Urea Inhibits NaK2Cl Cotransport in Human Erythrocytes

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Abstract

We examined the effect of urea on NaK2Cl cotransport in human erythrocytes. In erythrocytes from nine normal subjects, the addition of 45 mM urea, a concentration commonly encountered in uremic subjects, inhibited NaK2Cl cotransport by 33±7%. Urea inhibited NaK2Cl cotransport reversibly, and in a concentration-dependent fashion with half-maximal inhibition at 63±10 mM. Acute cell shrinkage increased, and acute cell swelling decreased NaK2Cl cotransport in human erythrocytes. Okadaic acid (OA), a specific inhibitor of protein phosphatase 1 and 2A, increased NaK2Cl cotransport by nearly 80%, suggesting an important role for these phosphatases in the regulation of NaK2Cl cotransport. Urea inhibited bumetanide-sensitive K influx even when protein phosphatases were inhibited with OA, suggesting that urea acted by inhibiting a kinase. In cells subjected to shrinking and OA pretreatment, maneuvers expected to increase the net phosphorylation, urea inhibited cotransport only minimally, suggesting that urea acted by causing a net dephosphorylation of the cotransport protein, or some key regulatory protein. The finding that concentrations of urea found in uremic subjects inhibited NaK2Cl cotransport, a widespread transport pathway with important physiological functions, suggests that urea is not only a marker for accumulation of other uremic toxins, but may be a significant uremic toxin itself. (*J. Clin. Invest.* 1995. 96:2126–2132.) Key words: bumetanide • uremia • renal failure • volume regulation

Introduction

Urea is the main nitrogenous end product of protein metabolism and is the most abundant nitrogenous product that accumulates in acute or chronic renal failure. While the concentrations of blood urea and blood urea nitrogen are well accepted markers for the severity of renal failure, the role of urea in the pathogenesis of the uremic syndrome is controversial. In 1823, Prevost and Dumas reported elevated urea concentrations in the blood of nephrectomized or ureter-ligated dogs and suggested that urea may be the cause of uremic intoxication (1). However, subsequent studies have cast doubt in the role of urea as the cause of uremic syndrome. Bollman and Mann implanted the ureters of dogs into the ileum and noted that toxic symptoms did not develop despite blood urea concentrations of 800 mg/dl (2). Merrill, Legrain, and Hoigne found that uremic subjects improved clinically with dialysis even when blood urea concentrations were maintained by adding urea to the dialysate solutions (3). In contrast, Johnson infused urea and observed some toxic features of uremia above blood urea concentrations of 200–300 mg/dl (33–50 mM, reference 4). Grollman and Grollman maintained anephric dogs by dialysis for up to 39 d, but when urea was added to the dialysate they noted weakness and anorexia 4 d after nephrectomy, followed by vomiting, bloody diarrhea, hypothermia, deep stupor, and coma at 9 d (5). Despite the controversial nature of evidence, summarized previously (6–8), many regard urea as a benign marker of uremia.

Recently, it was reported that urea affects a number of volume-sensitive cation transport pathways in mammalian erythrocytes. Transport pathways which are stimulated by cell swelling such as the KCl cotransport were found to be stimulated by urea (9) whereas transport pathways which are inhibited by cell swelling, i.e., Na-H exchange, were found to be inhibited (9). It was proposed recently that cells sense their volume by changes in macromolecular crowding (10, 11). Even though urea does not alter cell volume, urea may bind to proteins, stabilize the protein structure, decrease macromolecular crowding, mimic cell swelling, and thus may affect transport pathways that are volume sensitive (12).

The NaK2Cl cotransport is a volume sensitive, secondary active ion transport pathway that is widely distributed in many different cell types. The NaK2Cl cotransport mediates the coupled transport of Na, K, and Cl ions across cell membranes (13, 14). The transport system is electrically neutral (15), saturable with respect to external Na, K, and Cl, inhibitable by loop diuretics such as furosemide and bumetanide, and specific in its requirement for substrates (13, 14). Because NaK2Cl cotransport is stimulated by cell shrinkage and inhibited by cell swelling in many different cell types (13, 14), we examined whether urea alters NaK2Cl cotransport in human erythrocytes. We examined whether low urea concentrations (~ 45 mM) which are commonly encountered in the blood of uremic subjects can also alter NaK2Cl cotransport. In addition, we explored the relationship of the state of phosphorylation to the urea-mediated inhibition of NaK2Cl cotransport. Our findings suggest that urea, in concentrations commonly encountered in uremic subjects, inhibits NaK2Cl cotransport in human erythrocytes.

Methods

Venous blood was collected from nine normal adult donors, five males, and four females with no known history of blood dyscrasias. Cells were

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washed three times in medium A containing (mM) NaCl 145, KCl 5, dextrose 5, and Tris/Mops 5, pH 7.4, at 37°C.

Alteration of cell volume

Cell volume was altered by either the osmotic or the nystatin method (16).

Osmotic method. Cell volume was altered by incubation of cells in anisotonic media. The osmolality of the medium was varied by adding sucrose (0–200 mM) to a hypotonic flux medium containing (mM) NaCl 95, KCl 5, dextrose 5, Tris/Mops 5, pH 7.4, at 37°C. Sucrose was added to hypotonic, rather than isotonic, media to achieve both swelling and shrinking of cells in media with constant external Cl.

