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Oxidative Activation of K-Cl Cotransport by Diamide in Erythrocytes from Humans with Red Cell Disorders, and from Several Other Mammalian Species

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Abstract. Red blood cells (RBCs) from different mammalian species were investigated for the presence of diamide-induced oxidative activation of K-Cl cotransport reported to be present in sheep but absent in human RBCs. K efflux was measured in RBCs from human with hemoglobin (Hb) A or S, glucose-phosphate dehydrogenase (G6PDH) and a cytoskeletal deficiency, and from rat, mouse and rabbit. RBCs were incubated with diamide (0–1.0 mM) in K-free Cl or NO_3 media of variable osmolalities (200–450 mOsM). Cl-dependent K efflux or K-Cl cotransport (estimated as the difference between K efflux rate constants in Cl and NO_3) was activated by diamide in a sigmoidal fashion. Relative maximum K-Cl cotransport followed the sequence: human HbA (1) < rabbit (1.8) < sheep (6.9) < human HbS (9.5) ~ rat (9.7). Relative diamide concentrations for half maximal activation of K-Cl cotransport followed the sequence: sheep (1.9) > human Hb A (1) > rabbit (0.75) > human HbS and rat (0.67). Cell swelling in 200 mOsM doubled K-Cl cotransport in diamide, both in human HbA and S cells but reduced that in rat RBCs. In contrast, cell shrinkage at 450 mOsM obliterated K-Cl cotransport in human HbA and S but not in rat RBCs. Human RBCs with G6PDH and a cytoskeleton deficiency behaved like HbA RBCs. In mouse RBCs, diamide-activated K-Cl cotransport was 30% higher in isotonic than in hypotonic medium. In human HbA and S, and in low or high K sheep RBCs fractionated by Percoll density gradient, diamide increased the activity of K-Cl cotransport, an effect inversely correlated with cell density. Analysis of pooled data reveals that K-Cl cotransport accounted for about 80% of all K flux in Cl. There was a statistically significant correlation between K-Cl cotransport and K efflux in Cl ($P < 0.00001$) and in NO_3 ($P < 0.00001$). In conclusion, a diamide-activated K-Cl cotransport was present in human RBCs and in all other mammalian RBCs tested, with a large inter-, and for human and sheep, intraspecies variability for its maximum activity.

Key words: K-Cl cotransport — Erythrocytes — Oxidation — Diamide — Red cell disorders — Sickle cell anemia — Mammalian species

Introduction

Sulfhydryl (SH, thiol) oxidizing compounds are widely used to study the role of SH-groups in an array of cellular functions. Thiol-oxidizing agents such as diamide (Beppu et al., 1994) and SH-blocking agents such as N-ethylmaleimide (Beppu et al., 1994) are most commonly used. Diamide is of particular interest due to its mild oxidizing and reversible action (Klonk & Deuticke, 1992). Diamide cross links integral membrane proteins (Deuticke, Lutkemeier & Poser, 1992; Beppu, Mizukami & Kikugawa, 1992; Terada et al., 1992; Trada et al., 1993; Yamaguchi, Saeki & Kimoto, 1993; Turrini, et al., 1994), in particular dimers and tetramers of band 3 (Giger et al., 1995; Khan & Saleemuddin, 1995), and of band 3 and hemoglobin (Hb) leading to the formation of high molecular weight aggregates and adducts (Giger et al., 1995). Diamide also polymerizes and cross links spectrin (Kumar & Gupta, 1992), one of the major red blood cell (RBC) cytoskeletal proteins. Depending on the experimental conditions, diamide increases RBC hemolysis (Miller et al., 1991), decreases high pressure-induced hemolysis (Yamaguchi et al., 1994*a*) and high pressure-induced vesiculization of spectrin-containing vesicles (Yamaguchi, Kajikawa & Kimoto, 1991), and

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alters the composition of the formed vesicles (Yamaguchi, Saeki & Kimoto, 1993; Yamaguchi, Yamada & Kimoto, 1994*b*). Diamide increases RBC rigidity (Deuticke et al., 1992) and decreases RBC deformability (Pestonjamasp & Mehta, 1991; Muralidharan, Tateishi & Maeda, 1994; Giger et al., 1995), effects that may underlie the observed increased phagocytosis of RBCs by monocytes (Turrini et al., 1992) through binding of thus exposed glycophorin A to macrophages via their glycophorin A receptor (Beppu et al., 1994). The conformational changes induced by diamide in membrane and cytoskeletal proteins decrease cell survival (Giger et al., 1995) and accelerate cell senescence (Beppu et al., 1992; Giger et al., 1995). Diamide also induces thermotolerance (Laski & Jozwiak, 1992) and lowers the long-lasting oxidative burst in pigment fed monocytes, an effect that may explain the depression in cellular immunity observed in malaria (Schwarzer et al., 1992).

Diamide affects transport of a variety of molecular and ionic species by either inhibiting or activating the transporters or altering the membrane environment. Thus diamide inhibits RBC transport of adenosine (Gero et al., 1991), aminophospholipids (Connor & Schroit, 1991), phosphate (Yamaguchi et al., 1993) and lipid-soluble compounds, such as quinine and quinidine (Murakami, Takada & Muranishi, 1992). Diamide inhibits swelling-activated taurine transport in skate hepatocytes (Ballatori & Boyer, 1992), and carnitine transport in mitochondria (Indiveri et al., 1992). On the other hand, diamide stimulates serotonin transport in platelets (Bosin & Kasper, 1992) and transport of hydrophylic nonelectrolytes such as sucrose and mannitol in human RBCs and their ghosts, and in inside-out vesicle preparations (Klonk & Deuticke, 1992). Diamide also stimulates the GSH signal-transduction pathway of human RBCs. Via this pathway, ''GSH can transduce its reducing power by a thiol/disulfide exchange mechanism that sequentially involves sulfur-rich proteins spanning across the erythrocyte membrane'' (Citriolo et al., 1993). Most recently, diamide is reported to stimulate the newly attributed flip mechanism of anionic membrane-intercalated amphiphiles by band 3 protein (Ortwein, Oslender-Kohnen & Deuticke, 1994).

Based on this information, diamide should be expected to affect either directly or indirectly transport pathways involved in volume regulation, in particular, swelling-activated pathways. However, previous work by us has shown that in sheep and human RBCs, diamide appears to activated K-Cl cotransport by a different mechanism than swelling (Lauf, 1988*b;* Lauf, Perkins & Adragna, 1985), and it is not known whether additivity between these two components is species-dependent.

