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p-Chloromercuribenzenesulfonic acid stimulation of chloride-dependent sodium and potassium transport in human red blood cells

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The organic mercurial *p*-chloromercuribenzenesulfonic acid (PCMBS) reversibly increases fluxes of sodium and potassium across the human red blood cell membrane. We examined the effect of different monovalent anions on cation fluxes stimulated by PCMBS. A substantial portion of the fluxes of both cations was found to have a specific anion requirement for chloride or bromide, and was not observed when chloride was replaced by nitrate, acetate or methylsulfate. The chloride-dependent component of the cation fluxes was only observed when the cells were exposed to PCMBS concentrations of 0.5 mM or greater. Furosemide (1 mM) did not inhibit the PCMBS-stimulated cation fluxes. The observed anion specificity is directly associated with the transport process rather than PCMBS binding to the membrane. A portion of the potassium transport stimulated by PCMBS appears to involve K^+-K^+ exchange; however, $Na^+ + K^+$ cotransport is not stimulated by this sulfhydryl reagent.

Introduction

Compounds which bind to sulfhydryl groups have long been known to alter the permeability characteristics of cell membranes [1]. The organic mercurial PCMBS increases fluxes of sodium and potassium across the human red blood cell membrane [1–3]. This effect of PCMBS is thought to be due to its interaction with sulfhydryl groups within the interior of the cell membrane [2], and is rapidly reversed by exposure of the cells to compounds containing free sulfhydryl groups, such as cysteine or dithiothreitol [2,3]. *N*-Ethylmaleimide, an alkylating agent which reacts in an irreversible manner with membrane sulfhydryl groups, also increases potassium fluxes across red cell membranes, although, unlike the case with PCMBS, sodium fluxes are not stimulated [1,4–7]. Although the mechanisms of the cation transport stimulated by these sulfhydryl reagents are not clear, recent evidence suggests that the potassium transport stimulated by *N*-ethylmaleimide may be related to pathways which function in the unaltered cell. Lauf and co-workers first reported in low-potassium sheep red cells that potassium transport stimulated by *N*-ethylmaleimide has a specific anion requirement for chloride or bromide [4], and later showed that this transport is partially inhibited by furosemide [8,9]. Untreated low-potassium sheep red cells also exhibit a chloride-dependent, furosemide-sensitive potassium transport; this process is stimulated by cell swelling [10].

Human red cells also exhibit chloride-dependent, furosemide-sensitive potassium transport

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Abbreviations: PCMBS, *p*-chloromercuribenzenesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

[11], part of which is due to a cotransport of $Na^+ + K^+$, or $Na^+ + K^+ + 2Cl^-$ [12]. Recently, several laboratories [5,7,13,14] have reported that *N*-ethylmaleimide has a similar effect in human red cells to that in low-potassium sheep cells, stimulating a chloride-dependent potassium transport which is partially inhibited by furosemide. *N*-Ethylmaleimide was also found to have an inhibitory effect on furosemide-sensitive sodium fluxes [5,7], leading Lauf et al. [5] to suggest that *N*-ethylmaleimide might possibly uncouple $Na^+ + K^+$ cotransport while at the same time stimulating $K^+ + Cl^-$ cotransport.

These results with *N*-ethylmaleimide led us to investigate the anion dependence of cation fluxes stimulated by PCMBS. Preliminary results of this study have been presented in abstract form [15].

Materials and Methods

Blood was drawn from normal human donors at the start of each experiment. Following removal of the plasma and buffy coat, the red cells were washed three times in ice-cold, isotonic (300 mosmol/kg) NaX (X = chloride, bromide, nitrate, acetate or methyl sulfate).