Nystatin method. Packed washed cell suspensions (0.4 ml) were incubated with 40 mg/liter nystatin in 10 ml of loading solution containing (mM) NaCl 10, KCl 140, sucrose 15–200, Hepes 2.5, pH 7.2, at 4°C. After a 15-min incubation at 0°C, the cells were washed once and resuspended in the same medium for an additional 15 min. Cells were then washed four times at 37°C with the same medium but without nystatin and with 0.1% albumin to elute nystatin. Next, the cells were washed four more times with the same medium without sucrose, pH 7.4, at 37°C. Finally, cells were washed three more times with medium A (for composition, see above). After saving aliquots for hemoglobin and hematocrit, cells were used for the flux assay. In extensive preliminary studies, we established that the intracellular Na and K concentrations remain unchanged with this procedure, regardless of the changes in cell volume. Thus, nystatin treatment resulted in isosmotic swelling or shrinking.

Relative cell volume

Relative cell volume (RCV)[1] was measured from hematocrits and hemoglobin concentration of lysates, as described previously, using the volume of cells in whole blood as unity (16–19).

K influx

Unidirectional K influx was measured using ^{86}Rb as a tracer for K, as described previously (16–19). Packed erythrocyte suspensions (50 μl) of known hematocrit (usually between 70 and 80%) were suspended in 1 ml of flux medium at 37°C. Ouabain (0.1 mM) was added to all media to inhibit the Na-K pump. When okadaic acid (OA) or urea was added, a preincubation period of 30–90 min was observed before adding ^{86}Rb, and the reagents were also present during incubation with ^{86}Rb. Cells were incubated with ^{86}Rb at 37°C for 60 min with the influx medium (medium A unless specified otherwise in figure legends). At the end of incubation, cells were washed three times with ice-cold unbuffered $MgCl_2$. Cell pellets were lysed with 0.1% (vol/vol) ice-cold Triton X-100, followed by protein precipitation with 5% (wt/vol) trichloracetic acid and centrifugation for 10 min at 3,000 *g*. The radioactivity of the supernatant solution was counted by Cerenkov radiation in a scintillation counter as described previously by Dunham and Ellory (20). The NaK2Cl cotransport was defined as the difference in K influx with and without 2 μM bumetanide, and expressed as $\mu mol \cdot loc^{-1} \cdot h^{-1}$. It should be noted that 2 μM bumetanide inhibited > 95% of the NaK2Cl cotransport. Higher concentrations of bumetanide failed to inhibit a greater fraction of NaK2Cl cotransport, but did inhibit a portion of KCl cotransport, as reported earlier (16), and thus obscured the effect of urea or shrinking on NaK2Cl cotransport.

Materials

^{86}Rb was purchased as the chloride salt from Dupont-NEN (Boston, MA). Bumetanide was a gift from Hoffman La Roche (Nutley, NJ). OA was purchased from LC Laboratories (Woburn, MA). All other reagents were purchased from Sigma Immunochemicals (St. Louis, MO) or J. T. Baker through VWR Scientific (Piscataway, NJ). Urea was dissolved in media on the day of the experiment. Urea-containing media were never stored for > 48 h. When reagents were dissolved in DMSO, the amount of DMSO added to flux media never exceeded 0.5% (vol/vol).

Presentation of data

Experimental data points were fitted to equations using linear or nonlinear regression with successive iteration using Sigmaplot (Jandel Scientific, Cortes Madera, CA). Results are presented as means±SD or means±SE as specified in Results or figure legends. The standard deviation (SD) of bumetanide-sensitive K influx was calculated as $\sqrt{(SD_1)^2 + (SD_2)^2}$ where SD_1 and SD_2 represent SDs of total and bumetanide-resistant influx, respectively. Because of variations in the absolute rates of K transport in cells from different donors, the results from experiments on different donors have not been pooled. Instead, representative studies are shown in the table and the figures. In each case, similar results were obtained in two or more separate experiments with cells from at least two other donors.

Results

Urea equilibration across erythrocyte membranes is mediated by a facilitated diffusion process and is extremely rapid (21, 22). We confirmed that RCV was unchanged 2 min after addition of 600 mM urea to erythrocyte suspensions (0.97±0.02 vs. 0.99±0.02, $n = 4$). Similarly, RCV was unchanged after 120 min of incubation with urea.

The effect of low urea concentration (45 mM) on bumetanide-sensitive K influx was examined in nine normal subjects (Fig. 1). This urea concentration corresponds to a blood urea concentration of 270 mg/dl or a blood urea nitrogen concentration of 126 mg/dl, which is commonly encountered in uremic subjects. The basal activity of NaK2Cl cotransport varied widely, from 59 to 424 $\mu mol \cdot loc^{-1} \cdot h^{-1}$. The variation was entirely due to interindividual differences; the bumetanide-sensitive K influx was remarkably constant in a given individual over months (not shown). Despite this wide variation in the activity of NaK2Cl cotransport, 45 mM urea consistently inhibited bumetanide-sensitive K influx in every subject by paired *t* test. A statistically significant difference in bumetanide-sensitive K influx was observed ($P < 0.01$ by paired *t* test) even in the two subjects with the least (15) percent inhibition. The average inhibition with 45 mM urea was 33±7% of the control value (252±44 vs. 184±38, Fig. 1).