In the present study, we report on the effect of diamide on ouabain-resistant (OR) K transport in RBCs from human with HbA and S, glucose 6-phosphate dehydrogenase (G6PDH)-deficient, and with a cytoskeletal deficiency; in low and high K (LK and HK, respectively) RBCs of sheep, and in rat, mouse, and rabbit RBCs. Our studies support previous observations on the existence of a reversible diamide stimulation of K-Cl cotransport in LK sheep RBCs (Lauf, 1988*a*) and explain the reported absence of such an effect in human RBCs (Deuticke et al., 1992; Ihrig et al., 1991). We conclude that in all the mammalian species studied, including human, diamide did activate K-Cl cotransport.

This work was published in abstract form elsewhere (Adragna & Lauf, 1992; Adragna & Lauf, 1993; Adragna & Lauf, 1996).

Materials and Methods

Chemicals

All solutes were of analytic grade: NaCl, $NaNO_3$, Na_2HPO_4/NaH_2PO_4, glucose, EDTA: ethylenediaminetetraacetic acid, and $MgCl_2$ (from Fisher Scientific, Fair Lawn, NJ); diamide: diazene dicarboxylic acid BIS [N,N-dimethylamide], DTT: dithiotreitol, ouabain, DMSO: dimethylsulfoxide (from Sigma, St Louis, MO), and ultrapure $RbNO_3$ and RbCl (Johnson Mathew Chemicals, Royston, UK).

Solutions

Isotonic NaCl phosphate buffer (PBCl) (295 mOsM) contained in (mM): 150 NaCl and 5 $NaPO_4$ buffer, pH 7.4. Isotonic NO_3 phosphate buffer (PBN) (277 mOsM), contained $NaNO_3$ instead of NaCl. Cl media of variable osmolalities contained (mM): 100 NaCl, 5 $NaPO_4$ buffer pH 7.4 and 0, 47.6, 98 and 238 mM sucrose as filler solute, to obtain 200, 250, 300 and 450 mOsM, respectively. NO_3 media of variable osmolalities contained (mM): 97 $NaNO_3$, 5 $NaPO_4$ buffer pH 7.4 and 0, 43.8, 87.6, and 220 mM sucrose to obtain 185, 231, 277 and 416 mOsM, respectively. Diamide, when present at variable concentrations (0–1.0 mM), was added from a freshly prepared 25 mM stock solution in isotonic (277 mOsM) $NaNO_3$ medium and, when present at a maximum activating concentration (0.25 mM), was added from a 25 mM stock solution in isotonic (277 mOsM) or hypotonic (185 mOsM) $NaNO_3$ medium. Ouabain was added from a 10 mM stock solution in DMSO to give a final concentration of 0.1 mM.

Red Blood Cells

Blood from sickle cell patients ($n = 4$, two of the patients were twins) and from a G6PDH-deficient patient (12-year-old male Caucasian, normal mean value 6.5 IU/g Hb, patient 1.1 IU/g Hb) was kindly provided by Dr. D. Rucknagel, director of the Sickle Cell Center at the University of Cincinnati. Blood from HbA subjects was obtained from volunteers at Wright State University (WSU). Blood with abnormal RBC cytoskeleton (clinically established case of poikilocytosis with partial band 4.1 deficiency) was donated by a local patient at Frederick A. White Ambulatory Center. Blood from mixed-breed sheep homozygous for the LK and HK gene was obtained by venipuncture and collected into heparinized tubes. Blood from 3 adult Sprague-Dawley rats and from a 5–6 lb male New Zealand White rabbit was obtained from the Laboratory of Animal Facilities at WSU. Blood from cd-1 out

bred mice was kindly provided by Dr. D. Rucknagel from the University of Cincinnati.

K Efflux Determination

The K efflux rate constants were determined as detailed elsewhere (Lauf, 1983). Briefly, an aliquot of whole blood from human and other mammal sources was separated to determine the optical density of 1 ml of packed cells from the hematocrit and optical density of a diluted sample. The remaining blood was spun and the plasma and buffy coat were discarded. Packed cells were washed four times with isotonic PBCl (*see* composition above). The hematocrit of the packed cells was determined (averaging about 80%) and aliquots of the suspension were added to cold isotonic or variable osmolality Cl (200–450 mOsM) or NO_3 (185–416 mOsM) buffers (*see* composition above) for volume and anion equilibration (final hematocrit, approximately 2%). Cell suspensions were split into two equal aliquots once more before spinning. Prewarmed Cl or NO_3 flux media of the same composition as above and containing diamide at the maximum activating (0.25 mM) or at variable (0–1.0 mM) concentrations, and 0.1 mM ouabain, where appropriate, were added to the packed cells (final hematocrit for human, rat and rabbit, 2.5% and for sheep, 5%). Cell suspensions were incubated at 37°C in a water bath. Samples to determine K and hemoglobin concentrations at equilibrium (Lauf, 1983) were separated immediately. Aliquots of cell suspensions were taken as a function of time, spun, and the supernatants were separated to read optical density to correct for hemolysis and K concentration by atomic absorption spectrophotometry (Perkin Elmer, 5000, Norwalk, CT). Cell volume was determined from the optical densities of the hemolyzed cell pellets, as described elsewhere (Lauf, 1988*b*).

Cell Separation by Density Gradients

Cells were separated into light and dense fractions through a Percoll gradient. In preliminary experiments, 1.5 M NaCl, 1.12 g/ml Percoll and water were mixed in different proportions to determine the maximum separation of human and sheep RBCs. The mixture that produced the highest column of cells, from top to bottom, was considered to provide maximum separation. A mixture containing a ratio of NaCl: Percoll:water of 1:9.0:1.3 which corresponds to 79.4% Percoll gave the best separation for most cells. For human RBCs, 6 ml of Percoll 1.12 g/ml (at a ratio of NaCl:Percoll:water of 1:8.5:1.6) and 0.6 ml of 50% hematocrit blood (*see below*) were the optimal proportions to achieve maximum separation of cells. Gradients were spun at 35,000 × *g*, 4°C for 10 min. In all experiments the upper half of the gradient is defined as light cells and the bottom half as dense cells.