For efflux experiments, the cells were preincubated in loading solutions containing 9 mM KX, 1 mM KH_2PO_4, 20 mM glycylglycine (pH 7.4 at 37 or 4°C), and sufficient NaX to adjust the osmolality to 300 mosmol/kg. The preincubations were carried out for 3 h at 37°C for loading with ^{86}Rb, and for 12 h at 4°C followed by 2 h at 37°C in the presence of 0.1 mM ouabain for ^{22}Na loading. Following the loading incubations, the cells were washed three times in isotonic NaX, then incubated (10 or 15 min, 37°C, 10% hematocrit) in the above solutions in the presence or absence of PCMBS. Following this, the cells were washed an additional three times in ice-cold, isotonic tetramethylammonium ($(CH_3)_4NX$) and then test-incubated 20 min at 37°C, 5% hematocrit. The ionic composition of the test-incubation media are given in the individual table and figure legends; all such media contained 10 mM glucose and 20 mM glycylglycine (pH 7.4 at 37°C). Samples of test-incubation suspensions were taken at 5, 10, 15 and 20 min and immediately centrifuged, and a portion of the supernatant from each sample as well as a portion of each whole suspension were counted for gamma radioactivity. The efflux rate constant for each incubation was then determined from the slope of the plot of $-\ln(1 - (cpm_{supe}/cpm_{ws}))$ versus time for the four samples; cpm_{supe} and cpm_{ws} represent counts per minute in supernatant and whole suspension, respectively. Data yielding lines with a regression coefficient of less than 0.99 were discarded.

For influx experiments, cells were first preequilibrated with chloride or nitrate by preincubation at 37°C in the above loading media for two successive 30 min periods. The cells were centrifuged and resuspended in fresh media following the first 30 min incubation. The cells were then washed three times in isotonic NaCl or $NaNO_3$, then incubated in the same media used in preincubation for 10 min at 37°C, 10% hematocrit in the presence or absence of 1 mM PCMBS. The cells were then washed three times in ice-cold, isotonic $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$, and added at a hematocrit of 10% to test-incubation media containing 75 mM potassium and either zero or 75 mM sodium. The influx period was then initiated by addition of an aliquot of ^{86}Rb to each incubation. At 2 and 12 min, samples from each incubation were taken into 5 vol. of ice-cold incubation medium, and the cells were then centrifuged and washed three times with 30 vol. of ice-cold, isotonic $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$ to remove extracellular isotope. Following the third wash, the cells were centrifuged in specially fabricated nylon tubes [16], and the separated cells were weighed, lysed and assayed for gamma radioactivity. Samples of media from each incubation were also counted and the specific activity of each medium calculated. Influxes were then calculated from these specific activities and the difference in cpm per unit weight of cells between the 2- and 12-min samples.

PCMBS, cysteine, dithiothreitol, DIDS and valinomycin were obtained from Sigma Chemical Co., St. Louis, MO. Furosemide was the gift of Hoechst-Roussel Pharmaceuticals, Somerville, NJ. Tetramethylammonium salts of chloride, bromide, nitrate, acetate and methyl sulfate were obtained from Eastman Organic Chemicals, Rochester, NY. Solutions of $(CH_3)_4NNO_3$ were routinely filtered through activated charcoal to remove a yellow

discoloration, a procedure that had no effect on the osmolality.

Results

Fig. 1 shows the effect of different concentrations of PCMBS on potassium and sodium efflux rate constants in cells containing either chloride or nitrate as the major intracellular anion. In this experiment, cells were preincubated in either chloride or nitrate media and then incubated 10 min in these same media in the presence of the PCMBS concentration indicated on the abscissa. They were then washed to remove excess PCMBS and incubated in sodium- and potassium-free flux media containing $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$. Exposure of cells to PCMBS concentrations of 0.3 mM or less caused only a mild stimulation of cation effluxes which was similar in chloride and nitrate cells. However, higher concentrations of PCMBS markedly stimulated both potassium and sodium effluxes in chloride, but not nitrate, incubations. Addition of 1 mM furosemide, 0.1 mM ouabain or 5 mM HCO_3 (as $NaHCO_3$) to the flux media had no effect on cation effluxes from cells

