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Activation of K-Cl Cotransport by Mild Warming in Guinea Pig Red Cells

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Abstract. Unidirectional, ouabain-insensitive K^+ influx rose steeply with warming at temperatures above 37°C in guinea pig erythrocytes incubated in isotonic medium. The only component of ouabain-insensitive K^+ influx to show the same steep rise was K-Cl cotransport (Q_{10} of 10 between 37 and 41°C); Na-K-Cl cotransport remained constant or declined and residual K^+ influx in hypertonic medium with ouabain and bumetanide rose only gradually. Similar results were obtained for unidirectional K^+ efflux.

Thermal activation of K-Cl cotransport-mediated K^+ influx was fully dependent on the presence of chloride in the medium; none occurred with nitrate replacing chloride. The increase of K^+ influx through K-Cl cotransport from 37 to 41°C was blocked by calyculin A, a phosphatase inhibitor.

The Q_{10} of K-Cl cotransport fully activated by hydroxylamine and hypotonicity was about 2. The time course of K^+ entry showed an immediate transition to a higher rate when cells were instantly warmed from 37 to 41°C, but there was a 7-min time lag in returning to a lower rate when cells were cooled from 41 to 37°C. These results indicate that the steepness of the response of K-Cl cotransport to mild warming is due to altered regulation of the transporter.

Total unidirectional K^+ influx was equal to total unidirectional K^+ efflux at 37–45°C, but K^+ influx exceeded K^+ efflux at 41°C when K-Cl cotransport was inhibited by calyculin or prevented by hypertonic incubation. The net loss of K^+ that results from the thermal activation of isosomotic K-Cl cotransport reported here would offset a tendency for cell swelling that could arise with warming through an imbalance of pump and leak for Na^+ or for K^+.

Key words: Temperature — Hyperthermia — Heatstroke — K-Cl Cotransporter — Phosphatase — Erythrocytes

Introduction

The Na-K pump maintains steady-state cell concentrations of Na^+ and K^+ — and consequently cell volume — by offsetting the inward net leakage of Na^+ and outward net leakage of K^+ (Tosteson & Hoffman, 1960). Little attention has been paid to the question of how well this balance is maintained with moderate elevations of body temperature and an attendant rise in Na^+ permeation (reviewed in Willis, 1997). If extrusion of Na^+ by the pump keeps pace with increased Na^+ entry (Boonstra et al., 1984; Willis, 1997), then stoichiometric coupling with K^+ would obligate increased K^+ uptake. If it fails to keep pace with Na^+ entry, Na^+ will accumulate within the cell. Either scenario predicts cell swelling during warming.

Mammalian cells possess numerous volume-responsive transport systems, including protection against swelling, such as the K-Cl cotransporter, but whether these may be directly activated — or inhibited — by warming has not been explored. We have investigated these issues in guinea pig red cells, using unidirectional flux determinations in a range of temperatures encompassing the upper limits that the cells might experience physiologically.

We chose guinea pig red cells for this study both because the guinea pig has long been a standard animal model with constantly maintained normal mammalian body temperature and because transport of Na^+ and K^+ in guinea pig red cells has been well studied. In particular, their Na-K pump has been characterized (Ellory & Willis, 1982; Willis & Ellory, 1983; Marjanovic & Willis, 1992), and they have been reported to possess Na-K-Cl cotransport (Hall & Willis, 1984), K-Cl cotransport (Hynes & Willis, 1987) and Na-H exchange (Zhao & Willis, 1993).

Materials and Methods

Collection and Handling of Blood

Cells were drawn by heart puncture into a heparinized syringe from guinea pigs that had been anesthetized with xylazine and ketamine. The animal was then killed while it was still under deep anesthesia by cutting the spinal cord and severing the carotid arteries. The proce-

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dures used in this study were approved by the Animal Use Committee of the University of Georgia.

The blood was diluted with simple incubation medium (in mM: 150 NaCl, 5 KCl, 10 glucose, 5 adenosine, MOPS buffer, 10, pH 7.4 and 0.1 EDTA), centrifuged and the 'buffy coat' of white cells removed. The red cells were then washed twice more in the same way, resuspended in the same medium to a 'hematocrit' of about 10 percent and held in ice until use.

Choice of Temperatures for Incubation

The main focus of this study was elevated temperatures. The rectal temperature of 5 guinea pigs was 37.4 ± 0.2°C. Accordingly, 37°C was used as a 'base' or control temperature, 41°C as a moderately elevated temperature representing that achieved in fever or exertion hyperthermia, and 45°C as close to a limiting high temperature. Since, K-Cl cotransporter appeared in preliminary studies to be operational at 37°C, lower temperatures (32 or 27°C) were also used in some cases to encompass a full range of activation. Samples of cell suspension (1 ml in 1.5 ml microcentrifuge tubes, 3–5 percent hematocrit) were suspended in separate water baths for each temperature used and were allowed to equilibrate a minimum of 5 min before flux determinations were initiated. Suspensions in incubation were monitored visually for settling and change in color (deoxygenation) and were frequently shaken to prevent both.)

In experiments where temperature was instantly changed (Fig. 3, Table 1), 10–15 ml of cell suspension was incubated in 20-ml glass centrifuge tubes. Initial samples were taken at 2-min intervals for 6 min for determination of initial rate of ^{86}Rb uptake. Part of the suspension was then removed to a clean, dry, temperature-equilibrated tube and diluted with an equal volume of warmer or colder medium whose temperature was predetermined to achieve the desired temperature after rapid mixing. This diluting medium was identical in every way to the original suspension medium (including specific activity of ^{86}Rb). The rate following temperature change was compared with that of cells at both the original and final temperature that were diluted to the same hematocrit without any temperature change.

Unidirectional Fluxes

K^+ *Influx*

For determination of K^+ influx ^{86}Rb (in the presence of 5 mM nonradioactive KCl) was used in most experiments except as noted below. Influx was initiated after temperature equilibration by rapid mixing of the suspension with 20 μl of solution containing the radioactive isotope. After incubation with isotope for 15–20 min, each sample of cells (about 30 μl) was washed 3–4 times in 1.2-ml ice-cold isotonic, buffered Na-free medium (107 mM $MgCl_2$, 10 mM Tris, pH 7.8) using short (15 sec, 13,000 × *g*) spins in a microcentrifuge to remove excess isotope. Cells were lysed in 5 percent trichloroacetic acid and the precipitate removed by centrifugation; the supernatant was diluted to 4 ml with water and its radioactivity counted in a liquid scintillation counter. Computation of influx was based on specific activity determined from a suitable standard made from stock isotope and on cell volume computed from absorption of hemoglobin at 540 nm of the suspension related to measured hematocrit.

