J. Membrane Biol. 131, 43–53 (1993)

The Journal of
Membrane Biology
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Cold Activation of Na Influx through the Na-H Exchange Pathway in Guinea Pig Red Cells

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Summary. Previous work showed that amiloride partially inhibits the net gain of Na in cold-stored red cells of guinea pig and that the proportion of unidirectional Na influx sensitive to amiloride increases dramatically with cooling. This study shows that at 37°C amiloride-sensitive (AS) Na influx in guinea pig red blood cells is activated by cytoplasmic H^+, hypertonic incubation, phorbol ester in the presence of extracellular Ca^{2+} and is correlated with cation-dependent H^+ loss from acidified cells. Cytoplasmic acidification increases AS Na efflux into Na-free medium. These properties are consistent with the presence of a Na-H exchanger with a H^+ regulatory site. Elevation of cytoplasmic free Mg^{2+} above 3 mM greatly increases AS Na influx: this correlates with a Na-dependent loss of Mg^{2+}, indicating the presence of a Na-Mg exchanger.

At 20°C activators of Na-H exchange have little or no further stimulatory effect on the already elevated AS Na influx. AS Na influx is much larger than either Na-dependent H^+ loss or AS Na efflux at 20°C. The affinity of the AS Na influx for cytoplasmic H^+ is greater at 20°C than at 37°C. Depletion of cytoplasmic Mg^{2+} does not abolish the high AS Na influx at 20°C.

Thus, elevation of AS Na influx with cooling appears to be due to increased activity of a Na-H exchanger (operating in a "slippage" mode) caused by greater sensitivity to H^+ at a regulatory site.

Key Words erythrocytes (red blood cells) · amiloride · hypothermia (cold) · cold-storage · Na-H exchange · Na-Mg exchange

Introduction

Failure of mammalian cells to maintain a Na gradient at low temperature has long been a subject of both practical and general interest (*see* Willis, 1987 for review). Both because of their relevance to cold storage in blood banking and because of their suitability for membrane transport studies, red blood cells are a highly favorable cell type for addressing this issue. Comparison of typically cold-sensitive cells, such as red cells of guinea pig and human, with more cold-tolerant cells, such as those of mammalian hibernators, has shown that one cause of the failure in the former is their inability to sufficiently reduce passive permeability (Kimzey & Willis, 1971; Willis, Zhao & Zhou, 1989).

A previous study showed that in guinea pig red cells, as temperature is lowered, the fraction of unidirectional Na influx that is sensitive to amiloride increases; at 37°C that fraction is virtually nil, by 20°C it amounts to more than 40% of all Na influx (Zhou & Willis, 1989). A later study of guinea pig red cells stored at 5°C for 3–9 days showed that amiloride reduced net gain of Na by 30% (Zhao & Willis, 1989).

Aside from sensitivity to amiloride, Na influx in guinea pig cells showed two other properties that indicated the presence of a Na-H exchange mechanism: increase with cytoplasmic acidification and increase with elevated cytoplasmic Ca^{2+} (Zhou & Willis, 1989). Other properties common to Na-H exchange have not been investigated in guinea pig red cells. Furthermore, there could be other amiloride-sensitive pathways of Na influx that might account for the rise with cooling. Notable among the latter is Na-Mg exchange, which is amiloride sensitive and in rodent cells can be quite large when measured either as Mg^{2+} loss or as Na influx (Gunther, Vormann & Holriegl, 1990; Xu & Willis, 1991; Willis, Xu & Zhao, 1992).

The initial objectives of the present study were therefore twofold: to confirm the presence of Na-H exchange in guinea pig red cells and to establish whether it or some other pathway accounted for the rise in amiloride-sensitive Na influx in cooled cells. The results indicate that a Na-H exchange mechanism is present and that it does account for the increase in Na influx at 20°C, through a regulatory site becoming more sensitive to cytoplasmic H^+.

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However, at 20°C the carrier operates in an uncoupled (or "slippage") mode, without net H^+ loss from unacidified cells.

