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Thiol-Dependent Passive K : Cl Transport in Sheep Red Blood Cells: IX. Modulation by pH in the Presence and Absence of DIDS and the Effect of NEM

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Summary. Recently we proposed that cytoplasmic acidification of low K^+ (LK) sheep erythrocytes may stimulate ouabain-resistant Cl^--dependent K^+ flux ($K^+ : Cl^-$ cotransport), also known to be activated by cell swelling, treatment with N-ethylmaleimide (NEM), or removal of cellular bivalent cations. Here we studied the dependence of K^+ transport on intracellular and extracellular pH (pH_i, pH_o) varied either simultaneously or independently using the Cl^-/HCO_3^- exchange inhibitor 4,4, diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). In both control and NEM-treated LK cells volumes were kept near normal by varying extracellular sucrose. Using DIDS as an effective pH clamp, both K^+ efflux and influx of Rb^+ used as K^+ congener were strongly activated at acid pH_i and alkaline pH_o. A small stimulation of K^+ (Rb^+) flux was also seen at acid pH_i in the absence of DIDS, i.e., when $pH_i \approx pH_o$. Anti-L_l serum, known to inhibit $K^+ : Cl^-$ cotransport, prevented the pH_i-stimulated K^+ (Rb^+) fluxes. Subsequent to NEM treatment at pH 6, K^+ (Rb^+) fluxes were activated only by raising pH, and thus were similar to the pH activation profile of K^+ (Rb^+) fluxes in DIDS-treated cells with pH_o varied at constant physiologic pH_i. Anti-L_l, which inhibited NEM-stimulated K^+ (Rb^+) fluxes, failed to do so in NEM-plus DIDS-treated cells. Thus, NEM treatment interferes with the internal but not with the external pH-sensitive site.

Key Words ouabain-resistant K fluxes · sheep erythrocytes · pH effects · DIDS · N-ethylmaleimide · cotransport · membranes

Introduction

Ouabain-resistant K^+ fluxes of sheep erythrocytes are volume-sensitive [13, 14], and are activated through sulfhydryl (SH) group alkylation by NEM [21, 33, 34] or iodoacetamide [1, 27] or SH oxidation by methylmethane thiosulfonate or diamide [25, 28], and by lowering cellular bivalent cations [24, 26, 30], presumably Mg^{2+} [15]. It is characteristic for these K^+ fluxes that they are Na^+-independent [21], Cl^--dependent and apparently electroneutral [3, 13, 21, 24, 25], hence called $K^+ : Cl^-$ cotransport, and are fully inhibited by furosemide only when K^+ ions are also present externally [22]. Furthermore, anti-L_l, an allo-antibody produced by immunization of high K^+ (HK) sheep with LK red cells, reduces $K^+ : Cl^-$ cotransport [11, 12, 32], suggesting that the L_l antigen may be a part of this pathway [23, 24].

We suggested that all above-mentioned interventions activate the same transport molecule [24] present in LK red cells in greater quantities than in HK red cells [16]. Germane to this model is that at least some of the NEM-reactive SH groups appear to have low pK_a values [1], perhaps due to ionizing effects of neighboring amino or imidazolium residues. A change in the ionization of these chemical groups may be involved in the process of activation of $K^+ : Cl^-$ cotransport during swelling [24]. Because the crucial SH groups reacting with NEM are located at the cytoplasmic membrane aspect [23], we expected that pH changes would alter $K^+ : Cl^-$ cotransport by affecting chemical groups primarily from the inside of the membrane.

The effect of pH variations on active and passive Rb^+ transport was studied in human red cells [2, 18], where there is evidence for a low pH (6.5) activated K^+ flux (*see* Duhm in 29; and 10) in low density ("young") red cells [5] and in red cells from patients with homozygous hemoglobin C disease [4]. In contrast, higher Na^+ and K^+ effluxes were reported in human red cells when pH_i was increased [8], although cell volume was not controlled and therefore possible volume effects could not be ruled out. In our study we maintained cellular volume, known to vary with pH [19] by adjusting the osmolarity with the impermeant solute sucrose maintaining the ionic activity constant. Furthermore, to study separate effects of pH at the cytoplasmic and the external sides of the membrane, we inhibited Cl/HCO_3 exchange [6, 35] and hence transmembrane pH equilibration through band 3 protein (capnophorin) with DIDS (pH "clamping," ref. [7]). High concentrations of the DIDS analogue SITS (4-acetamido-4′-

isothiocyano stilbene-2.2′-disulfonic acid) [14], as well as DIDS itself (*see below*), partially inhibit volume-dependent K^+ fluxes in LK sheep red cells, and in human red cells DIDS reduces monovalent cation fluxes mediated by band 3 protein [20].

In this study we report that in untreated LK cells, at near isosmotic volume, both internal and external pH-sensitive sites govern the normally slightly acid-stimulated $K^+ : Cl^-$ cotransporter. A further augmentation of the acid-stimulated K flux occurred when DIDS was present during the preincubation and flux period suggesting a DIDS effect very different from that seen in swollen LK sheep red cells at pH 7.4 (E. Delpire & P.K. Lauf, *unpublished data*). Activation of $K^+ : Cl^-$ cotransport by NEM is due to modification of the internal site without affecting the external site. A preliminary report of this work has been presented in abstract form [38].