In one series of experiments (Fig. 6), K^+ influx was measured in the same samples of cells as were used to measure K^+ efflux. This was accomplished by incubating the cells in K-free medium with 5 mM nonradioactive RbCl replacing KCl. To be parallel with K^+ efflux (*see* below), cells were collected at 5 min and at a later time (20–25 min), their Rb content determined by emission flame photometry and influx computed from the Rb^+ uptake between the two times. The cells were otherwise handled as in the radioactive isotopic experiments.

K^+ *Efflux*

To avoid a long period of preincubation required for loading cells with radioisotope, unidirectional efflux of K^+ was measured simply as loss of K^+ from cells into a K-free medium containing nonradioactive RbCl as a replacement for KCl. To do this, cell suspension made with a K-free medium with 5 mM RbCl, was introduced to the microcentrifuge tubes containing the same medium for incubation, samples were removed at timed intervals, centrifuged and the supernatant was drawn off. An aliquot of the supernatant was taken for analysis by flame emission photometry. Another aliquot was taken for estimation either of protein loss by Bradford assay (Bradford, 1976) or of hemoglobin loss by measurement of absorption at 540 nm. In experiments with calyculin and okadaic acid, requiring introduction of inhibitor in an ethanol carrier, controls were run with the same concentration of ethanol (0.5 percent final concentration), and 0.5 percent albumin was also included in the medium to reduce lysis. Efflux was calculated from the loss of K^+ to the medium between an initial sample taken at 5 min and a later sample taken after 20–25 min of incubation, corrected for K^+ loss due to lysis.

Inhibitors of K^+ Fluxes

Except where noted, cells were incubated in 100 μM ouabain, a concentration sufficient to eliminate K^+ influx through the Na-K pump in guinea pig red cells (Willis & Ellory, 1983). To differentiate fluxes through the Na-K-Cl cotransport system, the inhibitor of Na-K-Cl cotransport, bumetanide, was used. In trial studies with cells incubated in hypertonic medium (to minimize K-Cl cotransport and maximize Na-K-Cl cotransport), 10 μM bumetanide was found to be maximally inhibitory to K^+ influx, and in cells incubated in hypotonic medium (with maximized K-Cl cotransport) the same concentration caused no greater inhibition of influx than that expected for inhibition of Na-K-Cl cotransport. Furthermore, in the presence of 10 μM bumetanide there was no Na-dependent component of the difference in K^+ influx between hypertonic and hypotonic media.

As a method for minimizing K-Cl cotransport, in the presence of ouabain and bumetanide, hypertonic incubation was used. Media were made hypertonic by the addition of sucrose to an osmotic concentration of 450 mOsM. The adequacy of this concentration to eliminate K-Cl cotransport at all temperatures investigated was verified in preliminary trials.

Hereafter, the difference in K^+ flux in the presence and absence of bumetanide (with ouabain always present) is referred to as ''Na-K-Cl cotransport,'' and difference in K^+ flux in the presence and absence of hypertonicity (450 mOsM, with both bumetanide and ouabain always present) is referred to as ''K-Cl cotransport.'' The K^+ flux remaining in hypertonic medium in the presence of bumetanide and ouabain is referred to as ''residual'' K^+ influx or efflux.

Analytical Methods

Concentrations of Na^+ and K^+ were measured by flame emission photometry. Total cell Mg was determined by flame absorption spectrophotometry. Osmotic concentrations of media were verified by vapor pressure osmometry. Cell ATP was determined by the method of lu-

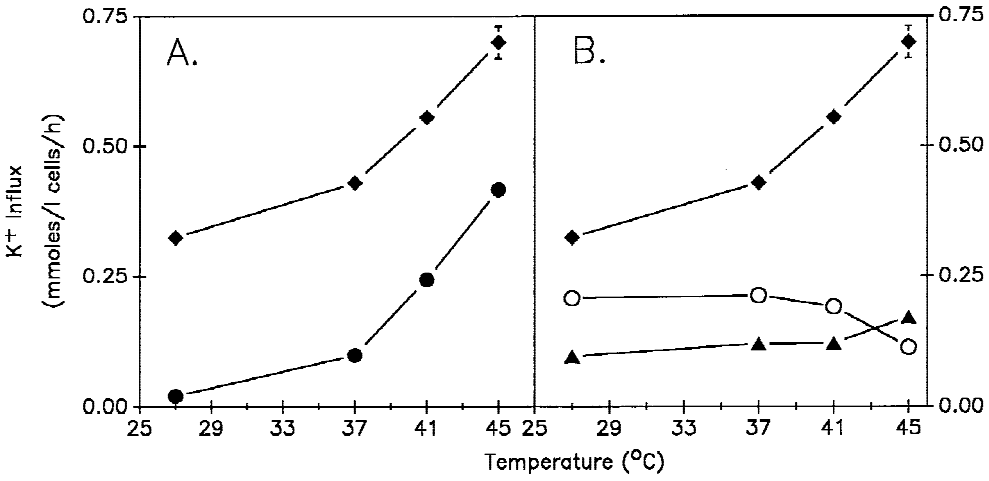

Fig. 1. Effect of temperature on components of ouabain-insensitive K^+ influx. (*A*) Diamonds, ouabain-insensitive influx; filled circles, K-Cl cotransport. K-Cl cotransport was computed by subtracting influx in hypertonic medium (with 10 μM bumetanide and 100 μM ouabain) from influx in isotonic medium containing ouabain and bumetanide. (*B*) Diamonds, ouabain-insensitive K^+ influx (same data as *A*, repeated for comparison); open circles, Na-K-Cl cotransport; filled triangles, residual K^+ influx. Cells were incubated as described in Materials and Methods. Na-K-Cl cotransport was bumetanide-sensitive component of influx in presence of ouabain; residual influx was that remaining in hypertonic medium (450 mOsM) in the presence of ouabain and bumetanide. Results are means ± SE (brackets shown where larger than symbols) of 3–6 experiments.

ciferin/luciferase assay (Brown, 1982) as previously described (Marjanovic & Willis, 1992). Cell pH was determined directly on lysates of cells as described previously (Zhao & Willis, 1993). Cell chloride was determined using a chloridometer as described previously (Zhao & Willis, 1993). Correction for chloride trapped in extracellular space in the red cell pellet was based on an extracellular space of packed cells of 6.7 percent found by introducing ^{86}Rb to a cell suspension and then immediately centrifuging the suspension (15 sec, 13,000 × *g*) on a layer of dibutyl pthalate that separates the cells from the medium. The pellets were lysed in 5 percent trichloroacetic acid and the radioactivity determined to obtain the volume of distribution of the Rb. Cell water content was determined on centrifuged pellets of red cells weighed wet in tared beakers with a microgram balance. The samples were then dried for a minimum of 1.5 hr at 90°C and 0.5 atm in a vacuum oven. (Preliminary trials showed these conditions to be sufficient to achieve full desiccation.) The samples were reweighed after drying and the water content was computed from the weight change.