Concentration dependence of urea-mediated inhibition of NaK2Cl cotransport. To further examine the concentration dependence of urea-mediated inhibition of NaK2Cl cotransport, we examined the effect of various urea concentrations on cotransport in a single individual. The degree of inhibition of cotransport in this subject with 45 mM urea (30%) was close to that for the entire group (33%). Fig. 2 shows the cotransport values at various urea concentrations (seven different experiments). Urea inhibited the bumetanide-sensitive K influx in a concentration-dependent manner. The data were fitted by a hyperbolic curve describing a single inhibitory site; attempts to fit the data with equations using two or more inhibitory sites did not improve the fit further. Greater than 90% of the bumetanide-sensitive K influx was abolished at urea concentrations > 300 mM. The concentration required for 50% inhibition (K_i) of cotransport activity was 63±10 mM (Fig. 2). In two other subjects, the calculated K_i values were 70±9 and 54±12 mM.

1. *Abbreviations used in this paper:* loc, liter of original cells; OA, okadaic acid; RCV, relative cell volume.

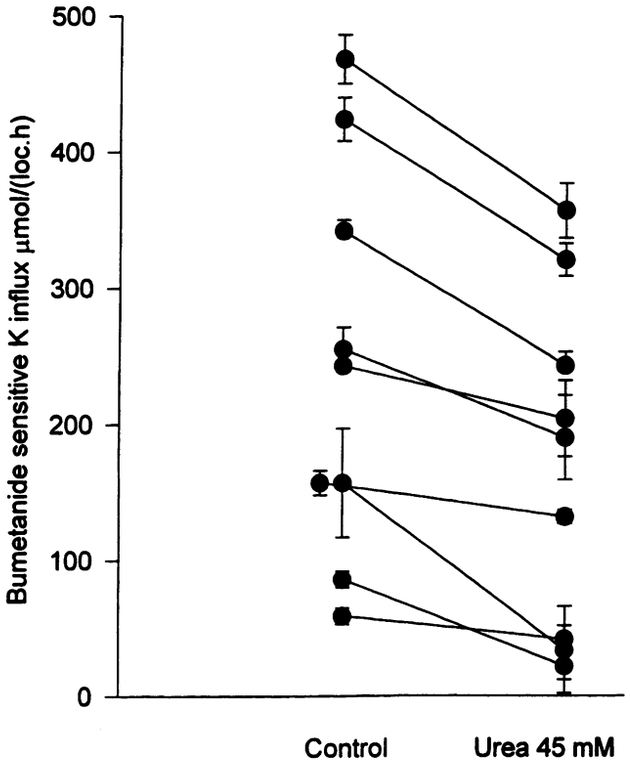

Figure 1. Effect of 45 mM urea concentration on bumetanide-sensitive K influx in nine different normal subjects. Each point depicts the average ±SD of triplicates obtained on a given day. The variation between triplicates did not exceed 6%. Despite a fivefold interindividual variation in basal bumetanide-sensitive K influx, urea inhibited cotransport in all nine subjects.

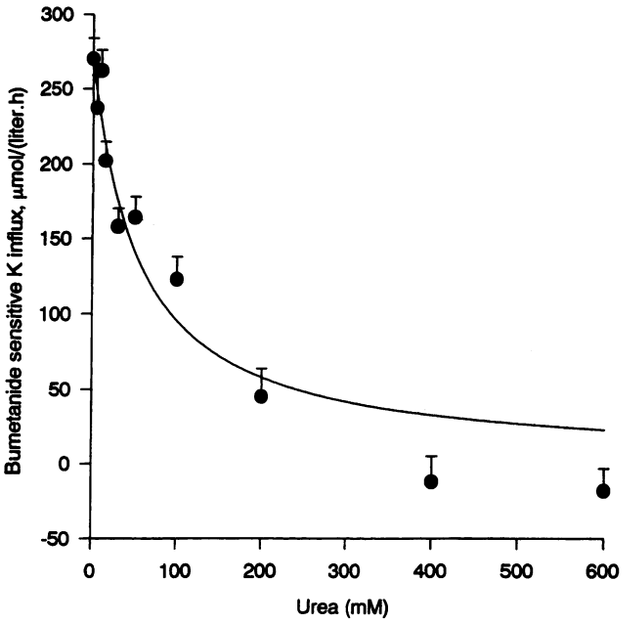

Figure 2. The effect of various urea concentrations on bumetanide-sensitive K influx in human erythrocytes. Cells were exposed to various urea concentrations for 60 min and bumetanide-sensitive K influx was measured as described in Methods. Depicted are means±SE from seven separate experiments from a single individual. Similar results were obtained in two other subjects. The solid line represents the theoretical line derived from fitting the data points to the equation, $J_u = J_c \cdot K_i/(K_i + U)$, where J_u and J_c represent bumetanide-sensitive K influx in the presence and absence of urea, K_i represents the urea concentration required to inhibit 50% of the bumetanide-sensitive K influx, and U represents the urea concentration in millimolars.

Reversibility of inhibition. In cells exposed to 500 mM urea for 90 min and then washed free of urea, bumetanide-sensitive K influx was not different from that in control cells not exposed to urea (Table I). Thus, the urea-mediated inhibition of NaK2Cl cotransport was entirely reversible.

Effect of urea analogues on NaK2Cl cotransport. Two urea analogues, acetamide and formamide (500 mM each), also inhibited bumetanide-sensitive K influx significantly (Table I).