Materials and Methods

Chemicals

N, N,-*bis* [2-hydroxyethyl] glycine (BICINE), 4,4′-diisothiocyanato stilbene-2,2′-disulfonic acid (DIDS), dimethylsulfoxide (DMSO), N-ethylmaleimide (NEM), 3-[N-morpholino] propanesulfonic acid (MOPS), ouabain and piperazine-N,N′-*bis* [2-ethanesulfonic acid] (PIPES) were obtained from Sigma Chemical, St. Louis, MO, Tris(hydroxymethyl)aminomethane (Tris) from Fisher Scientific, Pittsburgh, PA, and rubidium chloride grade 1 from Johnson Matthew Chemicals, Royston, England. All other chemicals used were reagent grade.

Blood

Just prior to the experiments, blood was drawn into heparinized tubes by venipuncture of either of two LK sheep of mixed Hampshire/Suffolk type. Initial samples were removed to determine hematocrit and hemoglobin (Hb). L_I-antibody containing anti-L serum was kindly supplied by Dr. Ben Rasmusen, University of Illinois, and Dr. P.B. Dunham, Syracuse University, Syracuse, NY. It was dialyzed against 155 mM NaCl and used at a final concentration of 2.5% (vol/vol).

Media

For pretreatment of cells prior to the flux experiments, the following media were used (concentrations in mM): 72 NaCl, 20 KCl, 5 glucose, *X* sucrose ("unbuffered wash media") or the same composition plus 10 buffer ("buffered wash media"). The solutions for the flux experiments were (in mM): 10 RbCl, 82 NaCl, 5 glucose, 10 buffer, 0.1 ouabain, *X* sucrose ("flux media"). After the flux incubation cells were washed in a medium buffered by 10 Tris-Cl, pH 7.4 (0°C), made 300 mOsm with $MgCl_2$. In order to avoid red cell volume changes with pH [19] and hence stimulation of ouabain-resistant K^+ transport by swelling [13], we varied the osmolalities of all media with sucrose maintaining the electrolyte concentration constant. Osmolalities were measured with an Advanced DigiMatic Osmometer, Model 3D11 (Advanced Instruments, Needham Heights, MA). The calculation of the osmolarity, required to balance pH_i-induced volume changes, was based both on the reported relationship of cell water content to pH [19] and the water content to solute concentration [9]. The buffers used as "flux media" and "buffered wash media" were PIPES for pH 7.4 and lower, BICINE for alkaline pH values and sometimes a mixture of 5 mM BICINE and 5 mM PIPES for pH 7.4. PIPES and BICINE were titrated to the desired pH with NaOH and made up to volume for 100-mM stock solutions. We preferred these buffers over Tris-MOPS buffer which inhibited Rb influx by about 50% at pH 7.4 (37°C) (*see* the Table).

Table. Effect of various buffers on Rb^+ influx

Buffer	Rb^+ influx (mmol · liter cells^{-1} · hr^{-1})	±SD	Relative volume
Bicine	0.31	0.006	0.92
PIPES	0.28	0.003	0.93
Bicine + PIPES	0.30	0.004	0.92
Tris + MOPS	0.16	0.001	0.93

$n = 4$.
Red blood cells (sheep 189Y) were washed in isotonic NaCl buffered with Tris-MOPS and then incubated in duplicates for 15 and 90 min, respectively, at 37°C in ouabain-containing media with different buffers at a concentration of 10 mM, all at pH 7.4 (37°C).

Cell Equilibration

To equilibrate cells with acidic media, the technique of titration with an air-CO_2 mixture was used [17]. Initially the titration was performed at about 23°C (Figs. 1–4), and in all subsequent experiments at 0–5°C. For alkaline internal pH values, cells were titrated with 284 mOsm Na_2CO_3. When the cellular hemoglobin (Hb) buffer had equilibrated with the bicarbonate buffer, the cells were washed three times in unbuffered media, Hb maintaining pH_i constant. The pH_o was measured in the third wash suspension with a pH meter. Further pH adjustment was rarely needed.

NEM Treatment

NEM treatment was performed in unbuffered medium with 2 mM NEM for 15 min at 37°C [33] on cells first titrated with CO_2-air to pH 5.8–6 and at a hematocrit of 5% (vol/vol). Treated and control cells were then washed three times.

DIDS Treatment

DIDS treatment was carried out for 45 min at 37°C at a hematocrit of 25% on cells brought to the desired pH by titration. A DIDS stock solution of 10 mM was prepared by dissolving DIDS just prior to use in buffered wash medium neutralizing with drops of 0.1 M NaOH and made up to volume at the desired pH. After 5

min pre-equilibration at 37°C, DIDS stock solution was added to give a final concentration of 0.1 mM DIDS. This concentration of DIDS was maintained in all following wash and flux incubation media. We justified the continuous presence of DIDS by assuming that the degree of covalent binding of DIDS varies with pH [6]. Control cells were similarly incubated but without DIDS. Internal pH variations were monitored by pH measurements in the cell suspensions prior to the addition of DIDS and the flux media (*see below*).