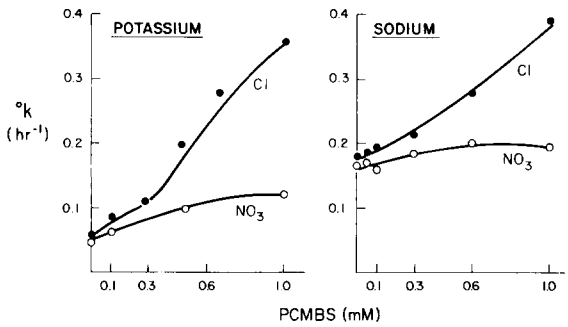

Fig. 1. Effect of exposure to different concentrations of PCMBS on rate constants for potassium (^{86}Rb) efflux ($^{\circ}k_K$, left panel) and sodium (^{22}Na) efflux ($^{\circ}k_{Na}$, right panel). Closed symbols indicate data for chloride-loaded cells, open symbols indicate data for nitrate-loaded cells. The cells were loaded with ^{86}Rb or ^{22}Na in either chloride or nitrate media as described in Materials and Methods, then incubated 10 min in the same media in the presence of the PCMBS concentration indicated on the abscissa. The cells were then washed three times in ice-cold, isotonic $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$, and test-incubated in media containing 10 mM glucose, 20 mM glycylglycine (pH 7.4 at 37°C), and sufficient $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$ to adjust the osmolality to 300 mosmol/kg. Each point on the plot represents the mean of two separate flux determinations.

treated with 1 mM PCMBS in either chloride or nitrate media. However, exposure of cells to 1 mM PCMBS in media already containing 1 mM furosemide yielded cation fluxes 20–30% lower than when the diuretic was not present (data not shown), suggesting furosemide interferes with PCMBS binding or reacts directly with PCMBS to form an inactive compound.

Table I shows the effects of several different monovalent anions on PCMBS-stimulated cation efflux rate constants. Cells were preequilibrated with the different anions, then incubated with 1 mM PCMBS for 10 min in the presence of the same anion. Test incubations were performed in tetramethylammonium media containing the same anion present during the preincubations. Control cells not exposed to PCMBS exhibited similar rate constants for potassium and sodium efflux regardless of the anion present. Following treatment with PCMBS, however, efflux rate constants in chloride and bromide incubations were more than double those in nitrate, acetate and methyl sulfate incubations. Results were qualitatively similar for both potassium and sodium.

Table II shows that the anion specificity of PCMBS-stimulated cation effluxes is associated with the actual transport process rather than with PCMBS binding to the membrane. Cells were preincubated in either chloride or nitrate media, then incubated 10 min in identical media containing 1 mM PCMBS. Next, half of each group of cells was washed and incubated in chloride media and half in nitrate media. These incubations were sufficiently long (30 min) to allow for complete reequilibration of the anions. Test incubations were then done in media containing the same anion present in the final preincubation. Cells containing chloride during test incubations exhibited high values for the potassium efflux rate constant regardless of whether chloride or nitrate was present during exposure to PCMBS. Similarly, cells containing nitrate during the flux period exhibited low values for the efflux rate constant regardless of which anion was present during PCMBS treatment.

Table III shows that the anion specificity of PCMBS-stimulated cation effluxes (Fig. 1) also holds true for influx. In control cells not exposed to PCMBS, potassium influx from sodium-free media containing ouabain was actually lower in

TABLE I

EFFECT OF DIFFERENT ANIONS ON RATE CONSTANTS FOR POTASSIUM AND SODIUM EFFLUX FROM CONTROL AND PCMBS-TREATED CELLS

Cells were first loaded with ^{86}Rb or ^{22}Na in chloride media, then equilibrated with the different anions by incubating for two successive 30-min periods at 37°C in media identical to that used for isotope loading except that chloride was replaced by X (X = Cl, Br, NO_3, acetate or methyl sulfate, $MeSO_4$). The cells were centrifuged and resuspended in fresh media following the first 30-min incubation. Next, the cells were washed three times in isotonic $(CH_3)_4NX$, then incubated 10 min at 37°C in the presence of the same anion and in the presence and absence of 1 mM PCMBS. After an additional three washes in ice-cold, isotonic $(CH_3)_4NX$, the cells were test-incubated in media containing 10 mM glucose, 20 mM glycylglycine (pH 7.4 at 37°C) and sufficient $(CH_3)_4NX$ to adjust the osmolality to 300 mosmol/kg. Each result is the mean of eight separate determinations, ±S.E.