Effect of altering cell volume on cotransport. The control bumetanide-sensitive K influx (in normal volume cells) was lower with the osmotic method (RCV 0.97) compared with the nystatin method (RCV 0.98, Fig. 3). While the precise reason for the large difference in control fluxes with the two methods is not clear, it may be related to the difference in external Cl (100 mM with the osmotic method vs. 150 mM with the nystatin method). Because of low Cl affinity, an increase in external Cl from 100 to 150 mM can stimulate bumetanide-sensitive K influx. Indeed, three other groups have reported that NaK2Cl cotransport-mediated K influx in human erythrocytes was 50–80% higher at an external Cl of 150 mM compared with the flux at an external Cl of 100 mM (23–25). Thus, most of the difference in baseline bumetanide-sensitive K influx with the two methods can be explained by the difference in external Cl concentrations.

With the nystatin method, alteration of cell volume had a striking effect on the bumetanide-sensitive K influx. Bumetanide-sensitive K influx nearly doubled in cells shrunken with the nystatin method compared with cells of normal volume (603±46 vs. 312±15 $\mu mol \cdot loc^{-1} \cdot h^{-1}$, $P < 0.001$, Fig. 3, *right, open bars*). Similarly, bumetanide-sensitive K influx was 64% lower in cells swollen with the nystatin method compared with cells at normal volume (111±54 vs. 312±15 $\mu mol \cdot loc^{-1} \cdot h^{-1}$, $P < 0.001$, Fig. 3, *open bars*).

Table I. Reversibility of Urea and Effect of Urea Analogues on Bumetanide-sensitive K Influx

	Bumetanide-sensitive K influx	*P* value (compared with control)
	µmol/(loc · h)	
Control	256±12	
Urea (500 mM)	−15±4	< 0.001
Urea (500 mM), then washed	260±14	NS
Acetamide (500 mM)	55±5	< 0.001
Formamide (500 mM)	27±3	< 0.001

Means±SD from four experiments from a single donor. Similar results were obtained in one other subject. With 500 mM urea, the K influx was consistently higher in the presence of bumetanide than in its absence, hence the negative value for bumetanide-sensitive K influx.

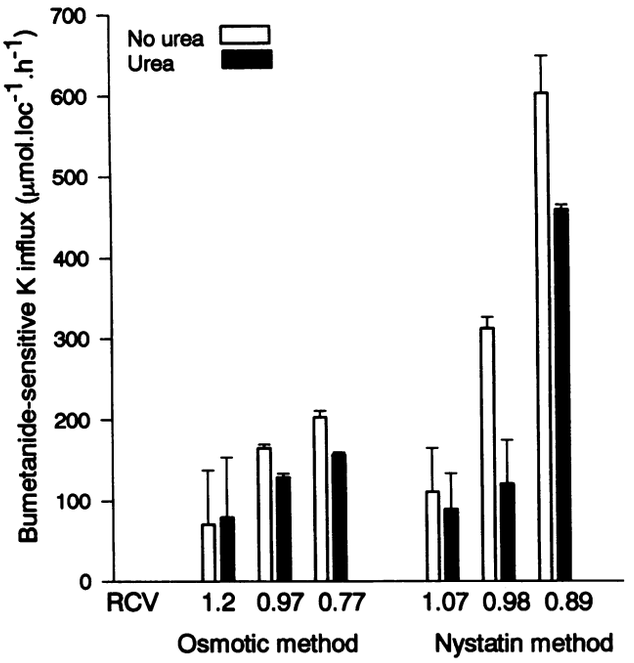

Figure 3. Effect of acute change of cell volume on NaK2Cl cotransport and the inhibitory effect of urea. When cell volume was altered with the osmotic method, the influx media contained 95 mM NaCl, 5 mM KCl, and sucrose (0–200 mM) to adjust the final osmolality to 200–400 mosmol/kg. When nystatin was used to vary the cell volume, the influx media contained 145 mM NaCl and 5 mM KCl. Thus, the external Na and Cl concentrations were lower with the osmotic method compared with the nystatin method and also lower compared with the concentrations used for Figs. 1, 2, 4, and 5.

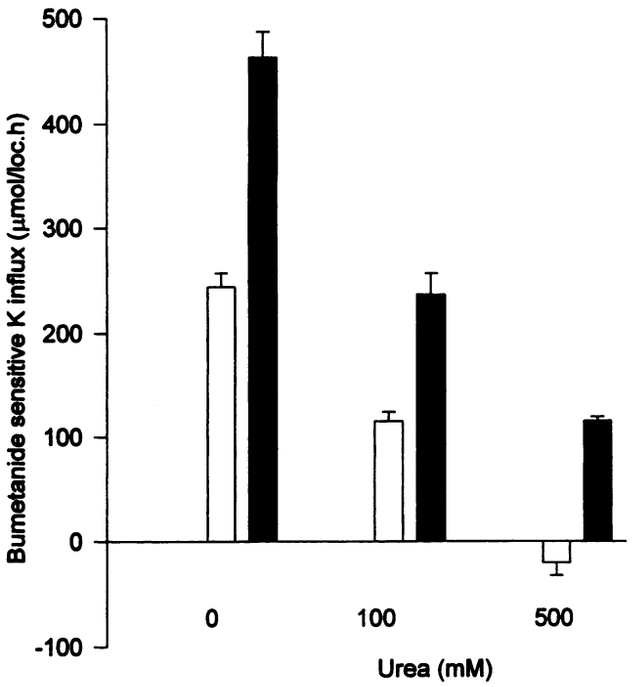

Figure 4. Effect of OA pretreatment on urea-mediated inhibition of bumetanide-sensitive K influx. Cells were pretreated with vehicle (DMSO 0.5% vol/vol, *open bars*) or 0.8 μM OA (*filled bars*) for 30 min. Subsequently, cells were incubated for 1 h in media containing 0 (*left*), 100 (*middle*), or 500 mM (*right*) urea in the continued presence of OA. At the end of this preincubation, ^{86}Rb was added and K influx was measured. Depicted are the means±SD of bumetanide-sensitive K influx from three separate experiments.