Flux Procedure

Cells were suspended at 0°C at an approximate hematocrit of 5% (vol/vol) in flux medium. To obtain K^+ at times $t = \infty$ samples were removed to determine Hb and K^+ concentration in the mixed cell suspensions. The remaining suspension was usually divided into 5-ml triplicates for each of the 5- and 30-min incubation time points at 37°C unless otherwise stated. Usually duplicate 5-ml samples, sometimes triplicates, were taken after 15 min incubation to determine relative cell volumes. At the end of each incubation the samples were centrifuged at 2–4°C and the supernatants were kept for analysis of Hb and K^+ release. Cells were then washed three times and assayed for Hb, Rb^+ and residual K^+ after suitable dilution with hemolyzing solution containing 3.125% (vol/vol) cation-free detergent (acationox, Lancer Division of Sherwood Medical, St. Louis, MO), 0.125% (vol/vol) NH_4OH and 4 mM CsCl.

Relative Cell Volumes

Relative cell volumes were based on determination of the exact hematocrit and Hb concentration after centrifugation of the cells and their resuspension at a high hematocrit. The values obtained were related to those measured for the original whole blood as reported earlier [21]. Remainders of the cell suspensions were centrifuged for 5 min in an Eppendorf 5412 centrifuge, the supernates completely removed, and the cell pellets frozen and thawed prior to pH_i determination.

Hemoglobin Determination

Hemoglobin absorbance was measured after dilution with hemolyzing solution at a wavelength of 527 nm in a Gilford 300-N microsample spectrophotometer. K^+ was determined by absorption and Rb^+ by emission flame photometry with a Perkin Elmer 5000 atomic absorption spectrophotometer (Norwalk, CT). pH was measured using an Orion Research ionalyzer 901 (Orion Research Incorporated, Cambridge, MA) and an Orion combination electrode 8102 or, for small volumes (pH_i in frozen-thawed cell pellets), with a Fisher Microprobe combination electrode or the electrode SA 2 U from World Precision Instrument (New Haven, CT). pH values are given as measured at about 23°C if not otherwise stated.

Calculations

Cellular K^+ and Rb^+ values were computed on the basis of the measured Hb absorbance in the same samples and expressed in millimoles per liter original cells. *Rb^+ influx* was calculated by linear regression analysis of Rb concentration in three samples taken each at 5 and 30 min. The slope (mmol · liter cells^{-1} · hr^{-1}) was taken as the influx. *K^+ efflux* was obtained by multiplying the internal K^+ concentration (average from 5 and 30 min) (mmol · liter cells^{-1}) with the slope obtained by linear regression analysis for K^+ in the medium expressed as $\ln(1 - K^t/K^{t=\infty})$ [21]. K^t is the K^+ concentration at time 5 or 30 min and $K^{t=\infty}$ that after infinite time obtained from analysis of the mixed cell suspension, with corrections made for K^+ released by spontaneous hemolysis of cells [21].

Statistics

Errors of flux values were obtained from the linear regression analysis. Test of significance of differences between fluxes from different conditions were performed using a t test according to Box 9.6 in ref. [37].

Results

Choice of Buffers

For the experiments over the range of 3 pH units we decided to use more than one buffer. The Table compares the results for ouabain-resistant Rb^+ influxes measured in media buffered to pH 7.4 with BICINE useful for the alkaline range, and with PIPES for the acidic range, with a mixture of the two, and Tris/MOPS buffer as references to previous work [24–26]. The difference between BICINE- and PIPES-buffered media was small but statistically significant. In contrast, Rb^+ influx in Tris/MOPS-buffered media was considerably reduced. Hence, we chose PIPES for the acidic and BICINE for the alkaline range of the experiments, and PIPES or sometimes a combination of the two near neutral pH values.

$K^+(Rb^+)$ Fluxes at Varied pH_i or pH_o in Presence of DIDS

LK red cells were titrated to various pH_i values prior to pretreatment with DIDS and then assayed for Rb^+ uptake, in the presence of DIDS, at a medium pH of 7.56 (measured at 23°C). As shown in Fig. 1, Rb^+ uptake increased with decreasing pH_i and was linear with time at least up to 48 min. Hence, we terminated all subsequent experiments at 30 min.

Intracellular pH was relatively constant during the flux incubation (Fig. 2*A*) with changes never exceeding 0.05 pH units during 30 min when the transmembrane pH gradients were largest. Also the relative cell volumes exhibited limited changes (Fig. 2*B*). Deviations from unity were common and in most cases are attributed to a loss of solute during