Whole blood from human HbA or S and from LK or HK sheep was divided into two aliquots, one to remain as whole blood (or unseparated cells) and the other to be separated into light and dense cells. Aliquots were spun down and the buffy coat was removed. The unseparated cells were kept on ice during the separation procedure. The aliquot to be separated was diluted to about 50% hematocrit with 300 mOsM NaCl and added to 1.12 g/ml Percoll as described above and spun down as before. The unseparated and separated cells were washed 4 and 3 times, respectively, with PBCl. After the last spin, cells were resuspended in cold 200 mOsM PBCl or 185 mOsM PBN, mixed to wash once more, and divided into two equal aliquots before spinning at 5,000 rpm. Thereafter, the protocol for K efflux rate constant measurement was followed (*see above*).

Statistics

Results are expressed as the mean ± standard deviation (SD) or ± standard error of the mean (SE). Statistical significance was assessed by two sample paired or unpaired *t*-test analysis with computer software.

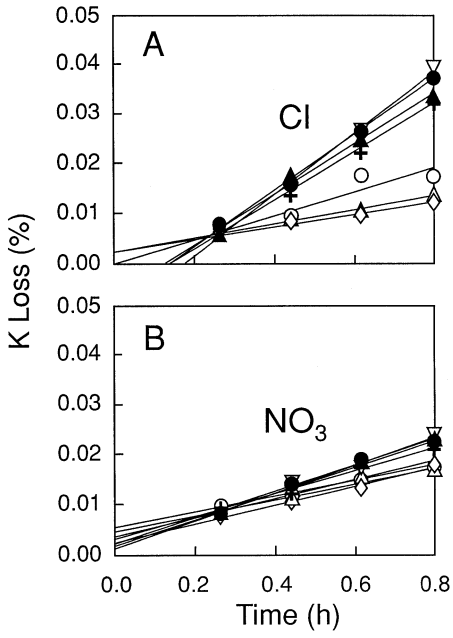

Fig. 1. K loss (%) from human HbA containing RBCs as a function of time at different diamide concentrations. Panel *A*, data in isotonic Cl medium (PBCl). Panel *B*, data in isotonic NO_3 medium (PBN). Similar results were obtained in six independent experiments with human HbA, HbS ($n = 2$), sheep, rat ($n = 3$) and rabbit RBCs. Symbols corresponding to different diamide concentrations are (mM): 0, underneath open triangles and diamonds; open triangle, 0.02; open circle, 0.05; bold cross, 0.1; closed triangle, 0.15; closed circle, 0.2; open inverted triangle, 0.25; open diamond, 0.25 + dithiotreitol (DTT).

Results

K-Cl Cotransport and Diamide Concentration in RBCs from Different Mammalian Species

In previous studies in sheep RBCs (Lauf, 1988*a*), diamide was used at concentrations as high as 2.0 mM, whereas in human RBCs 5 mM diamide was applied (Deuticke et al., 1992; Ihrig et al., 1991). To compare the effect of diamide on RBCs from the different species, K efflux rate constants were determined at increasing diamide concentrations. Figure 1 shows a representative experiment of K loss in Cl (panel *A*) and NO_3 (panel *B*) as a function of time and diamide concentration in human HbA RBCs. The diamide activation of K loss was dose-dependent and higher in Cl than in NO_3 (315% *vs.* 43%, respectively, at 0.25 mM diamide). At higher diamide concentrations the intercepts of the slopes (rate constants) of K loss in Cl increased to about 0.15 hr (~9 min) consistent with observations of a lag phase reported

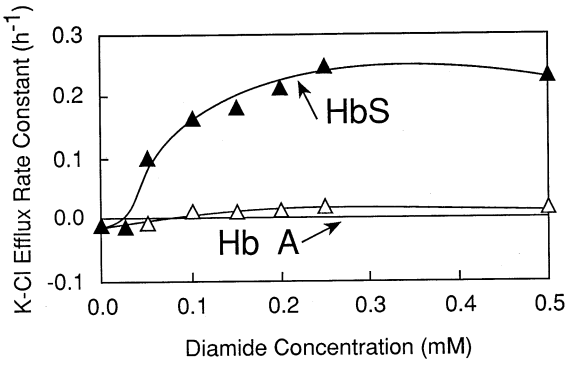

Fig. 2. Cl-dependent K (K-Cl) efflux rate constant as a function of diamide concentration in human HbA and HbS RBCs. Packed cells were washed four times with isotonic PBCl (*see* Materials and Methods). Aliquots of the suspension (approximately 80% hematocrit) were added to cold isotonic Cl (300 mOsM) or NO_3 (277 mOsM) buffers (*see* Materials and Methods) for anion equilibration (final hematocrit, approximately 2%). Cell suspensions were split into two equal aliquots before adding prewarmed Cl or NO_3 flux media of the same composition containing diamide at increasing (0, 0.025, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.5 mM) concentrations and 0.1 mM ouabain (final hematocrit 2.5%). Cell suspensions were incubated at 37°C and five samples were taken at approximately 10-min intervals for determination of K loss and calculation of K efflux rate constants as described in Materials and Methods. The dose-response curve for HbA is the average of two experiments with RBCs from two different donors.

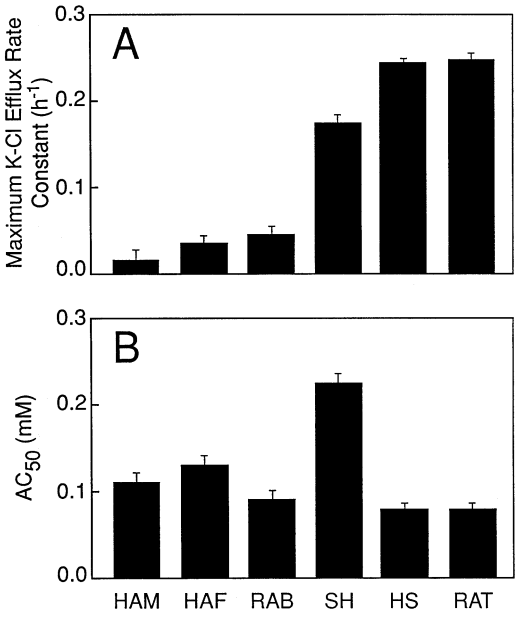

Fig. 3. Kinetic parameters of Cl-dependent K (K-Cl) efflux rate constant in different mammalian species. Panel *A,* K-Cl efflux rate constants determined at the diamide concentration yielding maximum K-Cl cotransport: human, 0.5 mM, rat and rabbit, 1.0 mM, and sheep, 2.0 mM. The maximum K-Cl efflux rate constant for each species, was calculated as the average of K-Cl efflux rate constants at saturating diamide concentrations on each dose-response curve. Results are expressed as mean ± SD. Panel *B,* 50% activating diamide concentrations (AC_{50} values). These values were obtained from the corresponding dose-response curves. Dose-response curves were obtained as described in legend to Fig. 2. HAM and HAF, human HbA, male and female, respectively. RAB, rabbit, HS human HbS.

by us earlier (Lauf, 1988*a*) and by Jennings and Al-Rohil (1990) for NEM-treated rabbit RBCs.