When cells were shrunken with the osmotic method, a small but significant increase in cotransport activity was noted compared with the activity in cells of normal volume (RCV 0.77, 203±5 vs. 167±8, $P < 0.002$ by paired t test). Conversely, when cells were swollen with the osmotic method (Fig. 3), bumetanide-sensitive K influx decreased significantly (79±75 μmol·loc^{-1}·h^{-1}, RCV 1.2) compared with cells of normal volume (167±8 μmol·loc^{-1}·h^{-1}, RCV 0.97, $P < 0.01$, Fig. 3).[2] While statistically significant changes in cotransport were observed with osmotic shrinking and swelling, the response of NaK2Cl cotransport to changes in cell volume was blunted with the osmotic method compared with the nystatin method.

Urea (100 mM) inhibited NaK2Cl cotransport in both normal volume cells and in shrunken cells (Fig. 3, *filled bars*), but the degree of inhibition was less in shrunken cells compared with cells at normal volume, with both the osmotic and the nystatin method. With the nystatin method, urea inhibited 23% of the bumetanide-sensitive K influx in shrunken cells (459±6 vs. 603±46) and 61% of that influx in normal volume cells (119±54 vs. 312±14).

2. Swelling activated the KCl cotransport (~ 400 μmol·loc^{-1}·h^{-1} at 5 mM external K), which was present both in the presence and absence of 2 μM bumetanide. Therefore, the relatively small bumetanide-sensitive K influx (79 μmol·loc^{-1}·h^{-1}), calculated as the difference of two large numbers (400–500 μmol·loc^{-1}·h^{-1}), had a large standard deviation.

Effect of OA on urea-mediated inhibition of NaK2Cl cotransport. Bumetanide-sensitive K influx was nearly doubled by pretreatment of cells with OA (0.8 μM, Fig. 4, *filled bars*, 463±24 vs. 244±13 μmol/(loc·h), $P < 0.001$ by paired t test). Urea decreased bumetanide-sensitive K influx in OA-pretreated cells, both at a concentration of 100 mM (237±20 vs. 463±24 μmol/(loc·h), $P < 0.001$) and at 500 mM urea (115±4 vs. 463±24 μmol/(loc·h), $P < 0.001$). While 500 mM urea completely abolished basal activity of NaK2Cl cotransport, it failed to inhibit cotransport completely in OA-pretreated cells. Nonetheless, urea clearly altered bumetanide-sensitive K transport under conditions where phosphatase activity was maximally inhibited by OA (Fig. 4).

Effect of urea on shrunken, OA-treated cells. Fig. 5 shows that cells shrunken by the osmotic method (in 600 mosmol/kg media) and exposed to OA showed upregulation of cotransport (498±12 vs. 319±48 μmol/(loc·h), $P < 0.001$). In shrunken, OA-treated cells, urea inhibited only 10% of the bumetanide-sensitive K influx (447±15 vs. 498±12) compared with 62% inhibition of basal cotransport (nonshrunken cells not exposed to OA, 121±58 vs. 319±48, $P < 0.001$).

Discussion

We have shown that urea inhibits NaK2Cl cotransport reversibly and in a concentration-dependent manner. Furthermore, the results shown here indicate that urea inhibits NaK2Cl cotransport significantly at relatively low concentrations.

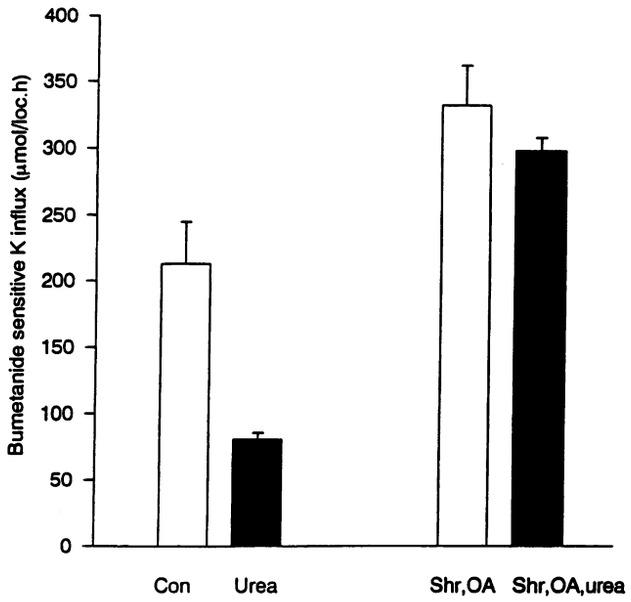

Figure 5. The combined effect of shrinking and OA pretreatment on urea-mediated inhibition of cotransport. Open bars represent cells not exposed to urea, whereas filled bars represent cells exposed to 100 mM urea. *Con* refers to cells incubated in medium A. *Urea* refers to cells preincubated for 30 min in medium A with added urea (100 mM). *Shr,OA* refers to cells which were preincubated for 30 min in medium A with added sucrose (100 mM) and OA (0.8 μM). *Shr, OA, urea* refers to cells which were preincubated for 30 min in medium A with added sucrose (100 mM) and OA (0.8 μM) for 30 min, and then exposed to 100 mM urea in the continued presence of OA for an additional 30 min. The influx media were identical to the medium used for preincubation and contained OA and urea where noted.