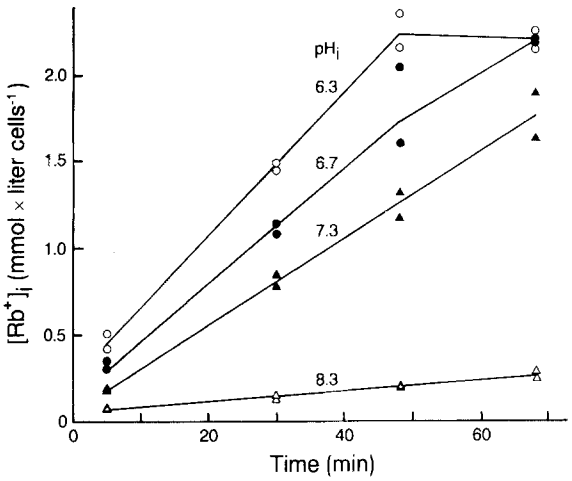

Fig. 1. Ouabain-resistant Rb^+ uptake in LK sheep red cells at 37°C with varying internal pH, (pH_i) and at constant medium pH (7.56, measured at 23°C) as a function of time. External Rb in this and all other experiments was 10 mM. Cells were first titrated to their respective pH with a CO_2-air mixture or with a Na_2CO_3 solution, then washed in unbuffered media and incubated with 10^{-4} M DIDS for 45 min at 37°C. The same DIDS concentration was also present in all subsequent wash and flux media. pH_i values given are the means of the values measured at 23°C from the 5- and 30-min time points after freeze-thawing packed cell samples. Sucrose was used in all experiments to vary osmolality with pH_i in order to maintain cell volumes close to that in untreated whole blood

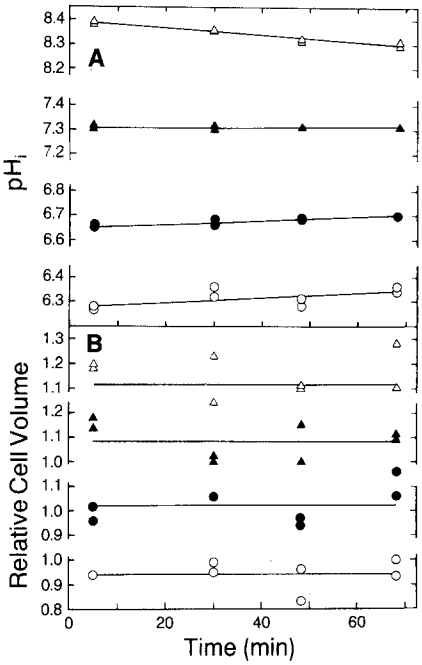

Fig. 2. Internal pH (*A*) and relative cell volumes (*B*) of LK sheep red cells in 37°C media (pH_o = 7.56) at various times. Data are from the experiment of Fig. 2. For *A*, the pH changes per hour were 0.085 (pH 8.3), 0.006 (pH 7.3), 0.043 (pH 6.7) and 0.059 (pH 6.3). Within the relatively large methodological errors no volume variations (*B*) were seen during the flux incubation period

pretreatment, particularly, the smaller cell volumes observed at pH_i below 6.3 can be explained in part by solute loss during the prolonged CO_2 titration performed in this experiment at room temperature.

Figure 3 shows that at pH_o = 7.54, both K^+ efflux and Rb^+ influx were biphasic functions of pH_i with maximal rates around pH 6.3. At lower pH values than 6.3, both K^+ and Rb^+ fluxes were reduced. For K^+, but not for Rb^+, the reduction will be explained in part by a varying loss of K_i^+ during the pretreatment of the cells. A similar reduction was seen also when the K_i^+ was more constant in cells from the same sheep (*see* Fig. 5, control, and Fig. 6). Conversely, when pH_i was kept constant at 7.46 and pH_o increased from 5.6 to 8.3, both K^+ efflux and Rb^+ influx rose linearly (Fig. 4). The data shown in Figs. 1–4 suggest that acidification at the cytoplasmic aspect of the membrane treated with DIDS prior to and during the flux experiments maximally activated $K^+(Rb^+)$ flux at $pH_i \cong 6.3$, while external acidification caused its inactivation.

Evidence for DIDS Activation of pH_i/pH_o Sensitive $K^+(Rb^+)$ Fluxes

As shown in Fig. 5, Rb^+ influx and K^+ efflux were studied as a function of pH ($pH_i \cong pH_o$) in cells equilibrated with the desired medium pH before the addition of DIDS. In the absence of DIDS both K^+ efflux and Rb^+ influx were maximal at pH 6.4. When no pH gradient was imposed after pH equilibration and DIDS treatment, $K^+(Rb^+)$ flux values at every pH value were higher than in untreated controls. The DIDS stimulation was most pronounced at the most acidic pH, an effect not explainable in terms of volume differences unless slight cell shrinkage augmented the DIDS action. (Note that the shape of the K efflux curve in the presence of DIDS differed in one point from that observed in the experiments of Figs. 3 and 6 and in experiments not shown, *see* Discussion.) Hence, *both* DIDS and acid pH_i stimulated K^+ and Rb^+ transport in LK red cells.