Figure 2 shows the dose-response relationship between the Cl-dependent K (K-Cl) efflux rate constant and the concentration of diamide in HbA and HbS RBCs. Diamide increased the K-Cl efflux rate constant in a sigmoidal fashion, in both HbA and HbS RBCs with saturation at concentrations above 0.2 mM. No K-Cl cotransport was observed in the absence of diamide in the two RBC samples; in fact, K loss was higher in NO_3 than in Cl, consistent with previous findings in human RBCs (Lauf, Adragna & Garay, 1984; Haas & Schmidt, 1985; Kaji, 1986). At 0.5 mM diamide, K-Cl cotransport was about 10-fold higher in Hb S than in Hb A cells.

Figure 3 shows the kinetic pharmacological parameters for K-Cl efflux estimated from dose-response curves in RBCs of human male HbA (HAM) and female HbA (HAF), rabbit (RAB), sheep (SH), human HbS (HS) and rat, at maximum diamide concentrations, 300 mOsM and 5% hematocrit. The maximum K-Cl efflux rate constant for each species, was calculated from the dose-response curve as the average of the K-Cl efflux rate constants at the saturating diamide concentrations. Under these conditions, the relative maximum Cl-dependent K rate constants in diamide were as follows: human Hb A (1) < rabbit (1.8) < sheep (6.9) < human Hb S (9.5) ~ rat (9.7) (panel *A*). The 50% activating concentration (AC_{50}) was about 0.1 mM diamide in all species tested with the exception of LK sheep RBCs where the AC_{50} was about twice and hence the apparent affinity 50% lower (panel *B*). The relative AC_{50} values were: sheep (1.9) > human hemoglobin A (1) > rabbit (0.75) > human Hb S (0.67) = rat (0.67).

Effect of Diamide on K-Cl Cotransport as a Function of Cell Volume

K-Cl cotransport is activated by cell swelling, chemical thiol modification and Mg_i removal (Lauf et al., 1992). In sheep and human RBCs, the two first types of activations are additive (Lauf, 1988*b;* Lauf, et al., 1985). However, it is not known whether additivity between these two components is species-dependent. Thus we designed a series of experiments to test this point. Figure 4 shows in a representative experiment, the K efflux rate constants as a function of medium osmolality in human HbA RBCs. Panel *A* shows the rate constants in Cl and NO_3 in control and diamide (0.25 mM)-treated RBCs. Increasing the osmolality of the NO_3 medium increased K efflux in a linear fashion in both control and diamide-treated cells, and K efflux was greater in di-

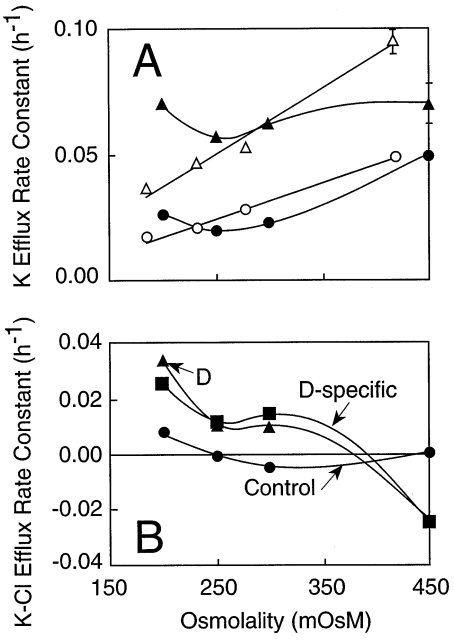

Fig. 4. K efflux in human Hb A RBCs as a function of medium osmolality. Panel A, K efflux rate constant in Cl (closed circles), NO_3 (open circles), Cl + 0.25 mM diamide (closed triangles), and NO_3 + 0.25 mM diamide (open triangles). Panel *B*, Cl-dependent K (K-Cl) efflux rate constant, in the absence (closed circles) and presence (closed squares) of 0.25 mM diamide. Diamide-activated K-Cl efflux rate constants were calculated as the difference between K-Cl efflux rate constant in the presence minus that in the absence of diamide (closed triangles). Prewarmed (37°C) Cl or NO_3 flux media of the same composition as above and containing diamide at the maximum activating concentration (0.25 mM) and 0.1 mM ouabain, were added to packed cells (final hematocrit, 2.5%). K efflux rate constants were determined as described in Materials and Methods.

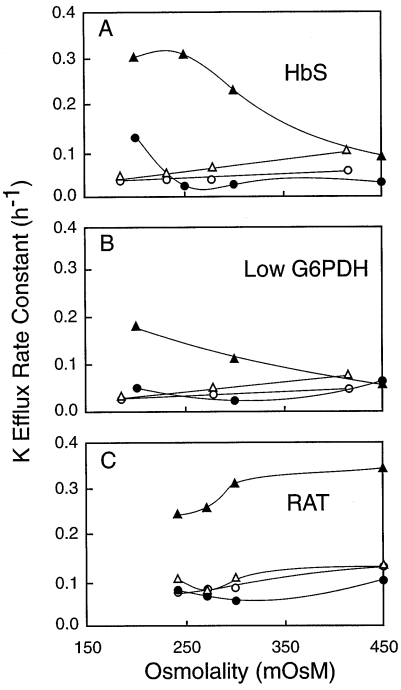

Fig. 5. K efflux rate constants in human and rat RBCs as a function of medium osmolality. Panel *A*, human HbS RBCs. Panel *B*, human glucose-6-phosphate-dehydrogenase (G6PDH) deficient RBCs. Panel *C*, rat RBCs. In each experiment, K efflux rate constants were determined as described in Materials and Methods. Symbols are as follows: Cl (closed circles), NO_3 (open circles), Cl + 0.25 mM diamide (closed triangles) or NO_3 + 0.25 mM diamide (open triangles).

amide-treated than in control cells at all osmolalities tested. In Cl, cell swelling activated K efflux above the levels found in cells with normal volume (~285 mOsM) both in control and in diamide-treated RBCs. However, cell shrinkage increased K efflux in control cells but not in diamide-treated cells where K efflux was not different from that at 300 mOsM.