Chemicals Used

Ouabain and bumetanide were obtained from Sigma Chemical, St. Louis, MO, and calyculin A was obtained from Calbiochem-Novabiochem C, La Jolla, CA. Bradford Assay kits were obtained from BioRad Laboratories, Hercules, CA.

Results

Effect of Temperature on Passive K^+ Influxes

Ouabain-insensitive K^+ influx, Na-K-Cl cotransport, K-Cl cotransport and residual K^+ influx were measured as described in Materials and Methods at temperatures between 27 and 45°C. The results showed that total passive, ouabain-insensitive K^+ influx rose steeply at temperatures above 37°C (Fig. 1). The only measured component pathway of passive K^+ influx that exhibited a behavior parallel with that of total ouabain-insensitive K^+ influx was K-Cl cotransport (Fig. 1*A*); Na-K-Cl cotransport declined gradually over the same range and residual influx rose only slightly (Fig. 1*B*) with a Q_{10} of about 1.4 over the full range of temperature.

K-Cl Cotransport

Dependence on Chloride and on Phosphatase. The increase in K-Cl cotransport caused by warming from 37 to 41°C was of about the same magnitude as that caused by incubation of cells in hypotonic medium (210 mOsM) at 37°C (Fig. 2*A* and *B*). (Both control and calyculin-containing medium in Fig. 2*B* contained 0.5% ethanol; this may have contributed to a greater relative rise at 41°C seen in isosmotic controls.) As with activation by hypotonicity, the activation of K-Cl cotransport by warming was entirely dependent upon the presence of chloride ion; there was no thermal or swelling activation in media with nitrate replacing chloride (Fig. 2*A*).

Swelling activation of K-Cl cotransport is dependent upon dephosphorylation of the carrier or of some regulator thereof (reviewed by Hoffmann & Dunham, 1995), and, accordingly, is inhibited by inhibitors of phosphatases such as okadaic acid and calyculin (Jennings & Schulz, 1991; Kaji & Tsukitani, 1991; Starke & Jennings, 1993). Possibly because of its poor permeation (Nambooripad & Jennings, 1996), okadaic acid only weakly inhibited K-Cl cotransport at 37°C (*data not shown*). Calyculin (25 nM), however, caused 93 percent

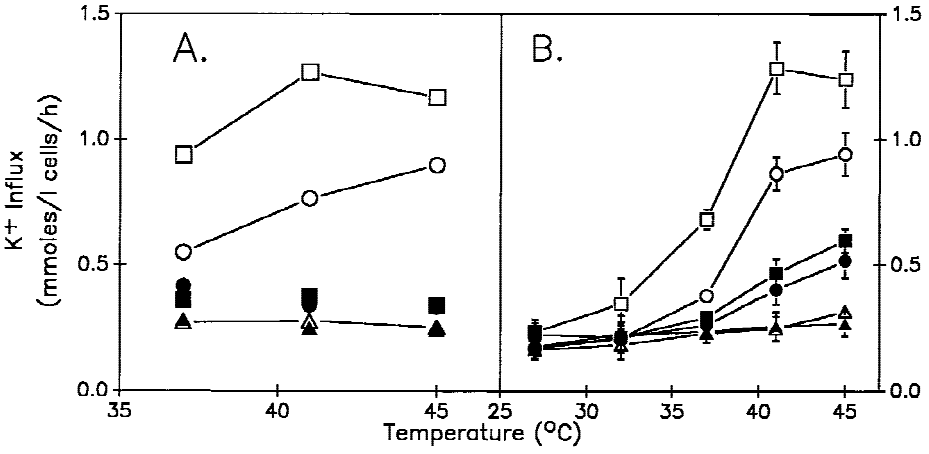

Fig. 2. Properties of K^+ influx through K-Cl cotransporter at elevated temperatures. (*A*) Chloride dependence. Cells were preincubated in standard medium or medium in which nitrate was substituted for chloride and were washed several times in the corresponding medium. Open symbols, with chloride; filled symbols, chloride-free. Squares, hypotonic incubation (210 mOsM); circles, isotonic; triangles, hypertonic incubation (450 mOsM). Lines are drawn to connect corresponding open symbols. All cells were incubated with ouabain and bumetanide. Results shown represent means of triplicate determinations in a single experiment, representative of three similar experiments. (*B*) Inhibition by calyculin. Cells were preincubated as a 10 percent suspension in medium containing 0.5% ethanol and with or without 25 nM calyculin for 15 min at 27°C. They were then diluted to 3 percent in microcentrifuge tubes containing the medium for determination of flux and were held at the flux temperature for 5 min before isotopic influx was started by the introduction of isotope. Open symbols, no calyculin; filled symbols, with 25 nM calyculin. Triangles, hypertonic; circles, isotonic; squares, hypotonic. Cells are incubated in presence of ouabain (100 μM) and bumetanide (10 μM). Means of 5 experiments ± SE are shown.

inhibition of volume-sensitive K^+ influx at 37°C (i.e., difference between ouabain-and-bumetanide-insensitive influx in hypotonic and hypertonic medium), and it blocked all of the rise in K^+ influx in isotonic medium caused by warming from 37 to 41°C (Fig. 2*B*). At 41 and 45°C, however, calyculin did not lower K^+ influx to the same minimal level as that found in hypertonic media at those temperatures (Fig. 2*B*). Thus, while calyculin blocked about 65 percent of isotonic K-Cl cotransport at 41°C, there was a remaining fraction of 35 percent that was unaffected. (In one experiment, raising calyculin concentration to 100 μM had no greater effect.)