To distinguish between effects caused in DIDS-treated cells with varied pH_i *versus* cells with pH varied on both sides (i.e., $pH_i \approx pH_o$), K^+ effluxes and Rb^+ influxes were measured in cells treated accordingly. Figure 6 shows that DIDS-treated cells have a pronounced pH_i-dependent K^+ efflux stimulation regardless of whether or not a transmembrane pH gradient was present. The fact that in cells with $pH_i \approx pH_o$, both K^+ and Rb^+ fluxes appeared to be lower in the acidic and slightly higher in the extreme alkaline range as compared to cells where pH_o = 7.38 (Fig. 6), is consistent with the acid-pH_o-inhibi-

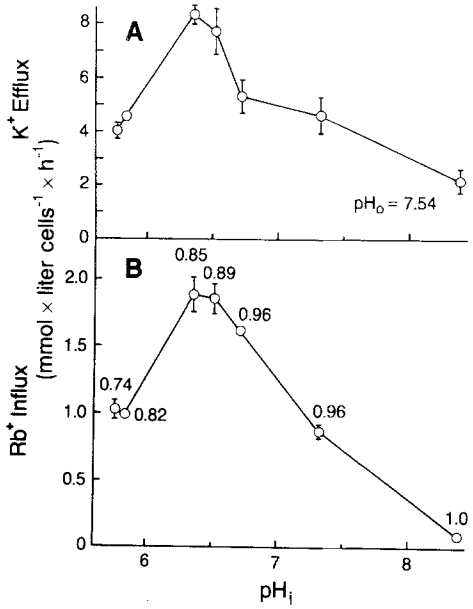

Fig. 3. Ouabain-resistant K^+ efflux (*A*) and Rb^+ influx (*B*) of cells with varying pH_i and constant pH_o. The cells were equilibrated at various pH values followed by DIDS treatment and incubation in a flux medium of pH 7.54 (23°C). Abscissa: pH_i values measured after the experiment in packed, freeze-thawed cells. The average internal (mid-flux) K concentrations were in mmol · liter water^{-1} (pH values in brackets): 1.8 (5.76), 2.4 (5.84), 8.3 (6.4), 9.3 (6.5), 11.3 (6.7), 13.2 (7.3), and 17.2 (8.4). Numbers indicate relative cell volumes. Vertical bars for SD when larger than symbols. Sheep LK5 was used. The scale of the ordinate in *A* is 40% of that in *B*

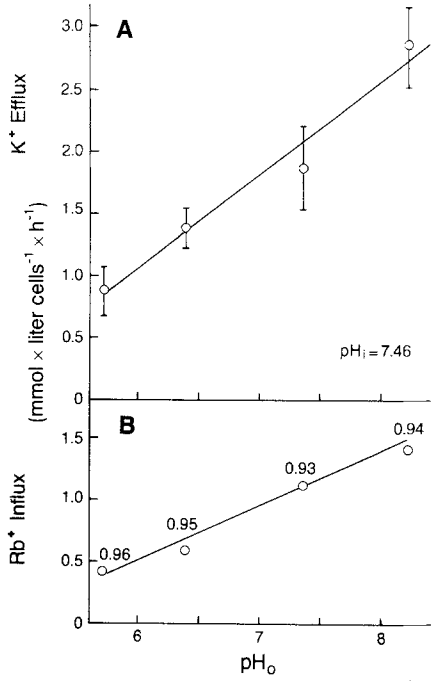

Fig. 4. Effect of varying medium pH (pH_o) on K^+ efflux (*A*) and Rb^+ influx (*B*) of cells with a constant pH_i. The cells were equilibrated to obtain a pH_i = 7.46 before DIDS treatment. The average internal K^+ concentration was 19.7 ± 0.4 mmol · liter cell water^{-1}. Numbers indicate relative cell volumes. Vertical bars for SD only when larger than symbols. Sheep LK5 was used

tory and alkaline-pH_o-activating effect when pH_i was kept at 7.46 (Fig. 4).

Effect of Anti-L_l on pH and DIDS-Sensitive $K^+(Rb^+)$ Fluxes

The Cl^--dependent $K^+(Rb^+)$ transport of low K^+ sheep cells is inhibited by the anti-L_l antibody [11–13, 21, 30]. Therefore, this antibody was used to show that the pH and DIDS effects observed were on the K : Cl pathway. In the absence of DIDS, the L_l-antibody inhibited K^+ efflux ($0.001 > P$) although not significantly at pH 8.9 where the efflux was smallest (Fig. 7*A*). The effect of anti-L_l on Rb influx was much more difficult to discern because of the comparatively smaller Rb influx values. Nevertheless, anti-L_l reduced Rb^+ influx significantly at pH 7.3 ($0.001 > P$) (Fig. 7*B*). Hence a major part of the K^+ efflux was anti-L_l sensitive and therefore involved the Cl^--sensitive K^+ pathway. When the fluxes were stimulated in the presence of DIDS (with a maximal effect shifted to even lower pH values than seen with other animals in Figs. 1 and 6, and in experiments not shown) by an internally raised proton (and chloride) concentration (pH_o = 6.3 and 5.7), both K^+ and Rb^+ fluxes were inhibited by anti-L_l ($0.001 > P$) to levels of untreated cells also exposed to anti-L_l ($0.001 > P$). Hence DIDS treatment stimulated the Cl^--sensitive K^+ pathway and did not activate a new or some other existing pathway.

pH-Dependence of K (Rb) Fluxes after NEM Treatment, and the Effects of DIDS and Anti-L_l