The Cl-dependent K efflux rate constants as a function of medium osmolality are shown in panel *B*. The control curve shows a well-known behavior of K-Cl cotransport at the osmolalities tested, i.e., swelling of RBCs in hypotonic medium induced K efflux higher in Cl than in NO_3 only at <250 mOsM, whereas shrinkage of the cells in hypertonic medium obliterated the Cl-dependent flux (Lauf et al., 1985; Brugnara, 1989). In diamide-treated RBCs, K-Cl efflux between 200–300 mOsM was higher and approximately paralleled the behavior of control cells. In contrast, due to the higher flux in NO_3 than in Cl at 450 mOsM (panel *A*), no K-Cl cotransport was observed in hypertonic medium for diamide-treated RBCs. Panel *B* also shows the resulting diamide-activated (specific) Cl-dependent K efflux as a function of medium osmolality which almost overlapped with the flux in medium with diamide. Thus, when appropriate and for simplicity, K-Cl cotransport will be shown in diamide-containing medium instead as the diamide-activated flux.

Figure 5 compares K efflux rate constants in Cl and NO_3 as a function of medium osmolality for control and diamide-treated human HbS (panel *A*), and G6PDH deficiency (panel *B*) RBCs, and for rat RBCs (panel *C*). The reason for using G6PDH-deficient RBCs is that in these cells the GSH content is decreased compared to normal subjects. A reduced GSH content is associated with activation of K-Cl cotransport (Lauf, 1988*a*). Thus a higher K-Cl cotransport activity should be expected in RBCs from G6PDH-deficient patients. The figure shows that a decrease in medium osmolality below 300 and 250 mOsM, increased the K efflux rate constants in Cl in the presence and absence of diamide (panels *A* and *B*), respectively. An increase in medium osmolality above 300 mOsM decreased K efflux rate constant in Cl + diamide without further change for K efflux in the control (Cl − diamide) (panels *A* and *B*, except for the control in panel *B*). In contrast, in NO_3, K efflux rate constants increased linearly between 200 and 450 mOsM both in control and

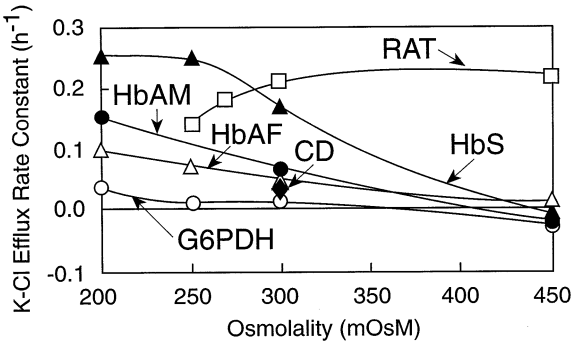

Fig. 6. Cl-dependent K efflux (K-Cl) rate constants in the presence of diamide and as a function of medium osmolality in RBCs from different mammalian species. K-Cl efflux rate constants were determined as described in Materials and Methods. Symbols are as follows: (HbA female (HbAF, open triangles), HbA male (HbAM, closed circles), HbS (HbS, closed triangles), G6PDH deficiency (G6PDH, open circles), cytoskeleton deficiency (CD, value only at 300 mOsM, closed diamond), and rat (open squares).

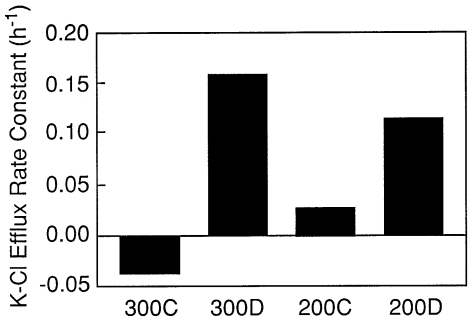

Fig. 7. Cl-dependent K (K-Cl) efflux rate constants as a function of osmolality in mouse RBCs. The protocol was as described in Materials and Methods, except that only isotonic (300 mOsM) and hypotonic (200 mOsM) conditions were tested in control (*C*) and diamide-treated (*D*) cells. In addition, due to the small amount of blood (4–5 ml/mouse/experiment) only two time points were taken to determine K efflux rate constants. The data represent the average of two independent experiments.

diamide-treated RBCs (panels *A* and *B*). Hence, the more swollen human HbA and S cells were, the more did diamide stimulate K-Cl cotransport. In rat RBCs, diamide also increased K efflux rate constants in Cl to levels similar to those observed in HbS, within the range of osmolalities tested (panel *C*). Furthermore, K efflux in Cl and NO_3 control (absence of diamide) behaved like in human HbS and G6PDH-deficient RBCs. In contrast, in the presence of diamide, K efflux rate constants in Cl actually increased with cell shrinkage. The two examples in panel *A* and *C* were chosen due to the significantly higher activity in HbS cells as compared to HbA (panel *A*), and the unlike behavior of rat red cells both in hypotonic and hypertonic medium with respect to the other species tested (panel *C*). In contrast to our expectations, panel *B* shows that cells with G6PDH deficiency were not different from untreated HbA neither in activity nor in behavior within the range of osmolalities tested (compare with Figs. 2–4). These results may indicate either that the GSH level was not low enough to affect the K-Cl cotransport activity or that in G6PDH-deficient cells other factors besides GSH regulate the activity of the system. Further studies are needed to test these possibilities.