Reversibility of Temperature Effect. Thermal activation of K-Cl cotransport at 41°C was fully reversible. When cells were preincubated at 41°C and then returned to 37°C for 10 min before flux determination, their influxes in isotonic and hypertonic media with ouabain and bumetanide were no different from those of cells preincubated and then incubated at 37°C (mean difference in hypertonically inhibitable component of 0.01 ± 0.01 for 3 experiments).

The time-course of entry of K^+ as measured by isotope is shown in Fig. 3 for cells incubated in isotonic medium with ouabain and bumetanide. Instantly warming cells from 37 to 41°C caused an apparently immediate increase in rate of uptake to that of cells incubated in 41°C continuously (Fig. 3*A*, Table 1). When cells were instantly cooled from 41 to 37°C the rate of K^+ entry ultimately returned to that of control cells at 37°C. However, the transition to the steady-state rate for 37°C occurred only after a delay of about 7 min (Fig. 3*B*, Table 1).

Q_{10} of Maximally Activated K-Cl Cotransport. The steep rise in K-Cl cotransport with warming (Fig. 1*A*) corresponds to a Q_{10} of 9.7 between 37 and 41°C. Q_{10} between 27 and 37°C was 4.9, and it was 3.8 between 41 and 45°C. The foregoing results seemed to indicate that such large values of Q_{10} represented an effect of temperature on the regulation of the K-Cl cotransporter rather than a direct effect of temperature on the transporter itself. To estimate the direct effect of temperature on K-Cl cotransport, independently of any modulation by regulatory factors, we sought a method for achieving a high level of activation of the carrier at each temperature. Among the methods that we tried, we achieved greatest activation at lower temperatures with a combination of hydroxylamine and hypotonicity. Using these conditions at several temperatures yielded a Q_{10} of K^+ influx of 2.6, both between 37 and 41°C and between 32 and 45°C (Fig. 4).

The K^+ influx in hypotonic medium with hydroxylamine, of course, included residual leak, as well as K-Cl cotransport. The residual leak (i.e., K^+ influx in hypertonic medium with ouabain and bumetanide) was estimated both with and without hydroxylamine present. While the results differed, subtracting either value from K^+ influx in hypotonic medium with hydroxylamine to obtain an estimate of maximal K-Cl cotransport yielded Q_{10} of 2–3 (Fig. 4).

Effect of Warming on Cytoplasmic Factors Known to Affect K-Cl Cotransport. Several cytoplasmic factors can influence the activity of the K-Cl cotransporter, such as cell Mg^{2+} concentration, ATP concentration, cytoplas-

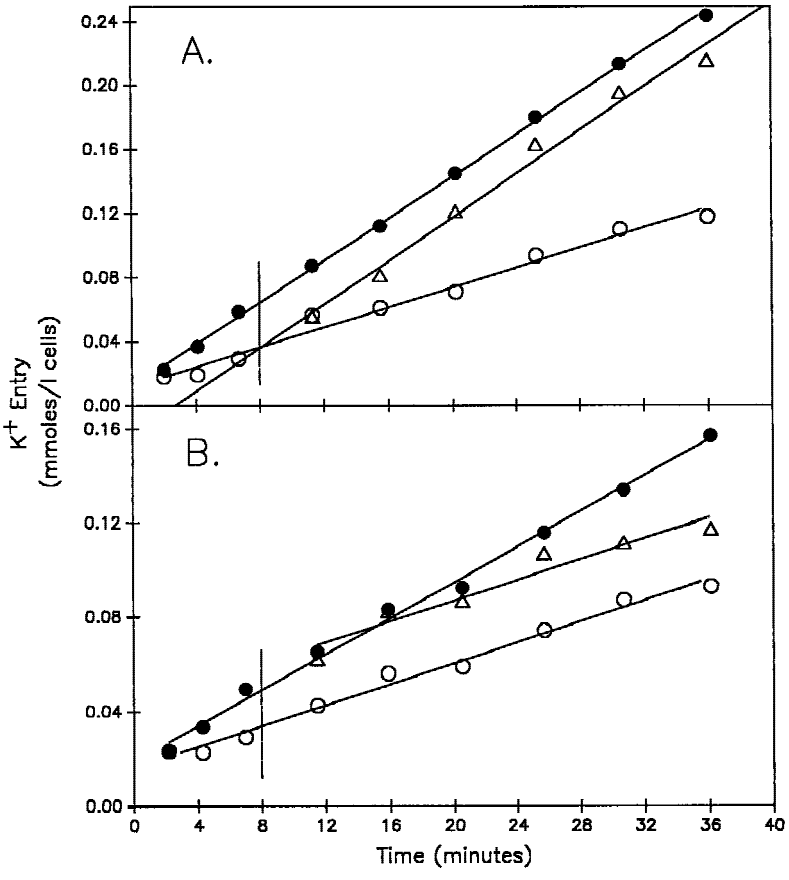

Fig. 3. Time-course of effect of rapid increase (*A*) or decrease (*B*) in temperature on ouabain-and-bumetanide insensitive K^+ influx. Cells were incubated in isotonic medium with ^{86}Rb, 100 μM ouabain and 10 μM bumetanide and samples were withdrawn at intervals as shown. Two suspensions of cells, one at 37°C and one at 41°C, were sampled in parallel. At the point marked by the vertical line, part of one of the suspensions was removed, placed in a pre-equilibrated container and an equal volume of medium was added at a temperature previously determined to cause a change in temperature either from 37 to 41°C (panel *A*) or from 41 to 37°C (panel *B*) after rapid mixing. The remainder of the same suspension was diluted by an equal volume of isothermal medium. The other suspension (i.e., 41°C in panel *A* and 37°C in panel *B*) was also diluted by addition of isothermal medium. Filled circles, 41°C throughout; open circles, 37°C throughout; triangles, temperature-shifted cells (panel *A*, rapidly warmed from 37 to 41°C; panel *B*, rapidly cooled from 41 to 37°C). Regression lines are shown for each set of points. Results are for a single experiment of each type, representative of 3. Group data for slope of curves and delay of onset of change in slope are shown in Table 1.