Anion	$^{o}k_K$ (h^{-1})		$^{o}k_{Na}$ (h^{-1})	
	control	PCMBS-treated	control	PCMBS-treated
Cl^-	0.046 ± 0.016	0.354 ± 0.054	0.088 ± 0.041	0.590 ± 0.059
Br^-	0.049 ± 0.013	0.396 ± 0.024	0.099 ± 0.043	0.608 ± 0.073
NO_3^-	0.051 ± 0.018	0.147 ± 0.019	0.092 ± 0.025	0.254 ± 0.036
Acetate	0.050 ± 0.014	0.130 ± 0.034	0.100 ± 0.021	0.226 ± 0.014
$MeSO_4$	0.049 ± 0.013	0.111 ± 0.016	0.090 ± 0.018	0.200 ± 0.034

chloride than in nitrate incubations, consistent with some previously reported results (e.g., Ref. 5). Addition of sodium to the medium resulted in an increased potassium influx in chloride (but not nitrate) incubations which is due at least in part to stimulation of $Na^+ + K^+$ cotransport [12]. In cells treated with PCMBS, however, the potassium influx in chloride incubations was approximately twice that in nitrate, though the sulfhydryl reagent did produce a 4-fold increase in influx (over control levels) in the nitrate incubations. External sodium had no effect on PCMBS-stimulated potassium influx in either chloride or nitrate.

Recent evidence has been presented that the band 3 protein is involved in PCMBS-stimulated potassium transport in human red cells, and that part of this transport may involve K^+-K^+ exchange [17]. In light of these findings, we examined the effect of external potassium and sodium on PCMBS-stimulated potassium efflux, as well as

TABLE II

EFFECT OF ANION SUBSTITUTION DURING PCMBS TREATMENT OF CELLS

Cells were loaded with ^{86}Rb in either chloride or nitrate media as described in Materials and Methods, then incubated with 1 mM PCMBS in identical media for 10 min. Each group of cells was then divided in two. Half of each group was then equilibrated with chloride and the other half with nitrate by incubating 30 min at 37°C, 2% hematocrit in the appropriate loading solution, then washing three times in ice-cold, isotonic $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$. Cells were then test-incubated in buffered tetramethylammonium flux media containing the same anion present in the previous incubation and wash. Values are the means of duplicate experiments.

PCMBS incubation	Wash and flux	$^{o}k_K$ (h^{-1})
Cl^-	Cl^-	0.243
Cl^-	NO_3^-	0.125
NO_3^-	Cl^-	0.261
NO_3^-	NO_3^-	0.143

TABLE III

EFFECT OF $(Na)_0$, ANION SUBSTITUTION AND PCMBS ON ^{86}Rb INFLUX

All influx values are the mean of two separate determinations and are expressed in mmol/kg cells per h. All test-incubation media contained 0.1 mM ouabain and 75 mM KCl or KNO_3; $(CH_3)_4NCl$ and $(CH_3)_4NNO_3$ were used to replace NaCl and $NaNO_3$, respectively, in sodium-free media to maintain the osmolality at 300 mosmol/kg. The cells were preequilibrated with chloride or nitrate as described in Materials and Methods, then incubated an additional 10 min in the presence or absence of 1 mM PCMBS. The cells were then washed three times in ice-cold, isotonic $(CH_3)_4NCl$ or $(CH_3)_4NNO_3$ prior to test incubations.