Figure 6 summarizes Cl-dependent K efflux rate constants in diamide-containing medium as a function of medium osmolality in human RBCs with HbA (female and male), with HbS, G6PDH and a cytoskeleton deficiency (CD, data at 300 mOsM only), and in rat RBCs. As said in the Introduction, diamide cross links integral membrane proteins and affects the membrane cytoskeleton. In recent years, the cytoskeleton has been implicated in the regulation of several ion transport pathways and this is not an exception for the K-Cl cotransporter (Lauf et al., 1992). In the present study, we investigated the effect of diamide in RBCs from a patient with poikilocytosis and partial band 4.1 deficiency (*see* Materials and Methods) with the expectation to find differences or abnormalities in the behavior of these cells. As noted in Fig. 5, human HbS and rat RBCs showed a marked difference in activity and behavior, respectively, as compared to the rest. Thus a decrease in osmolality from 450 to 300 mOsM and from 300 to 200 mOsM increased K-Cl cotransport in diamide-treated RBCs by more than 1.4- and 2.5-fold, respectively, for human HbA RBCs, and by more than 14.7- and 1.5-fold, respectively, for human HbS RBCs. Furthermore, at 200 and 300 mOsM, K-Cl cotransport in diamide was about fourfold higher in HbS than in HbA RBCs and virtually absent at 450 mOsM. In contrast, Cl-dependent K flux saturated in diamide-treated rat RBCs when the osmolality was increased above 300 mOsM but decreased below this value. This behavior indicates that rat RBCs are not a good model to study swelling-activated K-Cl cotransport in the presence of diamide. In contrast to our expectations, RBCs with the particular cytoskeleton deficiency described above behaved as normal cells.

We were interested in assessing the existence of a diamide-activated K-Cl flux in mouse RBCs because rat RBCs showed such a high activity for this transporter and because transgenic mice strains serve as new models for sickle cell anemia where K-Cl cotransport is known to be elevated. Figure 7 shows the Cl-dependent K efflux at 300 and 200 mOsM in control and diamide-treated mouse RBCs. As shown above in this study and reported earlier by us (Lauf et al., 1984) and confirmed by others (Haas & Schmidt, 1985; Kaji, 1985), RBCs from mammalian species other than LK sheep display higher K fluxes in NO_3 than in Cl medium under isotonic conditions and are activated in Cl more than in NO_3 under hypotonic conditions. The figure shows that

mouse RBCs are not an exception to these findings. Furthermore, K-Cl efflux in diamide was about 30% higher in isotonic than in hypotonic medium, as seen for rat RBCs (Figs. 5, panel *C*, and 6) and as reported in sheep RBCs (Lauf, 1988*a*).

Effect of Diamide on K-Cl Cotransport in Density-separated and Nonseparated RBCs

Numerous studies in the literature with human RBCs have shown important interindividual differences in both the composition of the RBC population and their K-Cl cotransport activity, particularly in sickle cell patients (*see* references in Lauf et al., 1992). Some of these studies show an increased activity of the system in dense RBCs. Furthermore, swelling-activated K-Cl cotransport decreases with cell aging (Lauf et al., 1992) and the mechanism of this process is not yet well understood. Thus to further test the suitability of sheep RBCs as a model for sickle cell anemia and to further assess the identity of the diamide-activated K-Cl cotransport vis-à-vis the swelling-activated component we designed a series of experiments to determine the diamide-activated K-Cl cotransport in density-separated and unseparated RBCs from human (HbA and HbS) and sheep (LK and HK). This is an ideal example to study intra- and interspecies differences because the two species possess marked intraspecies variants: HbA *vs.* HbS, and LK *vs.* HK. Cells were separated by density gradient in only two fractions, light and dense, as described in Materials and Methods. Figure 8 shows rate constants of K efflux in Cl and NO_3 from control and diamide-treated RBCs in unseparated (total), light and dense human HbA (panel *A*) and HbS (panel *B*) RBCs. In HbA cells, K efflux was significantly higher in diamide-treated than in control RBCs both in Cl and NO_3 in each of the three cell types (unseparated, light and dense). In HbS cells, diamide treatment increased K efflux in Cl but not in NO_3. However, the major difference between HbA and HbS cells was mainly quantitative (compare the magnitude of K efflux rate constant in all media and cell types). It is interesting to note that HbS dense cells displayed a sizable Cl-dependent K efflux rate constant both in the presence and absence of diamide whereas in HbA dense cells this component appeared only in diamide-treated cells.

Figure 9 summarizes Cl-dependent K efflux rate constants in control and diamide-treated RBCs from the two species selected, human and sheep, and from the intra-species subgroups, HbA/HbS and HK/LK. Panel *A* shows unseparated cells where panels *B* and *C* show light and dense cells, respectively, for each species/subgroup. In all cases, diamide increased the activity of K-Cl cotransport according to the following order: light cells > unseparated cells > dense cells. The three types of cells

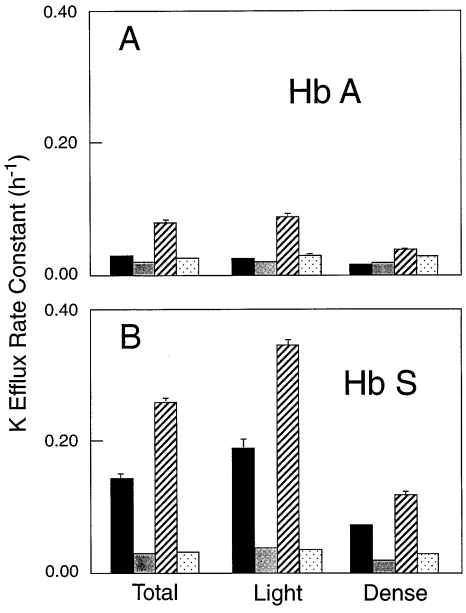

Fig. 8. K efflux rate constants in unseparated (Total) and density-separated (Light and Dense) human RBCs. Panel *A*, human HbA. Panel *B*, human HbS. Cl control, solid columns; NO_3 control, thin hatched columns; Cl + diamide, thick striped columns; NO_3 + diamide, dotted columns. *See* legend to Fig. 1 and Materials and Methods for further experimental details. Bars represent means ± standard deviations (SD).

had higher K-Cl cotransport activities in human HbS and LK sheep than in human HbA and HK sheep blood (except for HBAF in unseparated cells, which had a slight difference in the treatment, i.e., the cells were washed once in PBCl instead of being kept without washing on ice). These data indicate that human HbS and LK sheep behave more alike and that human HbA and HK sheep were the most alike. Furthermore, the major difference between human HbA and HbS occurred in control dense cells where K-Cl cotransport was negligible in the former but significantly different from zero in the latter (Panel *C*). Similarly, K-Cl cotransport was significantly elevated in unseparated control LK but not HK RBCs, as reported by us earlier (Fujise & Lauf, 1987). However, the increased K-Cl cotransport in dense LK but not HK RBCs is a finding documented for the first time in this study.