Table 1. Delay in change of rate of K^+ influx after a step change in temperature

Procedure	(n)	Identity	Slope ($\times 10^{-3}$) (mmole/l/min)	Delay (min)
Step warming	(3)	Control, 37°C	2.1 ± 0.5	
		Control, 41°C	4.0 ± 1.3	
		Shifted, 37 → 41°C	4.0 ± 1.0	0 ± 3
Step cooling	(3)	Control, 37°C	3.0 ± 0.4	
		Control, 41°C	5.1 ± 0.7	
		Shifted, 41 → 37°C	2.3 ± 0.3	7 ± 2

Slopes are computed from regression lines of all the data for controls (*see* Fig. 3) and of the last four data points for the temperature-shifted cells. The ''delay'' was calculated by subtracting the actual time of warming or cooling from the calculated time at which the regression curve for the warmed or cooled cells diverged from that of the control cells. (The ''control cells'' were those at the same temperature as the initial temperature of the temperature-shifted cells, i.e., 37°C for step-warmed cells and 41°C for step-cooled cells).

mic pH, chloride concentration and water content. Each of these factors was determined in red cells after incubation over a period of 30 min (corresponding to the ion flux determinations) and 2 hr. As shown in Table 2, none was observed to vary with temperature between 37 and 45°C.

Effect of Warming on Passive K^+ Efflux

The effect of warming above 37°C on K^+ efflux was similar to that for influx, in that the K-Cl cotransport component accounted for most of the rise (Fig. 5*A*). The pattern for efflux differed from that for influx in that the rise in residual efflux was somewhat steeper with warming (Q_{10} of 2 between 37 and 45°C; Fig. 5*B*) than was the case for residual K^+ influx (Fig. 1*B*); it also differed in that there was no detectable K-Cl cotransport under isotonic conditions at 37°C. No detectable K^+ efflux was observed as Na-K-Cl cotransport at 37, 41, or 45°C (Fig. 5*B*).

Efflux via K-Cl cotransport evoked by cell *swelling* was large at 37°C (i.e., the difference between efflux in hypertonic and in hypotonic media, both with bumetanide and ouabain, was 5.5 ± 1.5). At 41 and 45°C the difference between efflux in hypotonic and hypertonic medium was similarly large (7.5 ± 1.6 and 6.8 ± 1.7, respectively), and the differences among the three temperatures were not significant ($P > 0.05$ for all combinations of pairs between 37 and 45°C).

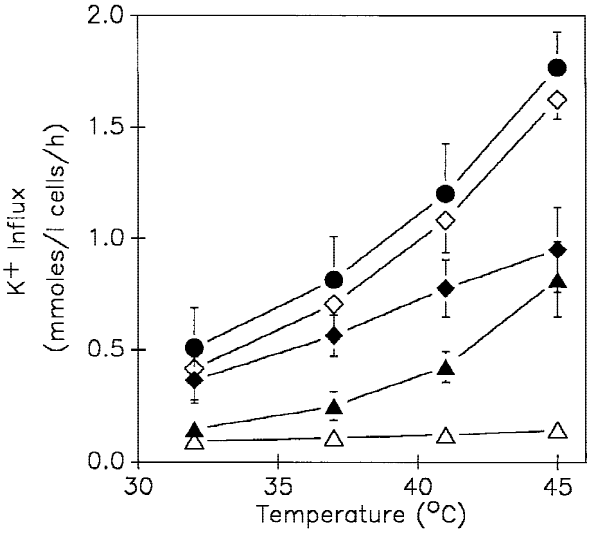

Fig. 4. Effect of temperature on K^+ influx in cells incubated in hypotonic medium with hydroxylamine. Combination of hydroxylamine and hypotonic incubation was used as a means of strongly activating influx through the K-Cl cotransporter at all temperatures. All cells were incubated with ouabain and bumetanide. Filled circles, influx in hypotonic medium (210 mOsM) with hydroxylamine (1 mM); Q_{10} is 2.6 for 32–45°C and for 37–41°C. Open triangles, residual K^+ influx in hypertonic medium. Filled triangles, residual K^+ influx in hypertonic medium with hydroxylamine. Open diamonds, ''maximal K-Cl cotransport,'' calculated as the difference between K^+ influx in hypotonic medium with hydroxylamine and in hypertonic medium *without* hydroxylamine. Q_{10} is 2.9 for 32–45°C and for 37–41°C. Filled diamonds, ''maximal K-Cl cotransport'' calculated as the difference between K^+ influx in hypotonic medium and in hypertonic medium, *both* containing hydroxylamine. Q_{10} is 2.1 for 32–45°C and 1.9 ro 37–41°C. Means ± SE for 3 cases are shown.

Table 2. Cell water content and cell concentrations of chloride, total magnesium, ATP and hydrogen ion at elevated temperatures

		37°C	41°C	45°C
	(*n*)	mmole/l cells		
$[Cl^-]_{cell}$				
<30 min	4	79.8 ± 3.3	82.2 ± 4.1	81.5 ± 5.4
2 hr	4	79.4 ± 3.4	80.4 ± 3.5	79.1 ± 3.5
$[Mg]_{cell}$				
<30 min	4	2.33 ± 0.04	2.41 ± 0.11	2.36 ± 0.11
2 hr	4	2.40 ± 0.15	2.37 ± 0.12	2.33 ± 0.18
$[ATP]_{cell}$				
<30 min	3	1.65 ± 0.08	1.58 ± 0.05	1.62 ± 0.02
2 hr	4	1.31 ± 0.08	1.69 ± 0.06	1.59 ± 0.06
pH_{cell}			pH	
<30 min	1	7.25	7.24	7.23
2 hr	4	7.28 ± 0.03	7.25 ± 0.03	7.24 ± 0.02
Cell water			g H_2O/g dry wt.	
<30 min	3	2.13 ± 0.05	1.99 ± 0.04	2.07 ± 0.20

Balance of K^+ Influxes and Effluxes at Elevated Temperature

Total K^+ Influx vs. Total K^+ Efflux

Cells were incubated in K-free medium with 5 mM RbCl replacing KCl at temperatures between 20 and 45°C. Efflux was measured as in Fig. 5 by sampling supernatants, and simultaneous Rb^+ uptake was determined in the pellets of the same samples by flame emission photometry. The ratio of influx to efflux (Fig. 6) was not statistically different from 1.0 between 27 and 45°C ($P > 0.05$, i.e., influx did not differ from efflux).