Conflicting results have been reported on the effects of acute change in cell volume on NaK2Cl cotransport in human erythrocyte. Four different groups of workers reported that furosemide-sensitive K transport was unchanged with acute changes in cell volume (26–29) or increased with cell swelling (29). All four studies were performed before KCl cotransport was demonstrated in human erythrocytes, used a nonspecific inhibitor, furosemide (1 mM), and did not distinguish NaK2Cl from KCl cotransport. Using a specific inhibitor (bumetanide, 2 μM), we showed that acute cell swelling inhibited, and acute cell shrinkage stimulated, NaK2Cl cotransport in human erythrocytes (Fig. 4). While volume sensitivity of NaK2Cl cotransport has been shown in a number of other cell types, the clear demonstration of this phenomenon in the human erythrocyte increases the importance of the human erythrocyte as a convenient and productive model system for the study of ion transport in general, and NaK2Cl cotransport in particular.

While statistically significant responses to cell swelling and shrinking were observed with both methods, the response was blunted with the osmotic method. The addition of sucrose to hypotonic media results in depolarization, an increase in the ratio of intracellular to external Cl, and alkanization of the cell interior (30), whereas with the nystatin (isosmotic method), swelling and shrinking of erythrocytes can be achieved without changes in intracellular Cl, pH, and voltage. Changes in voltage should not have a major effect on cotransport (15), and the effect of change in intracellular pH at constant external pH has not been studied. However, the increase in internal Cl (at a constant external Cl concentration) has been shown to inhibit NaK2Cl cotransport in the squid axon (31). While such an inhibitory effect of intracellular Cl on NaK2Cl cotransport has not been established in the human erythrocyte, the blunted response to cell shrinkage with the osmotic method in human erythrocytes (Fig. 3, *left*) may, in part, be due to the mitigating, inhibitory effect of an increase in intracellular Cl at a constant external Cl concentration. The more dramatic shrinkage activation of cotransport with the nystatin method (Fig. 3, *right*) may, in part, be attributable to the fact that intracellular Cl was unchanged with the nystatin method. The large (threefold) difference in bumetanide-sensitive flux in cells shrunken by the two methods remains unexplained and may be due a combination of factors, such as difference in intracellular Cl concentration, differences in the baseline fluxes (in normal volume cells) between the two methods and other, as yet unknown, factors. Nonetheless, the present findings show clearly that the NaK2Cl cotransport in the human erythrocyte responds to acute changes in cell volume, and are consistent with the suggestion made earlier that this cotransport system may help to maintain the steady state volume of human erythrocytes in vitro and in vivo (32, 33).

OA is a highly specific inhibitor of protein phosphatases 1 and 2A, two of the four major protein phosphatases that dephosphorylate serine and threonine residues (34). OA stimulated NaK2Cl cotransport significantly (Fig. 4), suggesting that the endogenous activity of protein phosphatase 1 or 2A keeps the cotransporter (or a key regulatory protein) in a partially dephosphorylated state under basal conditions and exerts a tonic inhibitory effect on the activity of the NaK2Cl cotransporter. Inhibition of this phosphatase by OA leads to net phosphorylation and upregulation of cotransport. The fact that OA alone, without kinase stimulators, was able to increase cotransport activity implies that a constitutively active kinase keeps the transporter (or a regulatory protein) partially phosphorylated and the cotransporter active under basal conditions.

Urea had only minimal effect on NaK2Cl cotransport in shrunken, OA-pretreated cells (Fig. 5), inhibiting only 10% of the bumetanide-sensitive K influx in shrunken, OA-pretreated cells compared with 62% of the basal cotransport. Shrinking leads to phosphorylation of the 195-kD cotransporter protein of the shark rectal gland (35) and OA pretreatment, by inhibiting phosphatase, should also lead to net phosphorylation. Thus, the finding that phosphorylation (of the cotransporter or a key regulatory protein) blocked subsequent inhibition by urea suggests that urea acts by dephosphorylating the cotransporter or a key regulatory protein, or that urea could not overcome the effect of increased crowding caused by shrinkage, and the cotransporter remained sufficiently phosphorylated in shrunken, OA-pretreated cells even with urea.

While urea was a less potent inhibitor of the OA-stimulated component of NaK2Cl cotransport (Fig. 4), it is of interest that urea was able to inhibit cotransport activity in OA-pretreated cells. The finding that urea was able to inhibit cotransport at all when protein phosphatase activity was maximally inhibited (by OA) suggests that the regulatory enzyme affected by urea was a kinase rather than the phosphatase. Thus, we propose that urea exerts its effect by inhibiting a constitutively active kinase,

thereby dephosphorylating the cotransporter or a key regulatory protein.