Materials and Methods

Animals

Guinea pigs were obtained from animal suppliers and were housed in an animal room with air temperature about 20°C and a light cycle of 14 : 10 L : D. The protocol for this study was approved by the UIUC Animal Care Committee.

Blood

All experiments in this study were done on red blood cells within a few hours of their being drawn by cardiac puncture. Animals were anesthetized with methoxyflurane and the blood drawn into heparinized syringes through sterile 22-gauge needles. The cells were washed three times by centrifugation (2500 × *g*, 5 min), aspiration of the supernatant and buffy coat, and resuspension in ice-cold medium containing (in mM) 150 NaCl, 7.5 KCl, 10 glucose, 5 adenosine and 10 MOPS buffer, pH 7.4. They were then held until use in ice-cold suspension of the same composition as the wash medium and approximately 10% hematocrit.

Na Influx

The procedures used for determination of Na influx were similar to those described previously (Zhou & Willis, 1989). Briefly, 1.5-ml Eppendorff microfuge tubes were preloaded with ice-cold medium with or without added amiloride and with 1–2 μC of ^{24}NaCl accounting for only 3.75 mM NaCl in final concentration. (^{24}Na was prepared from neutron bombardment of Na_2CO_3 in the UI TRIGA reactor, followed by neutralization and removal of carbonate, *see* Hall & Willis, 1984). The incubation medium was the same as the wash medium except that it usually contained 0.1 mM ouabain and, depending on the experiment, other agents such as DIDS (*see* below) or phorbol ester were also added.

Influx was initiated by addition of 0.3 ml of the ice-cold suspension of 10% red cells. Tubes were placed in water baths of appropriate temperature and samples taken at 5 min and 20 or 25 min. (The time course of isotopic uptake determined previously showed uptake to be virtually linear for over one-half hour after the first 5 min. Also, temperature equilibration occurs within 5 min.) The samples were immediately washed three times with ice-cold wash solution ($MgCl_2$, 107 mM, Tris 10 mM, pH 7.8) by centrifugation in a microcentrifuge at 15,000 × *g* for 15 sec, followed by aspiration of supernatant and resuspension in wash solution.

Isotope was extracted by lysis of the cells in 5% trichloroacetic acid. The radioactivity of the lysate was counted as Cerenkov radiation in a liquid scintillation counter. Appropriate standards were counted to determine specific activity of the flux medium.

Cell volume of the suspension was determined by absorption of hemoglobin of a diluted sample at 540 nm. For each animal this value was related to original cell volume by determination of hematocrit and corresponding hemoglobin absorption in parallel samples. From the cell volume in the actual flux tube and the specific activity of the radioactive Na, influx was calculated as mequiv/liter cells/hr.

Na Efflux

To determine efflux, red cells were loaded with ^{24}Na by incubation at 20% hematocrit in a loading medium containing (in mM) 75 NaCl (half of which was provided by the NaCl made radioactive in the reactor), 75 KCl, 10 glucose, 10 MOPS buffer (3-N-[morpholino]propane-sulfonic acid), usually pH 7.4 (but see below). The cells were quickly washed four times with loading medium. After the last wash, a suspension was prepared of 5% hematocrit in medium similar to the influx medium described above, except lacking isotope and containing 0.125 mM DIDS (4,4′-diisothiocyanatostilbene-2′2-disulfonic acid) for maximal inhibition for Cl-HCO_3 exchange (Escobales & Canessa, 1986; Canessa, 1989). The suspension was distributed in 1-ml aliquots into microfuge tubes which were then quickly placed at appropriate temperature. Loss of isotope from timed samples was terminated by centrifugation for 15 sec at 15,000 × *g*, followed immediately by transfer of 0.8 ml of sample to counting tubes. Counting was performed in a gamma counter because amiloride caused color quenching of Cerenkov radiation in the liquid scintillation counter.