Balance of K^+ Influx and K^+ Efflux in Absence of K-Cl Cotransport

To determine the impact of K-Cl cotransport on K^+ steady state at elevated temperatures, K^+ influx was measured using ^{86}Rb in 5 mM K^+ (as in Fig. 1) and this value was compared with K^+ efflux, in parallel cells treated as in Fig. 5. In these experiments (Fig. 7) the ratio of influx to efflux was close to 1 at both 37 and 41°C in control cells. However, in hypertonic medium or in isotonic medium with 25 nM calyculin, influx exceeded efflux at 41°C (ratio of 1.7 and 2.2, respectively), but not at 37°C (Fig. 7). The possibility that the imbalance of bidirectional K^+ flux at 41°C was caused by an increased influx through the Na-K-Cl cotransporter was ruled out in separate experiments, that showed that bumetanide-sensitive influx was not increased by hypertonicity at 41°C and that calyculin caused an increase that was the same at 37 as at 41°C (0.4 mmole/1/hr).

Net K^+ Uptake Through Na-K Pump vs. Net K^+ Loss Through K-Cl Cotransporter

To determine whether the loss of K^+ through the K-Cl cotransporter at an elevated temperature matched the increase of uptake of K^+ by the Na-K pump, measurements of unidirectional ouabain-sensitive K^+ influx and efflux were made simultaneously, and in parallel sets of cells unidirectional hypertonically inhibited ouabain-and-bumetanide-insensitive K^+ influx and efflux were measured simultaneously. Net uptake through the pump was taken as the difference between ouabain-sensitive K^+ influx and ouabain-sensitive K^+ efflux, and net loss through the K-Cl cotransporter was taken as the difference between hypertonically inhibited, ouabain-and-bumetanide insensitive K^+ efflux and influx (Table 3). Net K^+ uptake by the pump rose about 47 percent between 37 and 41°C (Table 3), with an increase of 0.63 mmole/1/hr that was possibly significant from 0 ($P < 0.05$). Net loss through the K-Cl cotransporter increased 8-fold over the same temperature range from 0.11 to 0.87 mmole/1/hr, a difference (0.76) that was highly signifi-

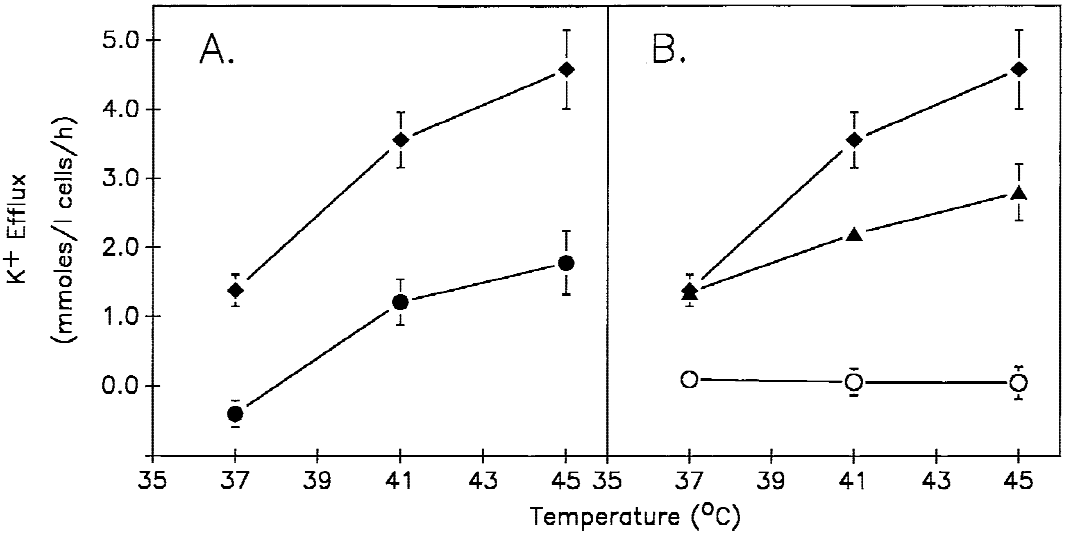

Fig. 5. Effect of temperature on unidirectional K^+ efflux. All cells were incubated with 100 μM ouabain. K^+ efflux was measured as K^+ loss into K-free medium with 5 mM RbCl substituted for KCl. Initial loss after 5 min was subtracted from loss at a later interval (20–25 min) and the difference was used to compute rate. Correction for K^+ loss due to lysis was based on measurement of loss of hemoglobin or protein. Cells were incubated with 0.5 percent albumin to minimize hemolysis. (*A*) Filled diamonds, ouabain-insensitive K^+ efflux; filled circles, K-Cl cotransport. K-Cl cotransport was computed by subtracting efflux in hypertonic medium (with bumetanide, 10 μM and ouabain 100 μM) from efflux in isotonic medium containing ouabain and bumetanide. (*B*) Filled diamonds, ouabain-insensitive K^+ efflux (same data as *A*, repeated for comparison); open circles, Na-K-Cl cotransport; filled triangles, residual K^+ efflux. Na-K-Cl cotransport was bumetanide-sensitive component of efflux in presence of ouabain; residual efflux was that remaining in hypertonic medium (450 mOsM) in the presence of ouabain and bumetanide. Results are means ± SE (error bars shown where larger than symbols) of 6 experiments.

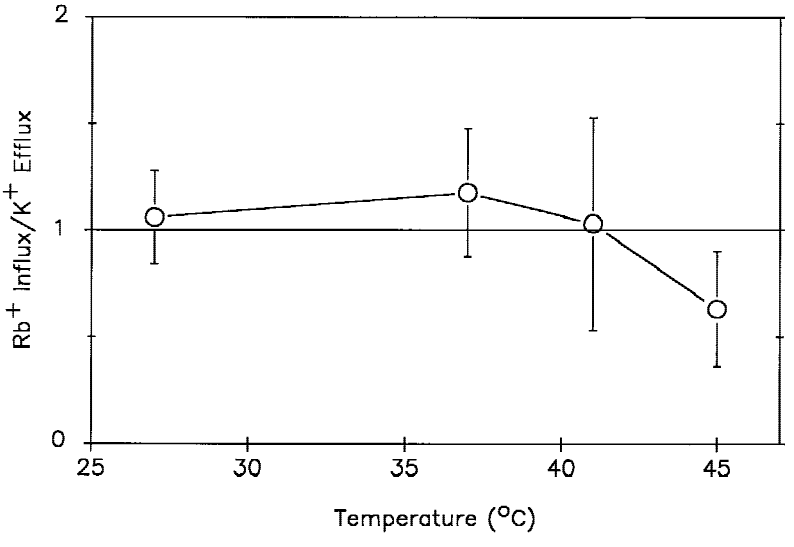

Fig. 6. Effect of temperature on balance of K^+ (Rb^+) influx and K^+ efflux. Efflux was determined as described in Fig. 5 except that no inhibitors were present. In the same samples pelleted red cells were washed three times in isotonic Tris-buffered $MgCl_2$ medium and influx determined as the uptake of Rb^+ as determined by flame emission photometry. Points represent means ± SE of 5 experiments.

cant from 0 ($P < 0.01$). The average capacity for increase in net loss of K^+ through the K-Cl cotransporter of 0.76 was therefore at least equal to average rise in net gain of K^+ through the pump (0.63).