$(Na)_0$, mM	Control		+ PCMBS (1 mM)	
	Cl^-	NO_3^-	Cl^-	NO_3^-
0	1.14	1.63	13.08	6.93
75	1.55	1.57	12.71	6.44

TABLE IV

EFFECT OF EXTERNAL CATIONS AND DIDS ON POTASSIUM EFFLUX RATE CONSTANTS IN CONTROL AND PCMBS-TREATED CELLS

Cells were loaded with ^{86}Rb as described in Materials and Methods, and where indicated, were treated with $1 \cdot 10^{-5}$ M DIDS during the final 90 min of this incubation. The cells were then washed and incubated 10 min in the presence and absence of 1 mM PCMBS. Following an additional three washes in ice-cold, isotonic $(CH_3)_4NCl$, the cells were test-incubated in media containing 10 mM glucose, 20 mM glycylglycine (pH 7.4 at 37°C), 0.1 mM ouabain and sufficient $(CH_3)_4NCl$, KCl or NaCl to adjust the osmolality to 300 mosmol/kg. Results shown are the means of two separate determinations for control cells and four separate determinations for PCMBS-treated cells, ±S.E. Statistical analysis using a paired *t*-test and combined data from DIDS-treated and non-DIDS-treated cells showed $^{o}k_K$ of PCMBS-treated cells in the KCl medium to be significantly greater than that of cells in $(CH_3)_4NCl$ and NaCl media with $P < 0.001$. Rate constants for PCMBS-treated cells in $(CH_3)_4NCl$ and NaCl media were not significantly different ($P > 0.1$). In control cells, $^{o}k_K$ in the NaCl medium was significantly lower than in the $(CH_3)_4NCl$ or KCl media ($P < 0.001$).

Medium	$1 \cdot 10^{-5}$ M DIDS	$^{o}k_K$ (h^{-1})	
		control	PCMBS-treated
$(CH_3)_4NCl$	0	0.038	0.255 ± 0.014
	+	0.038	0.249 ± 0.016
KCl	0	0.036	0.299 ± 0.008
	+	0.037	0.316 ± 0.014
NaCl	0	0.027	0.224 ± 0.010
	+	0.028	0.238 ± 0.006

the effect of DIDS, which inhibits anion transport via band 3 [18], on this efflux (Table IV). Ouabain was present in all of these incubations. In control cells not treated with PCMBS, potassium efflux was unaffected by external potassium (compared with $(CH_3)_4N^+$), but was reduced approx. 25% in sodium media. A similar inhibition of potassium efflux by external lithium (a sodium congener) has been previously noted, and this effect may be due to inhibition of outward $Na^+ + K^+$ cotransport [19]. In PCMBS-treated cells, a slight (but statistically insignificant) inhibitory effect of external sodium on potassium efflux was noted. A significant increase in potassium efflux, however, was observed in potassium media, suggesting the presence of a component of K^+-K^+ exchange.

Pretreatment of cells with $1 \cdot 10^{-5}$ M DIDS for 90 min had no effect on potassium efflux either in control or PCMBS-treated cells (Table IV). In this experiment, cells were exposed to DIDS only prior to PCMBS treatment, though identical results were seen when DIDS was also included in the flux media. DIDS exposure did, however, inhibit potassium efflux into potassium-free $(CH_3)_4NCl$ media by both control and PCMBS-treated cells in the presence of $1 \cdot 10^{-6}$ M valinomycin by 45–55% (data not shown). In the presence of this concentration of valinomycin, potassium efflux in potassium-free media is limited by, and thus reflects, the chloride conductance of the membrane [20,21]. Our observed degree of inhibition of chloride conductance by DIDS is consistent with previous studies in fresh human red cells [21], and these findings suggest that DIDS was not displaced from its binding site on band 3 by subsequent exposure of the cells to PCMBS.

The stimulatory effect of PCMBS treatment on cation effluxes under the conditions of our experiments was partially reversed by addition of cysteine or dithiothreitol to the test-incubation media (data not shown), in agreement with the results of Wiater and Dunham [7]. In four experiments, cells preincubated for 15 min with 1 mM PCMBS, then washed to remove excess PCMBS, exhibited a mean potassium efflux rate constant in $(CH_3)_4NCl$ media of 0.388 ± 0.027 (S.E., $n = 4$). Addition of 1 mM dithiothreitol to the test-incubation medium reduced this rate constant to 0.119 ± 0.033, a value only slightly higher than that observed in control cells never exposed to PCMBS (0.062 ± 0.021).