Efflux was determined over the period 5 min from addition of medium to 25 min by the relationship, Na efflux = $k[\mathrm{Na}]_i$, where k is the rate coefficient for efflux and $[\mathrm{Na}]_i$ is the cell Na concentration determined by flame photometry on a parallel sample taken from the suspension at the time of initiation of efflux. The value of k was determined as:

$$k = [\ln(1 - ((a_t - a_o)/(a_i - a_o))]/t,$$

where "a" represents activity, a_t is activity of sample at 20 or 25 min, a_o is activity of initial sample (i.e., 5 min), a_i is "infinity" or activity of entire suspension and t is time of flux measurement in hours.

Equilibration of Cytoplasmic H^+

Cells were acidified or alkalinized by incubating them in a 10% suspension at appropriate pH (6.0–8.2) in medium otherwise the same as flux medium for one-half to one hour at room temperature or for 10–20 min at 37°C. After these times DIDS was added to a concentration of 0.125 mM and the cells incubated for a further 20–30 min to inhibit later reequilibration through the Cl-HCO_3 exchanger. The supernatant was removed and the cells were resuspended and centrifuged in their respective experimental medium, whether isotopic flux medium (*see* below) or H^+-loss medium. For flux experiments the achieved pH was determined prior to flux incubation and at the end of flux incubation in parallel samples by lysing 0.1 ml packed cells in 1-ml distilled water and measuring pH with a pH meter.

H^+ Loss

Loss of H^+ was estimated by placing 5–10 ml of 10% suspension only lightly buffered with 0.2 mM MOPS in a water-jacketed chamber provided with a stirrer and pH electrodes. The external

pH was adjusted to be close to that of the cells. To begin the measurement, pH was raised to 7.4 mM by adding KOH through a burette driven by a calibrated shaft while medium was constantly stirred, and time was noted. The volume of KOH solution to maintain pH 7.4 was then recorded manually over time (usually about 5 min). For the hyperosmotic condition, sucrose was added in a predetermined amount to yield 450 mOsM. Osmotic concentration was verified with a vapor pressure osmometer. The pH electrode was calibrated for each use at the temperature of the measurement, using a commercial standard buffer.

Mg^{2+} Loading and Loss

Mg^{2+} concentration of the red cells was altered by incubating the cells at 37°C for 15 min with constant stirring with 3 μM A23187, a divalent cation ionophore, in medium containing (in mM) 145 KCl, 5 NaCl, 10 HEPES buffer, 0.3 EDTA, and concentrations of Mg^{2+} ranging from 0 to 20 mM. The cells were then washed three times with the same medium containing 0.1% albumin. They were then washed twice with a medium similar to the flux medium in which they would be incubated (but without isotope), and were finally resuspended in the influx medium.

Loss of Mg^{2+} was detected by collecting the flux medium at various intervals as supernatant and measuring Mg in an atomic absorption spectrophotometer.

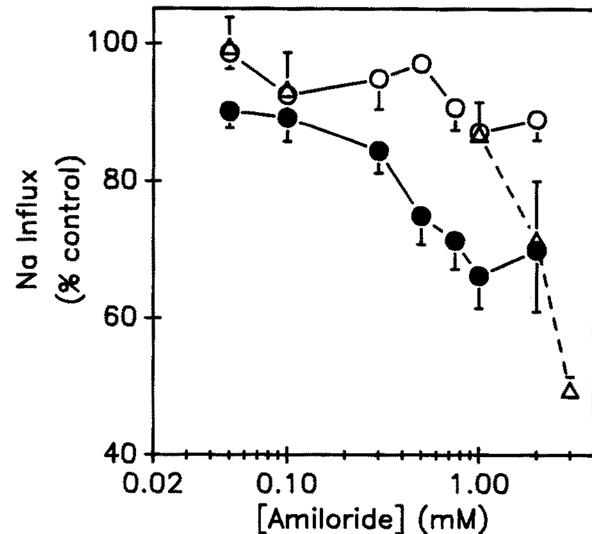

Fig. 1. Effect of amiloride on Na influx in guinea pig red blood cells. Cells were incubated at three temperatures in medium with 150 mM NaCl, 7.5 mM KCl, 10 mM glucose, 5 mM adenosine, 10 mM MOPS buffer (pH 7.4), 0.1 mM ouabain and various concentrations of amiloride. Open circles, 37°C; filled circles, 20°C; triangles, 5°C. Values shown represent means of 3–7 experiments with SE shown where larger than symbol.