Discussion

The results demonstrate that one specific component of ouabain-insensitive K^+ flux in guinea pig red cells — K-Cl cotransport — exhibits an unusually large and progressive increase with warming over a range of the temperature scale most relevant to a normally euthermic mammal — 32 to 41°C. The other measured components of ouabain-insensitive K^+ flux show either a very low thermal sensitivity (residual leak) or a plateau over the same range (Na-K-Cl cotransport).

Several investigators have noted a large temperature dependence of the activation of the carrier by swelling in

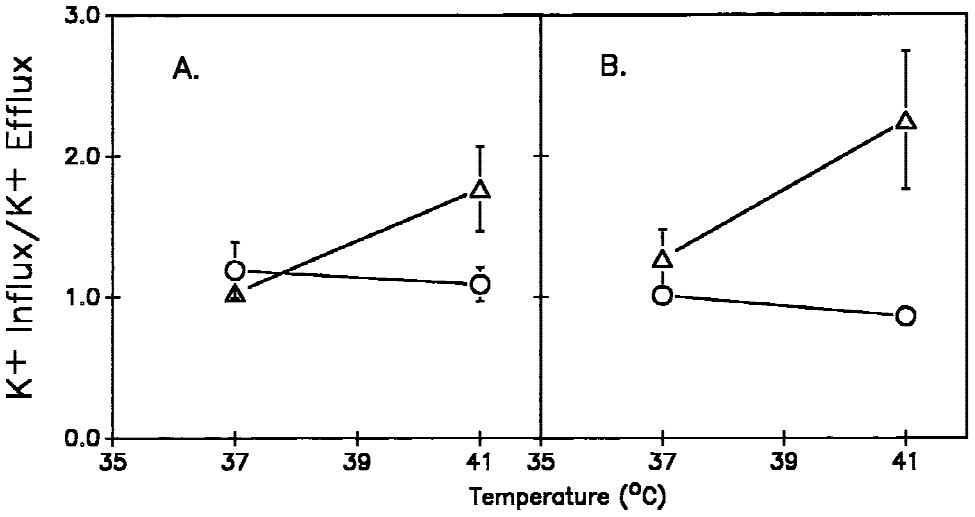

Fig. 7. Ratio of K^+ influx to K^+ efflux in guinea pig red cells at 37 and 41°C when K-Cl cotransport is inhibited. K^+ influx was measured by uptake of ^{86}Rb as described in Materials and Methods. K^+ efflux was measured in parallel cells as in Fig. 5. Media contained 0.5 percent albumin, but no ouabain or bumetanide. (*A*) Circles, control cells; triangles, cells incubated in hypertonic medium. Points represent means ± SE of five experiments. (*B*) Circles, control cells; triangles, cells incubated with 25 nM calyculin A. Points represent means ± SE of four experiments.

Table 3. Comparison of net fluxes of K^+ through the Na-K pump and K-Cl cotransporter at 37 and 41°C

	Net gain of K^+ via Na-K pump	Net loss of K^+ via K-Cl cotransporter
	mmole/l cells/hr	
37°C	1.35 ± 0.12	0.11 ± 0.07
41°C	1.98 ± 0.25	0.87 ± 0.18
Change due to warming	0.63 ± 0.25*	0.76 ± 0.19**

Results represent means of 9 experiments ± SE. In parallel sets of cells ouabain-sensitive influx and efflux and hypertonically inhibited K^+ influx and efflux were measured simultaneously. ''Net gain of K^+ via the pump'' was calculated from the difference of ouabain-sensitive influx and efflux. ''Net gain via K-Cl cotransporter'' was calculated as the difference of hypertonically inhibited influx and efflux.
*$P < 0.05$ for difference from 0.
**$P < 0.01$ for difference from 0.

red cells of human, sheep and rabbit over the range of 37 to 25°C (Lauf, 1983; Ellory, Hall & Stewart, 1985; Jennings & Al-Rohil, 1990). Those findings were extended by Jennings & Al-Rohil (1990), who showed that the thermal dependence of K-Cl cotransport was quite small (Q_{10} of about 1.5 between 25 and 37°C) in rabbit red cells that were already strongly activated by pre-exposure to N-ethyl maleimide (NEM). They interpreted this to mean that activation by swelling at low temperature (25°C) occurred more slowly and reached a lower maximum. We in turn have extended those results by demonstrating that even in isotonic medium a rise in temperature above 37°C strongly activates the system.

Mediation of Thermal Activation

More recently, the activation of the K-Cl cotransport system by cell swelling, though complex, has been shown to involve in part the dephosphorylation of the carrier itself or of a regulator of the carrier (for reviews *see* Lauf et al., 1992; Lauf, Adragna & Agar, 1995; Hoffmann & Dunham, 1995). The large thermal activation observed in the present study in the absence of cell swelling appears to depend upon alteration in the balance of these regulatory pathways, as distinct from a direct effect of temperature on the carrier itself. This proposition rests upon two of our observations: (i) The low temperature dependence of K-Cl cotransport when already highly activated by swelling and hydroxylamine (Fig. 4) and (ii) the delay in the time course of reversal (Fig. 3*B*). Both of these points are discussed below.

When, like Jennings and Al-Rohil (1990), we used NEM initially to activate K-Cl cotransport to obtain a Q_{10} at temperatures above 37°C in guinea pig red cells, we found that K^+ influx in NEM-treated red cells actually declined with warming (Willis & Anderson, 1995). This result is well explained by the findings of Lauf and Adragna (1995) of thermally activated inhibitory NEM binding sites. Accordingly, we sought other means for achieving high activation throughout the temperature range used in this study. Lauf (1990) had observed an extraordinary level of activation by hydroxylamine in LK sheep red cells at 37°C (greater than with NEM) and no further increase in hypotonic medium with that activator. In guinea pig red cells we found in trial experiments that the combination of hydroxylamine and hypo-

tonicity gave a higher K^+ influx at temperatures below 37°C than either agent alone.