Pretreatment of low K^+ sheep red cells with NEM at pH 6 stimulates Cl^--dependent K^+ transport measured near pH 7.4 [1], an effect that in sheep red cells is not transient of nature as reported for human red cells [36, *unpublished data*]. However, the pH dependence of the NEM-modified K transport had not been investigated as yet. To this end, pretreatment with NEM was carried out at or just below pH 6.0 as shown previously [1] followed by titration of the cells with CO_2-air or 284 mOsm Na_2CO_3 to various pH values and equilibration in buffered media at the same pH values for 45 min at 37°C (together with other samples undergoing DIDS treatment, *see below*). Figure 8 shows that with increasing pH K^+ efflux (Fig. 8*A*) and Rb^+

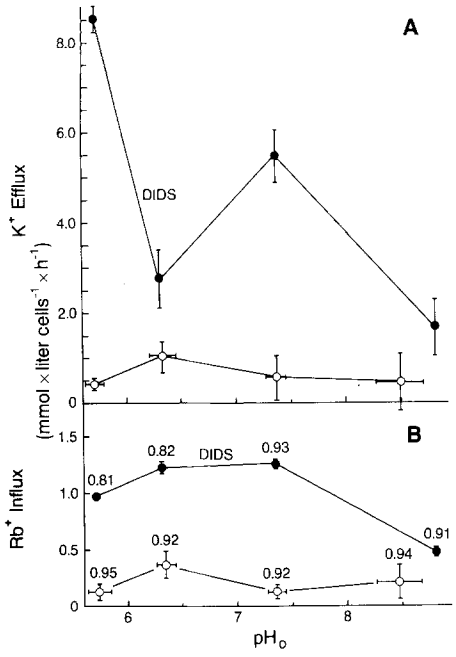

Fig. 5. Ouabain-resistant K^+ efflux (*A*) and Rb^+ influx (*B*) of cells with $pH_i \approx pH_o$. The cells were equilibrated at 37°C with buffered media of various pH_o values. The control curves (open circles) are the average of five K^+ efflux experiments and of six Rb^+ uptake experiments. The SD are given as vertical and horizontal bars. K^+ efflux was higher at pH 6.4 than at 5.8 ($0.02 > P > 0.01$) and 8.5 ($0.02 > P > 0.01$), and Rb^+ influx was higher at pH 6.4 than at 5.8 ($0.02 > P > 0.01$) and 7.4 ($0.01 > P > 0.001$). These differences were not due to volume effects as cell volumes were identical at pH 6.4 and 7.4 and only slightly larger at pH 5.8, where fluxes decreased. Flux values after pretreatment with DIDS of cells first equilibrated at respective pH_o (one experiment) are given by filled circles (Sheep LK5) with vertical bars for the SD values from linear regression of the flux slopes. All K^+ and Rb^+ flux values were higher ($0.01 > P$ or less) than in untreated controls. Numbers indicate values of relative cell volumes. Note that in *A* the scale on the ordinate is 40% of that in *B*

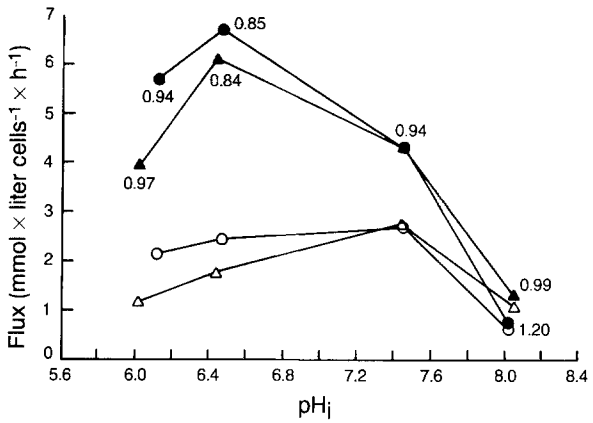

Fig. 6. Ouabain-resistant K^+ efflux and Rb^+ influx of DIDS-treated cells both with $pH_i \approx pH_o$ (triangles) and pH_o = constant (circles). Cells were equilibrated at the various pH values *before* DIDS treatment. The pH of the equilibration medium was either maintained during the flux incubation (▲ for K^+ and △ for Rb^+), or pH_o was constant at 7.38 ± 0.04 (● for K^+ and ○ for Rb^+). Numbers indicate relative cell volumes. Duplicate samples were used for 5- and for 30-min flux incubation. SD were smaller than the symbols. Sheep LK5 was used. When $pH_i \approx pH_o$, K_i was (in mmol · liter cell water^{-1}, pH in brackets): 6.9 (6.0), 7.2 (6.4), 10.1 (7.4), and 13.4 (8.1); when pH_o = 7.4, K_i was 5.9 (6.1), 6.6 (6.5), 10.0 (7.5), and 13.6 (8.0)

influx (Fig. 8*B*) of NEM-treated cells increased monotonically with pH. In contrast to the stimulatory action of DIDS in untreated controls (Figs. 3–6), in NEM-treated cells DIDS lowered both Rb^+ and K^+ fluxes.

At pH 7.4 anti-L_l inhibits the NEM-induced Cl^--dependent K^+ transport in LK red cells [23]. Anti-L_l inhibition in the presence and absence of DIDS was tested at pH 8.8 as shown in Fig. 9. This pH was chosen because it gave the highest NEM activation as shown in Fig. 8. Anti-L_l inhibited both control (untreated) as well as NEM-stimulated K^+ effluxes and Rb^+ influxes, but failed to exert any effect on cells exposed to both NEM and DIDS. Note that the inhibitory effects of DIDS on NEM-treated cells was similar in magnitude to the anti-L_l effect.