Results

Dose Response to Amiloride

At 37°C amiloride caused a maximum inhibition of Na influx of only about 12.9% at 1 mM from an uninhibited Na influx of 4.02 ± 0.38 mmol/liter cell/hr ($P < 0.05$), whereas at 20°C it caused a maximum inhibition of 34% at 1 mM from an uninhibited Na influx of 2.60 ± 0.25 ($P < 0.001$) (Fig. 1). At 5°C 1 mM amiloride caused only about 20% inhibition from an uninhibited influx of 0.84 ± 0.09, but the dose-reponse curve appeared shifted to the right in that at 3 mM inhibition was about 50% (Fig. 1), and in a smaller number of experiments it did not show a bottoming out within the soluble range (*not shown*). (In a single experiment, dimethyl amiloride, typically a more potent inhibitor of Na-H exchange, reduced Na influx by a maximum of 31% at 5°C.) Thus, cooling, at least between 37°C and 5°C, appears to enhance the fraction of Na influx that is inhibitable by amiloride. Since determinations can be made more reliably at 20°C than at 5°C, 20°C and 37°C were chosen as the two working temperatures for this study.

Properties of Amiloride-Sensitive Na Influx at 37°C

Na-H Exchanger

The first objective was to test more fully for the presence of a Na-H exchange mechanism in guinea pig red cells before investigating the effect of cooling. There are two approaches to this: (1) using common activators of Na-H exchange to see if they promote Na influx at 37°C and (2) verification of a Na-dependent H^+ loss. The latter point had not been tested in guinea pig cells and the use of activators had previously only included cell acidification and elevation of intracellular Ca^{2+} (Zhou & Willis, 1989).

Effect of Cell Acidification on Na Influx. In that earlier study, cell acidification did not cover a very wide range and did not take into account change in cytoplasmic pH during flux incubation. Accordingly, in this study both points were addressed—an effort was made to force pH to wider limits and cytoplasmic pH was estimated at the beginning and end of the flux period as described in Materials and Methods, and the mean of the two sets of values taken. In order to plot the values shown in Fig. 2, results were divided among four groups according to their observed cytoplasmic pH, i.e., below 6.8, 6.9–7.0, 7.1–7.4, and greater than 7.4. The results show a steep rise in amiloride-sensitive Na influx in