As shown in Fig. 4, influx in hydroxylamine and hypotonic medium yields a Q_{10} of only 2.6 between 37 and 41°C, which was the temperature range of steepest rise in untreated cells. In hydroxylamine-treated cells a Q_{10} of 2.6 is also obtained over the wider range of 32 to 45°C (i.e., there is no range of temperature with steeper increase).

Part of this influx was residual leak, and this must be subtracted from the total to determine influx via K-Cl cotransport. In this case, however, we have two choices for ''residual leak'' — K^+ influx in hypertonic medium or K^+ influx in hypertonic medium with hydroxylamine. The latter rose steeply with warming, and, if we assume that this means that hydroxylamine made the membrane leakier to K^+, we should use this as the baseline. To do so would yield a Q_{10} of about 2 for K-Cl cotransport. If, on the other hand, the steep rise means that hydroxylamine activates K-Cl cotransport even in hypertonic medium, we should use the original baseline (hypertonic medium without hydroylamine). Doing so yields a Q_{10} of about 3 for K-Cl cotransport.

In either case the relative increase with warming of the already highly activated carrier is far less than that of untreated cells isotonically incubated, and leads to the same conclusion as that of Jennings and Al-Rohil (1990), vis-a-vis the effects of cooling, i.e., an effect of temperature on regulation of transport rather than on the transporter itself. This conclusion, based on influx, is supported by the low-to-nil temperature dependence for volume-activated K^+ efflux above 37°C.

An argument based solely on an attempt to ''fully activate'' the carrier faces the objection that there is no way of knowing whether ''full activation'' was in fact achieved at each temperature. As was the case with NEM activation, temperature itself could have altered the efficacy of hydroxylamine or swelling. It was desirable, therefore, that this result be corroborated by another, independent approach.

That the large temperature effect between 37 and 41°C cannot be attributed mainly to a direct kinetic effect on the transporter itself is demonstrated further by the existence of a time lag before the rate of influx could 'relax' to the rate characteristic of 37°C after cooling from 41°C (Fig. 3*B*, Table 1). It should be recognized, however, that the rates illustrated in Fig. 4 and Table 1 necessarily included residual K^+ influx. For the sake of this discussion we have assumed that rate of flux through this pathway changes instantly with change in temperature in either direction. Because flux through this pathway is very small and because the change in this flux between 37 and 41°C is even smaller, we were unable to test this assumption directly. The quantitative estimate of the time lag also depends upon the new steady state having been achieved by the beginning of the interval used to compute the regression curve (*see* Table 1). While this procedure could underestimate the time lag that would be computed from a two-state model (Jennings & Al-Rohil, 1990), we attempted to verify that the final rate had been achieved by comparison of the slope of the last four points of the temperature-shifted cells with the slope of parallel control cells exposed continuously to the final temperature (Fig. 4 and Table 1).

This time lag is reminiscent of those seen with activation of the K-Cl cotransporter (Jennings & Al-Rohil, 1990; Parker, Colclasure & McManus, 1991) — and the inactivation of Na-H exchange (Parker et al., 1991) — following rapid swelling. In the two-state model of Jennings and Al-Rohil (1990) the time lag in activation of the K-Cl cotransporter was attributed to the effect of swelling being a decrease of the rate constant for inactivation — later postulated to be a kinase mediated step (Jennings & Schulz, 1991; Parker et al., 1991) — rather than an increase in the rate constant for (phosphatase-mediated) activation.

The present results with thermal activation imply the opposite — a greater effect of warming on activity of a phosphatase proximal to the carrier than on activity of an equivalently proximal kinase (Fig. 3*B*, Fig. 4). By this interpretation, the slower effect of cooling from 41 to 37°C (Fig. 4*B*) would be attributable to a slow depletion of activated carrier by a kinase-mediated forward reaction whose rate has been decreased less by cooling than that of the phosphatase-mediated back reaction.

Bize and Dunham (1994) have noted that while the phosphatase has not previously been regarded as a regulated component in this system, staurosporine appears to activate K-Cl cotransport indirectly by blocking a kinase that phosphorylates a proximal phosphatase. Hence, for regulation of the K-Cl cotransporter by inputs other than cell volume, phosphatase, or the kinases that regulate the phosphatase, are plausible candidates. Two Src-family kinases, Fgr and Hck, have recently been identified as likely candidates of the negatively regulating phosphatase-kinases (Defranchesi et al., 1997).

The balance between kinase-mediated inactivation and phosphatase-mediated activation has been postulated as the final common pathway for several other determinants of activity of the K-Cl cotransporter, such as Mg^{2+}, ATP and thiol-reactive agents (Lauf et al., 1995), peroxide (Bize & Dunham, 1995) and PO_2 (Cossins et al., 1994; Honess, Gibson & Cossins, 1996). Temperature could impact directly on the proximal phosphatase (or kinase) activity. Alternatively or additionally, it could operate indirectly by altering one or more of those more distal determinants of K-Cl cotransport activity. The rapid transition observed with warming (Fig. 3*A*), however, places a constraint on such an hypothesis, and among several candidate cytoplasmic factors investi-

gated we observed no apparent changes that could account for activation (Table 2).

Physiological Significance of Thermal Activation

Two possible consequences of the activation of K-Cl cotransport at elevated temperatures are apparent: cause of hyperkalemia and prevention of cell swelling. Hyperkalemia is a common but variable feature of passive hyperthermia in intact mammals (reviewed by Francesconi et al., 1997). Cell swelling could result either from a competent Na-K pump responding to increased temperature and possibly increased Na^+ leak, thus obligating enhanced K^+ uptake, or from an incompetent Na-K pump failing to meet a rise in cell Na^+. Increased K-Cl cotransport was more than sufficient to offset any pump-mediated rise in cell K^+ observed in this study (Table 3), but that does not preclude the possibility of excess K^+ loss. The balance studies (Fig. 7) suggest that on average under the conditions of this study net K^+ loss did not occur with warming. However, in vivo and over longer intervals, the factors that might lead to insufficient pump activity (reduced ATP delivery, lowered cell pH) are the very ones that would accentuate the loss of K^+ through the K-Cl cotransporter. In such a situation, the benefits of prevention of cell lysis by swelling would be pitted against the dangers of hyperkalemia.

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