Urea has been known to inhibit many enzymes, and at least two different mechanisms have been identified to explain the enzyme inhibition by urea. First, urea may inhibit enzymes via an effect on macromolecular crowding. Urea-mediated inhibition of enzymes including pyruvate kinase and lactate dehydrogenase was observed in tissues with high protein concentrations, was reversed by counteracting organic osmolytes like trimethylamines, and attributed to macromolecular–solvent interactions (36). However, urea may also inhibit enzymes by direct, competitive inhibition. The inhibition of enzymes such as xanthine oxidase was observed in dilute solutions with low protein concentrations and cannot be attributed to effects on macromolecular crowding (37). It is of interest that urea concentrations required for direct inhibition of enzymes were high ($K_i > 200$–1200 mM, reference 37), whereas the inhibition of NaK2Cl cotransport reported here was observed at low urea concentrations (15–45 mM, $K_i = 63$ mM). This finding provides suggestive, but not conclusive, evidence against direct inhibition (and by inference, in favor of macromolecular–solvent interaction). However, the present studies do not allow us to distinguish definitively whether urea inhibits the putative regulatory kinase in the erythrocyte by reducing macromolecular crowding or by a direct inhibitory effect independent of macromolecular crowding.

Regardless of the mechanism, the findings presented here indicate that urea, in concentrations commonly encountered in patients with renal failure (45 mM, corresponding to blood urea concentrations of 270 mg/dl, or blood urea nitrogen concentration of 126 mg/dl), can inhibit NaK2Cl cotransport in human erythrocytes. Previous workers have shown that erythrocytes obtained from uremic subjects exhibited a lower activity of NaK2Cl cotransport compared with cells from normal subjects (38 and for review see reference 39). The earlier studies were performed in washed erythrocytes suspended in urea-free media, therefore the inhibition of cotransport was presumably secondary to membrane defects. Our results suggest that the inhibition of cotransport in uremic erythrocytes may be more severe than previously appreciated, due to the additional effect of blood urea on cotransport activity.

The NaK2Cl cotransport pathway has been identified in a wide variety of cell types and subserves a number of vital physiological functions such as transepithelial salt reabsorption and secretion, regulation of cell volume, extrarenal potassium homeostasis, maintenance of ion gradients in excitable cells such as the axon and the cardiomyocyte, and maintenance of cell water during cellular growth and differentiation (13, 14, 40). The present studies indicate that urea, at concentrations observed in uremic subjects, inhibits NaK2Cl cotransport. If urea inhibits NaK2Cl cotransporter in other cells as well, the global inhibition of NaK2Cl cotransport may cause defects in physiological functions in uremia. Thus, it is possible that certain defects in uremia, such as obligatory salt loss, abnormal extrarenal potassium homeostasis, and neuropsychiatric abnormalities (6, 8), may in part be due to inhibition of NaK2Cl cotransport. Taken together with the recent finding that urea is a potent activator of KCl cotransport in mammalian (including human) erythrocytes (9, 19, 41) and a powerful inhibitor on NaK2Cl cotransport in cultured mouse thick ascending limb cells (42), these findings suggest the possibility that urea, in addition to being a marker for uremic state, may act as a uremic toxin itself.

Acknowledgment

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References

1. Prevost, J.-L., and J.-A. Dumas. 1823. Examen du sang et de son action dans divers phenomenes de la vie. *Ann. Chim. Phys.* 23:90–104.

2. Bollman, J. C., and F. C. Mann. 1927. Nitrogenous constituents of blood following transplantation of ureters into different levels of intestine. *Proc. Soc. Exp. Biol.* 24:923–924.

3. Merrill, J. P., M. Legrain, and R. Hoigne. 1953. Observations on the role of urea on uremia. *Am. J. Med.* 14:519–523.

4. Johnson, J. W., W. W. Hagge, and R. D. Wagoner. 1972. Effect of urea loading in patients with far-advanced renal failure. *Mayo Clin. Proc.* 47:21–27.

5. Grollman, E. F., and A. Grollman. 1959. Toxicity of urea and its role in the pathogenesis of uremia. *J. Clin. Invest.* 38:749–754.

6. Schreiner, G. E., and J. F. Maher. 1961. Uremia: Biochemistry, Pathogenesis and Treatment. Charles C. Thomas, Springfield, IL.

7. Johnson, J. W. 1953. Does elevated blood urea participate in the pathogenesis of the uremic syndrome. *Semin. Nephrol.* 3:265–272.

8. May, R. C., R. A. Kelly, and W. E. Mitch. 1991. Pathophysiology of uremia. *In* The Kidney. 4th ed. B. M. Brenner and F. C. Rector, editors. W. B. Saunders Co., Philadelphia. 1997–2018.

9. Parker, J. C. 1993. Urea alters set point volume for K-Cl cotransport, Na-H exchange and Ca-Na exchange in dog red blood cells. *Am. J. Physiol.* 265:C447–C452.

10. Zimmerman, S. B., and B. Harrison. 1987. Macromolecular crowding increases the binding of DNA polymerase to DNA: an adaptive effect. *Proc. Natl. Acad. Sci. USA.* 84:1871–1875.

11. Minton, A. P., G. C. Colclasure, and J. C. Parker. 1992. Model for the role of macromolecular crowding in regulation of cellular volume. *Proc. Natl. Acad. Sci. USA.* 89:10504–10506.

12. Parker, J. C. 1993. In defense of cell volume? *Am. J. Physiol.* 265:C1191–C1200.

13. Haas, M. 1994. Na-K-Cl cotransporters. *Am. J. Physiol.* 267:C869–C885.

14. Parker, J. C., and P. B. Dunham. 1989. Passive cation transport. *In* Red Blood Cell Membranes. P. Agre and J. C. Parker, editors. Marcel Dekker Inc., New York. 507–561.

15. Kracke, G. R., and P. B. Dunham. 1987. Effect of membrane potential on furosemide-inhibitable sodium influxes in human red blood cells. *J. Membr. Biol.* 98:117–124.