Comparative Analysis

To obtain a set of general reference values, we pooled all data of all controls and diamide-treated RBCs, unseparated and density-separated, from human HbA (one female and one male) and HbS, and from LK and HK sheep RBCs. Mean K efflux rate constants were (Mean ± SE, $n = 30$): 0.085 ± 0.015 in Cl, 0.020 ± 0.002 in NO_3, and 0.065 ± 0.014 (h-1) for the difference. Hence,

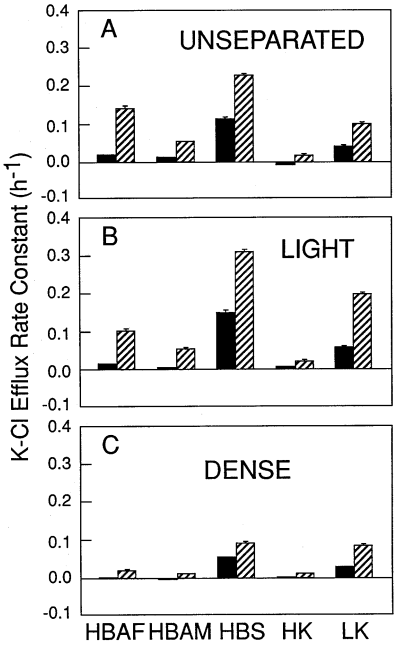

Fig. 9. Cl-dependent K efflux rate constant in unseparated and density-separated human and sheep red blood cells. Panel *A*, whole blood, panel *B*, light cells, panel *C*, dense cells. Solid bars, control; thick striped bars, diamide. HBAF and HBAM, human female and male HbA, respectively. HBS, human HbS. LK and HK, low and high K sheep RBCs, respectively. For further details see legend to Fig. 8.

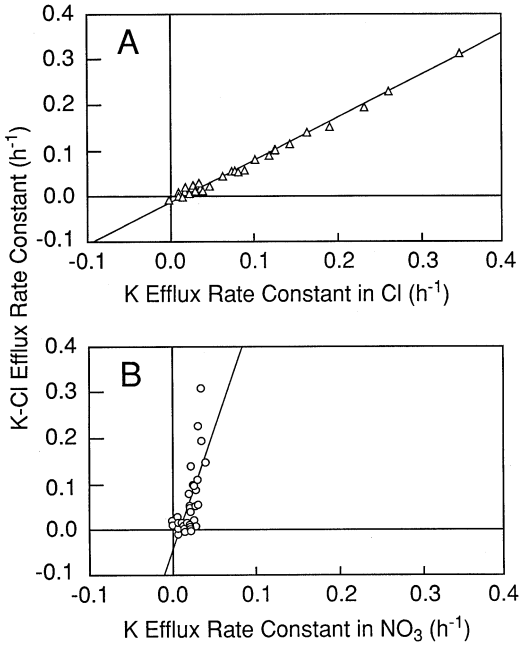

Fig. 10. Correlation between K-Cl efflux rate constants pooled from control and diamide-treated RBCs and their respective K efflux rate constants in Cl (panel *A*, open triangles) or NO_3 (panel *B*, open circles). Data corresponds to unseparated and density-separated human and sheep RBCs. The regression line for K-Cl cotransport *vs.* K efflux rate constants in Cl was $y = 0.908x - 0.013$, $P < 0.00001$, $r^2 = 0.992$, $n = 30$ and in NO_3 $y = 5.183x - 0.041$, $P < 0.00001$, $r^2 = 0.480$, $n = 30$. For further details see legend to Fig. 8. Diamide concentration, 0.25 mM.

K efflux of all samples was about four times higher in Cl than in NO_3 and K-Cl cotransport accounted for about 80% of the flux in Cl, suggesting that the measurement of K flux in Cl is a good predictor of K-Cl cotransport activity. Figure 10 *A/B* shows a plot of Cl-dependent K efflux rate constants as a function of K efflux rate constants in Cl or NO_3 for the above sample. The equation describing the regression lines for K-Cl cotransport *vs.* K efflux rate constant in Cl was $y = 0.908x - 0.013$ ($P < 0.00001$, $r^2 = 0.992$, $n = 30$) and in NO_3 $y = 5.183x - 0.041$ ($P < 0.00001$, $r^2 = 0.480$, $n = 30$). Since a regression line allows the prediction of values of y given the values of the independent variable x, these data indicate that the flux in Cl is indeed a better predictor of K-Cl cotransport activity than the flux in NO_3. However, the K flux in NO_3 also showed a significant correlation with K-Cl cotransport activity in this sample, i.e., with control and diamide-treated human and SRB cells of different ages.

Figure 11 panel *A* shows all data divided in two groups, control and diamide in unseparated cells. K efflux rate constants in Cl were 2.4-fold higher in diamide-treated RBCs than in controls. Likewise, the Cl-dependent component was 2.9-fold higher whereas the rate constants in NO_3 were not different in the two groups. These data indicate that diamide, at the dose used (0.25 mM), activated only K-Cl cotransport. Linear regression analysis, as shown in Fig. 10 panels *A* and *B*, indicated that the flux in Cl predicted better than in NO_3 the activity of K-Cl cotransport in these samples. Slopes (r^2, p) in Cl for control and diamide-treated cells were 0.847 (0.990, $P < 0.00001$) and 0.925 (0.993, $P < 0.00001$), respectively, and in NO_3 3.967 (0.671, $P = 0.0002$) and 5.215 (0.379, $P = 0.0145$), respectively. Panel *B* shows Cl-dependent K efflux rate constants calculated from above data in unseparated (total) and separated (light and dense, *see* Materials and Methods) RBCs. Diamide increased the activity of K-Cl cotransport by 3.1-, 2.9- and 2.7-fold over the level of the control in unseparated, light and dense RBCs, respectively. In the three groups shown in this panel, the threefold increase in K-Cl cotransport in diamide-treated RBCs over the controls was due to an increase in K efflux in Cl rather than in NO_3 medium.