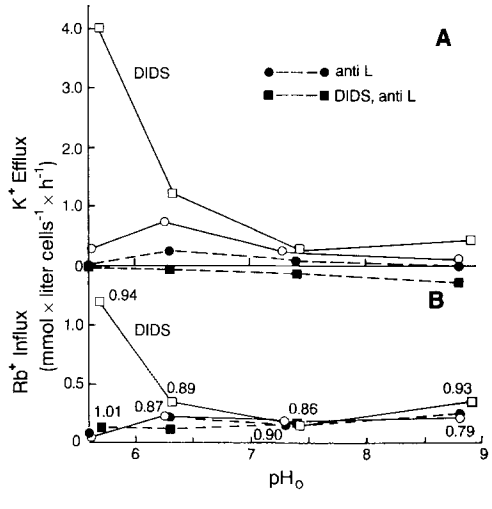

Fig. 7. Ouabain-resistant K^+ efflux (*A*) and Rb^+ influx (*B*) in cells with $pH_i \approx pH_o$. Control cells (open circles) and cells equilibrated at 37°C were at the respective pH prior to DIDS treatment (open squares). Fluxes were also determined in the presence of a serum containing the anti-L_l antibody (dashed lines, filled circles for controls and filled squares for DIDS-treated cells). Sheep LK189Y was used. The SD values were not larger than the points. Numbers indicate relative cell volumes. Note that the scale on the ordinate in *A* is 50% of that in *B*

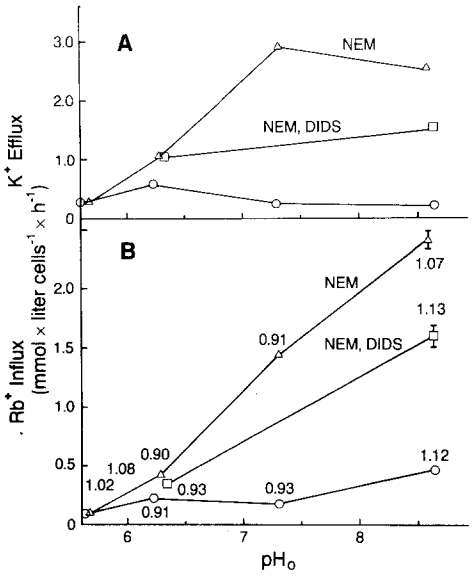

Fig. 8. Ouabain-resistant K^+ efflux (*A*) and Rb^+ influx (*B*) for cells with $pH_i \simeq pH_o$. The cells were equilibrated at the pH indicated of controls (circles), of cells pretreated with NEM at pH 6 prior to equilibration in absence (triangles) or presence of DIDS (squares). Average internal K concentration during flux was 19.9 ± 2.1 mmol · liter water^{-1}. Numbers indicate relative cell volumes. Vertical bars for SD are given only when larger than the symbols. Sheep LK5 was used. The scale on the ordinate in *A* is 50% of that in *B*

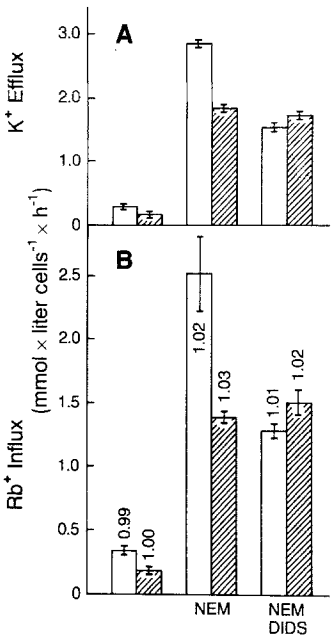

Fig. 9. The effect of anti-L on ouabain-resistant K^+ efflux (*A*) and Rb^+ influx (*B*) $pH_i \approx pH_o$ in controls, in cells pretreated with NEM at pH 6, and with NEM at pH 6 and DIDS at pH 8.8. Controls (open bars) and anti-L treated cells (hatched bars). Prior to flux cells were equilibrated at pH_o = 8.8. Numbers indicate relative cell volumes. Vertical bars are for SD values. Sheep LK189Y was used. The scale of the ordinate in *A* is 50% of that in *B*

Discussion

The present study originated in the working hypothesis [24] that the Cl^--dependent K^+ transport system possesses NEM-reactive SH groups with pK_a values determined by neighboring amino or imidazolium groups which themselves may have pK_a values in the acid titratable range studied here. Indeed, we have evidence for the presence of such groups since exposure of LK cells to diethylpyrocarbonate eliminated the NEM-stimulated K : Cl cotransport [29].