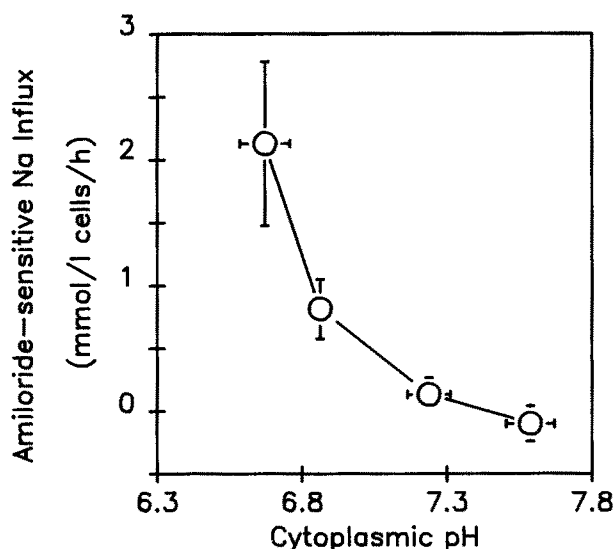

Fig. 2. Effect of cytoplasmic pH on Na influx at 37°C. Cells were equilibrated in media with pH set to various values as described in Materials and Methods; pH was then "locked" by blocking Cl-HCO_3 exchange with 0.125 mM DIDS, and washed and incubated in medium with pH 7.4. Cytoplasmic pH was determined in parallel cells just before and just after incubation for determination of Na influx and the mean between the two was used. Results were divided according to measured cytoplasmic pH into four groups: less than pH 6.8, pH 6.8–6.99, pH 7.0–7.39, pH 7.4 and greater. Results shown represent means of eight cases with SE of flux determination indicated by vertical bars and SE of pH indicated by horizontal bars.

cells acidified below 7.2, rising to about 2.2 mmol/liter cells/hr at about pH 6.6. We were unable to achieve cell pH values much below this range and so did not find a plateau in the response. Judging from the steepness of the rise it would have been above 3 mmol/liter cells/hr.

Effect of Phorbol Ester on Na Influx. In lymphocytes and other cells Na-H exchange is stimulated by presence of phorbol ester in the medium (Grinstein & Rothstein, 1986; Rosoff, 1988), but this response has seldom been tested in red blood cells (*see* Discussion). We observed that when phorbol ester, PMA (phorbol 12-myristate 13-acetate), was added to the medium to a concentration of 0.01 mM there was no increase in Na influx (Table 1), but when 2 mM Ca^{2+} was present as well, amiloride-sensitive Na influx at 37°C rose to over 2 mmol/liter/hr. External Ca^{2+} by itself exerted no effect.

Effect of Hypertonicity on Na Influx. Osmotic shrinkage is a well-known activator of Na-H exchange in many cells including red cells (Parker & Castranova, 1984; Grinstein & Rothstein, 1986). To test for its effect in guinea pig red cells we used sucrose to make medium hypertonic to a concentration employed previously for red cells (450 mOsM, Jennings, Douglas & McAndrew, 1986). This concentration greatly elevated Na influx, to 6.5 mmol/liter/hr at 37°C, and the effect was entirely abolished by amiloride (Table 2).

Table 1. Effect of phorbol ester on amiloride-sensitive Na influx in guinea pig red cells[a]

Conditions	N	Amiloride-sensitive Na influx (mmol/liter cell/hr)	
		37°C	20°C
Control	4	0.43 ± 0.16	1.27 ± 0.24
+Phorbol ester	4	0.72 ± 0.36	1.46 ± 0.21
+Ca	3	0.80 ± 0.34	1.28 ± 0.25
+Ca + Phorbol ester	3	2.20 ± 0.48[b]	1.30 ± 0.25

[a] Cells were incubated in same medium as described in Fig. 1 except that 0.2 mM EGTA was added, with or without 0.01 mM phorbol ester (phorbol 12-myristate) 13-acetate and with or without 2 mM $CaCl_2$. Means ± SE of 3–4 cases are shown.
[b] $P < 0.01$ for the comparison with control.

Table 2. Effect of osmotic shrinkage on amiloride-sensitive Na influx in guinea pig red cells[a]

Conditions	Amiloride-sensitive Na influx (mmol/liter cell/hr)	
	37°C	20°C
(A) Control cells	0.22 ± 0.19	0.91 ± 0.15
(B) Shrunken cells	6.47 ± 0.84[b]	2.07 ± 0.44[c]
Ratio B/A	29.4	2.3

[a] "Control cells" represent cells incubated in isotonic medium as in Fig. 1 with and without 1 mM amiloride. "Shrunken cells" represent cells of the same individuals that were incubated in medium made hypertonic by addition of sucrose to 450 mOsM. Means ± SE of five cases are shown.
[b] $P < 0.01$ for the comparison with control cells at 37°C.
[c] $0.01 < P < 0.05$ for the comparison with control cells at 20°C.