Discussion

Our findings show that a substantial portion of the potassium and sodium fluxes stimulated by PCMBS in human red cells has a specific anion requirement for chloride or bromide. In contrast, such anion specificity of PCMBS-stimulated potassium influx was not observed by Wiater and Dunham [7]. In that study, the cells were preincubated with PCMBS for 15 min, then washed prior to flux determinations, a method similar to that used in the experiments presented in this paper. However, the highest concentration of PCMBS employed by Wiater and Dunham [7] was

0.3 mM. As shown in Fig. 1, we found no significant stimulation of chloride-dependent cation effluxes following 10-min incubations with PCMBS unless the concentration of this reagent was 0.5 mM or more. This concentration effect is therefore the most likely explanation for the apparent discrepancy between the results of the two studies.

The anion specificity of PCMBS-stimulated cation fluxes is similar to that found for potassium fluxes stimulated by *N*-ethylmaleimide in both human [5,7] and low-potassium sheep [4,6,8] red cells. Unlike PCMBS, however, *N*-ethylmaleimide does not stimulate sodium fluxes [1,4–7], and its effect on potassium fluxes is not reversed by dithiothreitol [7,8]. This suggests that the two sulfhydryl reagents have different sites of action, though the subsets of membrane sulfhydryl groups which react with the two compounds have considerable overlap [22].

Lukacovic et al. [17] have presented evidence that the band 3 membrane protein is involved in PCMBS stimulation of cation fluxes in human red cells. They found that addition of PCMBS (but not *N*-ethylmaleimide) to phospholipid vesicles containing reconstituted band 3 had a mild stimulatory effect on tracer potassium efflux; this effect of PCMBS was not observed in vesicles without band 3. Because disulfonic acid stilbenes such as DIDS inhibit anion transport via band 3 [18,21], we tested for an effect of DIDS on PCMBS-stimulated cation fluxes, though none was found. Still, this does not rule out band 3 as either a site for PCMBS binding, a mediator of PCMBS-stimulated cation transport, or both. Pretreatment of cells with DIDS would be expected to inhibit PCMBS entry into the cells [17], and thus prevent its interaction with sulfhydryl groups on the internal surface of the membrane. In this regard, the lack of an effect of DIDS on PCMBS-stimulated cation fluxes is consistent with the interpretation of Sutherland et al. [2] that the sulfhydryl groups responsible for PCMBS stimulation of cation transport are located within the interior of the cell membrane.

The finding that a portion of PCMBS-stimulated cation fluxes has a specific anion requirement for chloride or bromide raises the question of whether these fluxes are related to cation transport processes in the unaltered cell which have a similar anion specificity [11,19,23,24]. The latter processes in human red cells are inhibited by 'loop' diuretics such as furosemide and bumetanide [11,12,19,23,24], whereas cation fluxes stimulated by PCMBS were found to be unaffected by furosemide. Still, it is possible that PCMBS reacts with membrane sulfhydryl groups necessary for binding of the diuretic. The finding that PCMBS-stimulated potassium influx in chloride media was unaffected by external sodium (Table III), however, argues against PCMBS stimulation of $Na^+ + K^+$ cotransport. In addition, the lack of effect of DIDS or HCO_3 on PCMBS-stimulated cation fluxes suggests that they are not due to cation-proton exchanges such as have been described in dog [25] and *Amphiuma* [26] red cells; these systems are also inhibited when chloride is replaced by nitrate.

One aspect of PCMBS-stimulated potassium transport which resembles that in untreated cells is the apparent presence of a component of K^+-K^+ exchange (Table IV), which was also noted by Lukacovic et al. [17]. A substantial component of chloride-dependent potassium transport in duck red cells has been found to be due to K^+-K^+ exchange [27], and a recent preliminary report suggests that this may be the case in human red cells as well [28]. Still, much additional investigation is needed to establish the relationship between cation fluxes activated by sulfhydryl reagents such as PCMBS and *N*-ethylmaleimide and the transport processes present in the unaltered cell.

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