Our initial studies with DIDS (Figs. 1–4) suggested two possibilities: First, $K^+(Rb^+)$ transport in LK sheep is under the control of two titratable groups, a low pH_i activator and a low pH_o inhibitor site; second, low pH_i and high pH_o each separately activate two different $K^+(Rb^+)$ transport pathways. Although not exclusive, the evidence accumulated throughout this study is consistent with the first proposition: DIDS activated primarily the inside, proton-stimulated K^+ efflux as born out from a comparison with control fluxes in the absence of DIDS (Fig. 5) and from the experiments where a comparison was made between pH_i and pH_o varied simultaneously as opposed to pH_i alone (*compare* Figs. 4 and 6). As pointed out in Results, the DIDS effect on K^+ efflux varied in one point (*compare* Figs. 3 and 6 with Figs. 5 and 7). Moreover, the peak of the activation was shifted to lower pH values in the experiment of Figs. 5 and 7 as compared with those of Figs. 3 and 6. Although different animals as well as the small number of points may contribute to these discrepancies, they are insignificant in light of the overwhelming evidence of pH_i acid- and DIDS-stimulated K^+ transport in all experiments. In the absence of DIDS, i.e., when changing both pH_o and pH_i, there was a small acid activation of both K^+ and Rb^+ fluxes with a maximum around pH 6.3. Further lowering pH decreased these fluxes. However, with DIDS present, either low pH_i at normal pH_o alone (Figs. 3 and 6), or both low pH_i and pH_o (Figs. 5–8) stimulated both fluxes by several-fold. This effect was variable between experiments and animals with respect to the overall shape of the activation curves with DIDS present (*compare* Figs. 3 and 6 *versus* 5 and 7).

The individual K^+ and Rb^+ fluxes were greater in acid than alkaline pH in presence and absence of DIDS. Also, the ratio of K^+/Rb^+ fluxes increased when pH_i or pH on both sides turned acid and was approximately constant when pH_i was fixed (and pH_o varied). Provided that the $K^+(Rb^+)$ fluxes are

due to electroneutral cotransport with chloride, they should be proportional to the products of the transported solute concentrations on both sides of the membrane. The ratio of K^+ and Rb^+ fluxes as expected then should vary in the directions observed in our experiments. As the chloride ratio in sheep red cells should vary with pH similar to that of human red cells [19] the measured ratios are in agreement, within errors of the methods. Since at very low pH_i, Cl_i^- is known to increase, the decrease of K^+ efflux seems to be unrelated to Cl_i^- and must be due to other variables caused by pH values < 6. The absolute magnitude of the peak value for K^+ efflux and its relation to pH may shift to a higher pH value after correction for the effect of varying the chloride ratio. In contrast, both Rb_o^+ and Cl_o^- being constant, increasing Cl_i^- may reduce and thus shift the absolute Rb^+ influx peak into a more acidic direction, causing a better congruence of both K^+ efflux and Rb^+ influx peaks.

By virtue of anti-L_l inhibition, the DIDS-activated and proton inside-sensitive K^+ pathway can be attributed to Cl^--dependent K^+ transport activated by cell swelling [13, 14], SH-group reagents [21, 24, 25, 33] or removal of bivalent cellular cations through A23187 [26]. The total inhibition of K^+ flux activated by DIDS and low pH_i (Fig. 7) is in sharp contrast to the always partial inhibition by anti-L_l observed in control, swollen and NEM-treated cells at physiologic pH [23, 24]. Possibly, low pH_i and DIDS altered chemically the surface expression of the L_l antigen and/or the antibody-combining site or both so that the ensuing antigen-antibody reaction was stronger resulting in complete inhibition of the pH_i- and DIDS-induced K^+ flux. Alternatively, antibody heterogeneity has to be considered whereby acid pH and DIDS promote binding of an antibody population with a higher inhibitory activity/affinity toward K^+ transport. Either of these possibilities as well as that of a reaction between DIDS and antibody at alkaline pH may account for the failure of anti-L_l to inhibit DIDS-treated cells at high pH_o.

The present studies shed new light on the nature of the NEM-stimulated pathway: NEM treatment at pH 6 abolished the stimulation by internal acidification but did not modify the response of $K^+(Rb^+)$ fluxes to rising external alkalinization, thus mimicking the pH_o stimulation at constant pH_i in LK red cells treated with DIDS, but not with NEM (*compare* Figs. 4 and 8). This interesting parallelism suggests that NEM altered the pH dependence at the cytoplasmic side. It is unlikely that this loss of the NEM effect is related to a transient nature of the NEM effect reported recently for human red cells [36] since a similar pH dependence of the NEM action was also seen in human red cells (*see* Duhm in [31]). In NEM-treated cells DIDS was inhibitory and there was no further effect of anti-L_l, suggesting reaction of DIDS with amino groups crucial for the inhibitory effect of anti-L_l. Nevertheless, an additional external site unreactive with anti-L_l and stimulating $K^+(Rb^+)$ fluxes at alkaline pH cannot be ruled out at this time.

In summary, ouabain-resistant K^+ transport in sheep LK red blood cells is governed by two cooperative sites generating under (isosmotic) control conditions peak flux rates in the pH range 6.4–7.3. These fluxes are augmented in the presence of DIDS. With pH varied on both sides (i.e., without "pH clamping") the external and the internal site were rate limiting for K^+ transport at acid and at alkaline pH, respectively. The effect of NEM then is to modify the internal site to the extent that both K^+ effluxes and Rb^+ influxes are controlled only by the external pH-sensitive site.

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