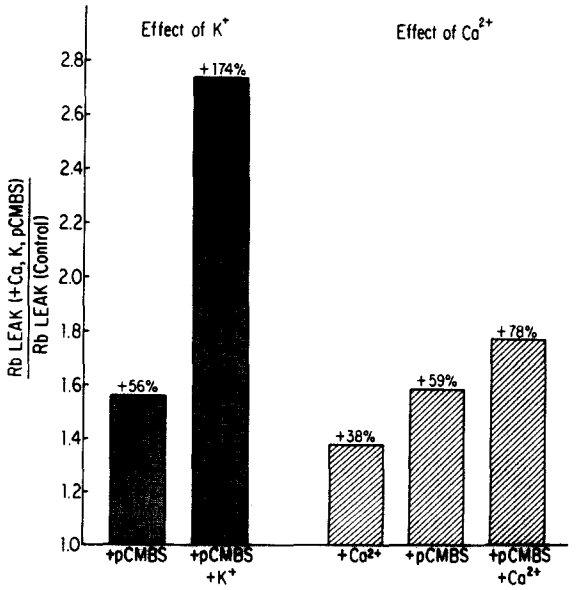

Fig. 3. Effect of pCMBS on cation influx into reconstituted band 3 vesicles. All of the points in these experiments were taken at 15 min; the extravesicular concentrations were 36 mM Na_2HPO_4 plus 5 mM KCl. Intravesicular KCl (5 mM) was added with the protein suspension before sonication as described in Methods. pCMBS (1 mM) or $CaCl_2$ (0.5 mM), when present, were added to the extravesicular buffer.

Band 3 in the reconstituted vesicles has lost its sidedness, as Lukacovic et al. [10] showed. Furthermore, the lipid composition in our phosphatidylcholine vesicles differs from that in the normal band 3 environment. Although it is not possible to make a quantitative comparison between the effects of pCMBS on whole cells, shown in Fig. 1, with that on vesicles shown in Fig. 3, it seems that there is a much greater effect in whole cells. We conclude that the present findings are necessary, but not sufficient, to show band 3 to be the site for the pCMBS induced cation leak in whole cells. That is, pCMBS does induce a cation leak in band 3; in order to be sure that this is the only induced leak, it would be necessary to show that there was no *N*-ethylmaleimide-insensitive, pCMBS-sensitive SH group on any other red cell transmembrane protein that also controlled a cation leak.

Comparison of cation fluxes in reconstituted band 3 vesicles and whole cells

Control studies show that our phosphatidylcholine vesicles, in the absence of band 3, were essentially impermeable to $^{86}Rb^+$ with and without added pCMBS. The baseline K^+ leak in our reconstituted band 3 preparation, determined by the method used by Lukacovic et al. [10] to measure sulfate uptake is 7.8 nmol K^+/nmol band 3, min or $1.4 \cdot 10^3$ molecules K^+/red cell per s. Karlish and Stein's value of the Rb^+ leak flux in the absence of substrates other than monovalent cations is equivalent to 120 molecules/red cell per s and this figure needs to be increased by a factor of about 20 to $2.4 \cdot 10^3$ molecules/red cell per s in the presence of Mg^{2+} and ATP (Karlish et al. [19]). This is in good agreement with the K^+ leak in intact red cells which we have calculated from the data given by Hall et al. [20] to be $2.8 \cdot 10^3$ molecules/red cell per s. Since the red cell is very tight to cations, these calculations indicate that our preparation of reconstituted band 3 vesicles does not induce a large cation leak into the system.

In connection with these comparisons, we carried out some additional exploratory experiments on our band 3 vesicles whose results are also shown in Fig. 3. In one experiment, 0.5 mM Ca^{2+} was found to stimulate the cation leak. When 1.0 mM pCMBS and 0.5 mM Ca^{2+} were added together, the effect was not much less than the sum of the two added separately, revealing no synergy in the action of these two substances at these concentrations. This observation may be related to the well known observation that intracellular Ca^{2+} stimulates K^+ leakage, a phenomenon which has also been observed in red cell vesicles (see Refs. 21 and 22). The observation, in two experiments, that the addition of intravesicular KCl stimulates the pCMBS induced cation leak by a factor of about three was entirely unexpected. It is a phenomenological observation that is very difficult to rationalize. The presence of 5 mM KCl on the inside of the vesicle does alter the environment and may even induce a small membrane potential gradient, depending upon the relative permeability of the monovalent ions in the system. But it is difficult to accept these differences as explanations for the phenomenon since band 3 is not sided in this preparation. The results of our experiments on the net cation leak in whole red cells, in agreement with those of Sutherland et al. [17], indicate that the native pCMBS-induced leak in the red cell does not discriminate between Na^+ and K^+. Yet Fig. 3 shows that intravesicular K^+ increases the pCMBS-induced Rb^+ leak flux in our reconstituted band 3 preparation. In descriptive terms, this is more like a K^+-K^+ exchange than any other process that comes to mind. Is it related to the K^+-K^+ exchange in the native red cell? Does it have any bearing on the suggestion of Fossel and Solomon [23] that band 3 and the ($Na^+ + K^+$)-ATPase are closely related in the native membrane? Is there ($Na^+ + K^+$)-ATPase present in our preparation which has escaped detection because it is shorn of its phosphorylating activity? These are questions that warrant further investigation of the cation leak properties of the reconstituted band 3 preparation.

Acknowledgements

This research was supported in part by USPHS grant HL14820 and GM00782. This work was done during the tenure of a research fellowship (MFL) from the American Heart Association, Western Mass. Division, number 13-401-812.

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