Na Activation of Na Influx in Hypertonically Incubated Cells. Since Na-H exchange is conceived in other systems to operate as an exchange carrier, one ought to expect the Na influx component associated with it to show saturation kinetics. However, since amiloride-sensitive Na influx is only variably present at 37°C in untreated red cells of guinea pig, and since there may be other amiloride-sensitive systems present as well, it was desirable to investigate Na sensitivity when the pathway was as fully activated as possible.

Since osmotic shrinkage appeared to exert the largest effect presumably attributable to this pathway, we investigated the effect of $[Na]_o$ in hyperton-

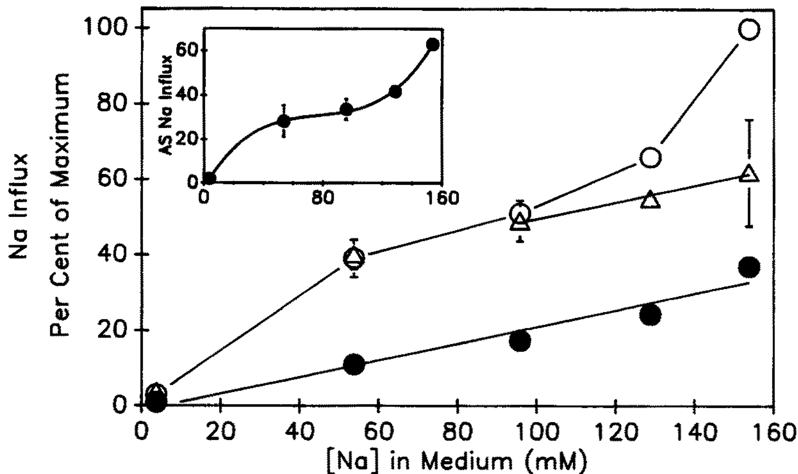

Fig. 3. Dependence of Na influx on external Na concentration in hypertonically incubated red cells of guinea pig. Cells were incubated in 450 mOsM solution as in Table 2, except NaCl was variably substituted with choline chloride. Results shown are of a single experiment representative of three similar ones. Ordinate represents Na influx as percent of the maximum influx in control cells in 154 mM Na, which was 10.8 ± 2.1 (N = 3). Open circles, control (i.e., no amiloride); filled circles, 1 mM amiloride; triangles, 5 μM amiloride. Curves for 1 mM amiloride and for 5 μM amiloride above 95 mM are Na linear regression lines. Means ± SE of three experiments are shown. *Inset:* Amiloride-sensitive Na influx (i.e., difference between control cells and cells with 1 mM amiloride at each concentration of external Na).

Table 3. Na-dependent H^+ loss in acidified and hypertonically incubated guinea pig red blood cells[a]

Conditions	N	Na-dependent H^+ loss (mmol/liter cells/hr)	
		37°C	20°C
Control	(12,4)	2.2 ± 1.1	not detectable
Acidified	(11,9)	10.4 ± 3.51[b]	4.2 ± 1.0[b]
Hyperosmotic	(5,5)	10.7 ± 3.2[b]	1.2 ± 0.9

[a] Initial rate of loss of H^+ from red cells was determined as described in Materials and Methods by titration with KOH in lightly buffered medium. In each experiment loss in medium with no Na (i.e., with K replacing Na) was subtracted from loss in medium with 150 mM NaCl. Control cells were unacidified and incubated in isotonic medium. Means ± SE are shown.
[b] $P < 0.01$ for comparison of value with zero.

ically incubated cells at 37°C (Fig. 3). Amiloride-sensitive Na influx (*inset* in Fig. 3) exhibited both a plateau between 50 and 100 mM $[Na]_o$, and a sharp rise between 120 and 150 mM. Further, this sharp rise or "tail" was abolished by only 5 μM amiloride (i.e., Na influx was reduced to the rate expected from uninhibited flux at lower $[Na]_o$), even though 5 μM amiloride had no inhibitory effect at lower $[Na]_o$.