Discussion

This study shows for the first time diamide-activated K-Cl cotransport in human, rat and mouse RBCs and contrasts previous reports in human (Deuticke et al.,

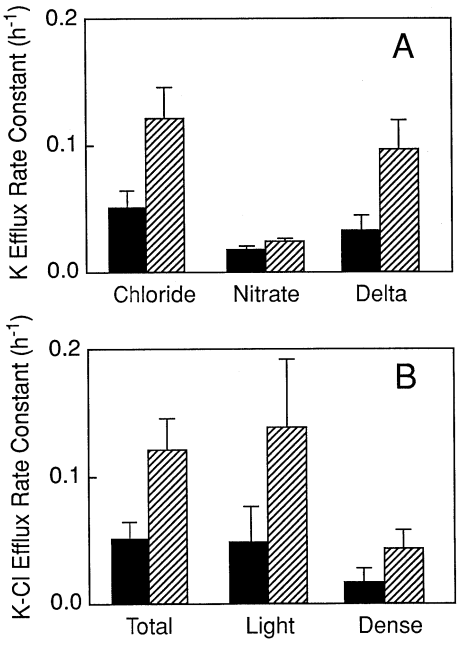

Fig. 11. Average K efflux rate constants pooled from all controls and diamide-treated unseparated and density-separated RBCs from human HbA (one female and one male) and HbS subjects, and from LK and HK sheep RBCs. Panel *A*, mean values (Mean ± SE, $n = 15$) of K efflux rate constants in Cl and NO_3, and of the difference between the fluxes in Cl and NO_3 (Delta) for control (solid columns) and diamide-treated (thick striped columns) RBCs. Panel *B*, Cl-dependent K efflux, calculated as the difference between the rate constant in Cl minus that in NO_3, in unseparated (TOTAL), light, and dense RBCs. Control (solid columns, mean ± SE, $n = 5$), diamide-treated (thick striped columns, mean ± SE, $n = 5$). For further details *see* legend to Fig. 8.

1992; Ihrig et al., 1991). The discrepancy with the study in human RBCs where diamide was found to activate F fluxes in a Cl-independent manner may be due to the high diamide concentration used (5 mM). It is possible that at this high concentration nonspecific diamide effects took place at the membrane or cytoskeleton level such as those mentioned in the Introduction (Pestonjamasp & Mehta, 1991; Yamaguchi et al., 1991; Deuticke et al., 1992; Beppu et al., 1992; Terada et al., 1992; Kumar & Gupta, 1992; Terada et al., 1993; Yamaguchi et al., 1993; Turrini et al., 1994; Muralidharan et al., 1994; Giger et al., 1995; Khan & Saleemuddin, 1995).

One of our major findings is the significant quantitative and qualitative difference in diamide-activated K-Cl cotransport between and within the species studied. The apparently anomalous behavior of rat RBCs as a function of medium osmolality (Figs. 5*C* and 6) may be caused by activation and inhibition of Na-K-2Cl cotransport by shrinkage and swelling, respectively. An argument against a possible activation of Na-K-2Cl cotransport by cell shrinkage is that the Cl-dependent K efflux rate constant was not higher at 450 mOsM than the value observed at 300 mOsM (Fig. 6). On the other hand, decrease of K-Cl and increase of Na-K-2Cl cotransports could lead to an apparent constancy of Cl-dependent flux with hypertonicity. However, human RBCs also possess a Na-K-2Cl cotransport system which is activated by cell shrinkage yet only rat RBCs appear to behave differently when cell volume is changed in the presence of diamide. Further studies are needed to clarify this point.

Previous studies by Brugnara (1989) on human HbA and CC RBCs show activation by NEM of ouabain- and bumetanide-resistant K efflux at alkaline pH and in a range of 200–400 mOsM. Furthermore, NEM also activates K-Cl cotransport in all RBC fractions that are density-separated through a Stractant gradient. Our results on the effect of diamide in human HbA and S under isotonic conditions (Figs. 2, 3*A* and *B*), as a function of cell volume (Figs. 4–6) and in density-separated cells (Fig. 8 and 9) agree with Brugnara's findings despite differences in the approach between the two studies. First, NEM and diamide activate K-Cl cotransport by different mechanisms of action: NEM is an irreversible SH-blocker (Beppu et al., 1994), whereas diamide is a mild and reversible SH-oxidizing agent (Beppu et al., 1994). Second, the hemoglobin abnormalities in CC and SS cells correspond to a single amino acid mutation where lysine or valine, respectively, replace glutamic acid. Third, density separation in our study was obtained with Percoll gradient whereas Brugnara (1989) used a discontinuous Stractan density gradient.

Our study expands on a question in Brugnara's study on the role of the positively charged hemoglobin C in maintaining an elevated K-Cl cotransport activity in dense RBCs (Brugnara, 1989). Further data on the also positively charged HbS appears to support this conclusion (Olivieri et al., 1992). However, the elevated K-Cl cotransport activity in dense LK sheep RBCs with no positively charged hemoglobin seems to be inconsistent with this conclusion unless one assumes that cell age affects hemoglobin net charge. This seems to be true for anemic sheep Hb type AB, where DEAE cellulose chromatographic separation of its blood shows a peak corresponding to HbC in addition to the characteristic peaks of HbA and B (Blunt & Huisman, 1975). Similar results are observed in 15-day-old goat (Blunt & Huisman, 1975). Thus, young cells may express HbC. However, no signs of anemia were observed in the sheep used in the present study, as judged by the hematocrit values. This point, while settled for human hemoglobins (Olivieri et al., 1992), needs further investigation in sheep.

The strong correlation between K-Cl cotransport and K efflux in Cl was to be expected since this is a Cl-dependent flux. However, in NO_3 K-Cl cotransport is completely inactive in LK SRBCs and practically nonfunctional in human RBCs, consequently no such a correlation was thought to be found. Thus, the unexpected significant correlation between K-Cl cotransport and K efflux in NO_3 is another point for which no clear expla-

nation is available at present (Figs. 10 and 11). It is possible that in NO_3, diamide activates some additional pathways (channels?) in some of the mammalian species studied.

In conclusion, a diamide-activated K-Cl cotransport was found in mammalian red cells with a large inter-, and for human intraspecies variability of the maximum diamide-activated Cl-dependent K flux component. In light of the variability in the magnitude and behavior of K-Cl cotransport in the different species and subgroups, the relationship between diamide-activated K-Cl cotransport and hemoglobin structure or function deserves further attention. Our data are consistent with the hypothesis that the activation of K-Cl cotransport by diamide involves the oxidation of adjacent thiols to dithiols.

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