16. Kaji, D. M. 1986. Volume-sensitive K transport in human erythrocytes. *J. Gen. Physiol.* 86:719–738.

17. Cheng, J.-T., T. Kahn, and D. M. Kaji. 1984. Mechanism of alteration of Na-K pump of erythrocytes from patients with chronic renal failure. *J. Clin. Invest.* 74:1811–1820.

18. Kaji, D. M., and Y. Tsukitani. 1991. Role of protein phosphatase in activation of KCl cotransport in human erythrocytes. *Am. J. Physiol.* 260:C178–C182.

19. Kaji, D. M., and C. Gasson. 1995. Urea activation of K-Cl cotransport in human erythrocytes. *Am. J. Physiol.* 268:C1018–C1025.

20. Dunham, P. B., and J. C. Ellory. 1980. Stimulation of the sodium-potassium pump by trypsin in low potassium type erythrocytes of goats. *J. Physiol. (Lond.).* 301:25–37.

21. Brahm, J. 1983. Urea permeability of human red cells. *J. Gen. Physiol.* 82:1–23.

22. Macey, R. I. 1984. Transport of water and urea in red blood cells. *Am. J. Physiol.* 246:C195–C203.

23. Dunham, P. B., G. W. Stewart, and J. C. Ellory. 1980. Chloride-activated passive potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA.* 77:1711–1715.

24. Chipperfield, A. R. 1985. Influence of loop diuretics and anions on passive potassium influx into human red cells. *J. Physiol. (Lond.).* 369:61–77.

25. Kaji, D. M., and T. Kahn. 1985. Kinetics of Cl-dependent K influx in human erythrocytes with and without external Na: effect of NEM. *Am. J. Physiol. 249 (Cell Physiol. 18)*:C490–C496.

26. Dunham, P. B., and M. A. Benjamin. 1984. Cl-dependent cation transport in mammalian erythrocytes. *Fed. Proc.* 43:2476–2478.

27. Lauf, P. K., C. M. Perkins, and N. C. Adragna. 1985. Cell volume and

metabolic dependence of NEM-activated K^+Cl^- flux in human red cells. *Am. J. Physiol.* 245:C124–C128.

28. Adragna, N. C., and D. C. Tosteson. 1984. Effect of volume changes on ouabain-insensitive net outward cation movements in human red cells. *J. Membr. Biol.* 78:43–52.

29. O'Neill, W. C., and R. B. Mikkelsen. 1987. Furosemide-sensitive Na and K transport and human erythrocyte volume. *Biochim. Biophys. Acta.* 896:196–202.

30. Freedman, J. C., and J. F. Hoffman. 1979. Ionic and osmotic equilibria of human red blood cells treated with nystatin. *J. Gen. Physiol.* 74:157–185.

31. Breitweiser, G. E., A. A. Altamirano, and J. M. Russell. 1990. Osmotic stimulation of $Na^+ - K^+$-Cl^- cotransport in squid giant axon is $[Cl]_i$ dependent. *Am. J. Physiol.* 258:C749–C753.

32. Duhm, J., and B. O. Göbel. 1984. Role of the furosemide-sensitive Na^+/K^+ transport system in determining the steady-state Na and K content and volume of human erythrocytes in vitro and in vivo. *J. Membr. Biol.* 77:243–254.

33. Mairlbäurl, M., and J. F. Hoffman. 1992. Internal magnesium, 2,3 diphosphoglycerate, and the regulation of the steady-state volume of human red blood cells by the Na/K/2Cl cotransport system. *J. Gen. Physiol.* 99:721–746.

34. Cohen, P., C. F. B. Holmes, and Y. Tsukitani. 1990. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* 15:98–102.

35. Lytle, C., and B. Forbush III. 1992. The Na-K-Cl cotransport protein in shark rectal gland. II. Regulation by direct phosphorylation. *J. Biol. Chem.* 267:25438–25443.

36. Yancey, P. H., M. E. Clarke, S. C. Hand, R. D. Bowlus, and G. N. Somero. 1982. Living with water stress. Evolution of osmolyte systems. *Science (Wash. DC).* 217:1214–1222.

37. Rajagopalan, K. V., I. Fridovich, and P. Handler. 1963. Competetive inhibition of enzyme activity by urea. *J. Biol. Chem.* 236:1059–1065.

38. Corry, D. B., M. L. Tuck, A. S. Brickman, N. Yanagawa, and D. B. N. Lee. 1986. Sodium transport in red blood cells from dialyzed uremic patients. *Kidney Int.* 29:1197–1202.

39. Kaji, D. M., and T. Kahn. 1987. The Na-K pump in chronic renal failure. *Am. J. Physiol.* 252:F785–F793.

40. Haas, M., W. F. Schmidt III, and T. J. McManus. 1982. Catecholamine-stimulated ion transport in duck red cells. Gradient effects in electrically neutral (Na + K + 2Cl) cotransport. *J. Gen. Physiol.* 80:125–147.

41. Dunham, P. B. 1995. Urea activation of K-Cl cotransport in sheep red blood cells: evidence for two mechanisms of swelling. *Am. J. Physiol.* 268:C1026–C1032.

42. Kaji, D. M., and J. C. Parker. 1993. Urea acutely inhibits NaK2Cl cotransport in mouse medullary thick ascending limb cells. *J. Am. Soc. Nephrol.* 4:868*a*. (Abstr.)