Na-Dependent H^+ Loss at 37°C. In order to determine whether there was H^+ efflux matching Na influx through a putative Na-H exchange, DIDS-treated red cells were placed in lightly buffered medium, and the acidification of the medium was determined by titration with KOH (Table 3). To discriminate Na-H exchange from baseline loss of H^+, loss in Na-free medium (with K replacing Na) was subtracted in parallel measurements. (Amiloride is itself a buffer and being lipid soluble also serves to equilibrate differences in H^+ concentration across the cell membrane [Benos, Reyes & Shoemaker, 1983]. Hence, it is an unsuitable discriminator.)

In unacidified cells there was no statistically significant Na-dependent H^+ loss at 37°C (Table 3), but in acidified cells and in unacidified cells hypertonically incubated there was a large and significant ($P < 0.01$) loss on the order of 11 mmol/liter/hr.

Na-Mg Exchanger

Effect of Cytoplasmic $[Mg^{2+}]$ on Na Influx. Presence of Na-Mg exchange in red blood cells has in earlier studies been identified as Na dependent (or amiloride blocked) net Mg^{2+} loss from Mg-loaded cells into Mg-free medium (e.g., Gunther, Vormann & Forster, 1984; Feray & Garay, 1986; Ludi & Schatzmann, 1987; Gunther, Vormann & Hollriegl, 1990). Although net Na uptake has been shown in one or two instances, measurements of correlated unidirectional Na influx have not been done (except for those in a companion study to this one, Xu & Willis, 1991; Willis et al., 1992). Nevertheless, one should expect an activation of Na influx by intracellular Mg^{2+} corresponding to that of Mg^{2+} loss, as observed, for example in human and chicken red blood cells.

To test this, guinea pig red cells were loaded with Mg^{2+} by treatment with the ionophore, A23187, with various concentrations of Mg^{2+} in the medium from 0 mM (with 0.2 mM EDTA) to 10 mM. The cells were then washed and Na influx determined with and without amiloride (Fig. 4). Elevation of cytoplasmic Mg^{2+} caused a strong elevation of Na influx rising with 10 mM Mg^{2+} to above 40 mmol/liter cells/hr (Fig. 4). With 1 mM amiloride about half this increase

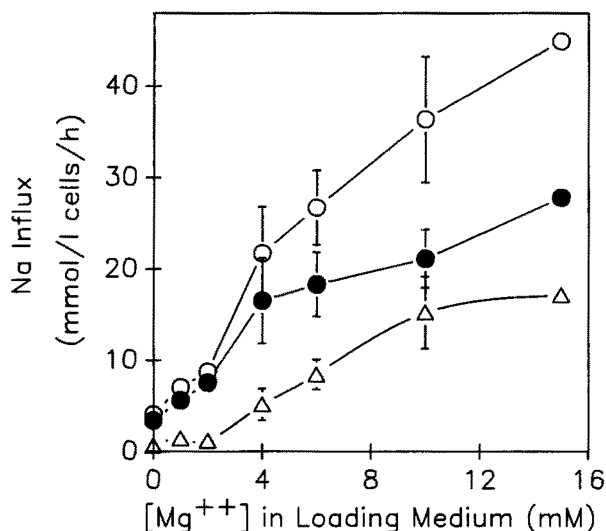

Fig. 4. Effect of cytoplasmic Mg^{2+} on Na influx in guinea pig red cells. Cells were equilibrated in medium with 145 mM KCl, 5 mM NaCl, 0.3 mMEDTA, 10 mM HEPES buffer (pH 7.4) and various concentrations of Mg^{2+} from 0 to 15 mM and 3 μM A23187 ionophore at 37°C. Cells were then washed as described in Materials and Methods and incubated in same medium as described in Fig. 1. Open circles, control (no amiloride); filled circles, 1 mM amiloride; triangles, amiloride-sensitive Na influx (difference between influx with and without amiloride). Results shown for 0 to 10 mM Mg^{2+} represent means ± SE for 3–5 experiments; results shown for 15 mM Mg represent only a single experiment.

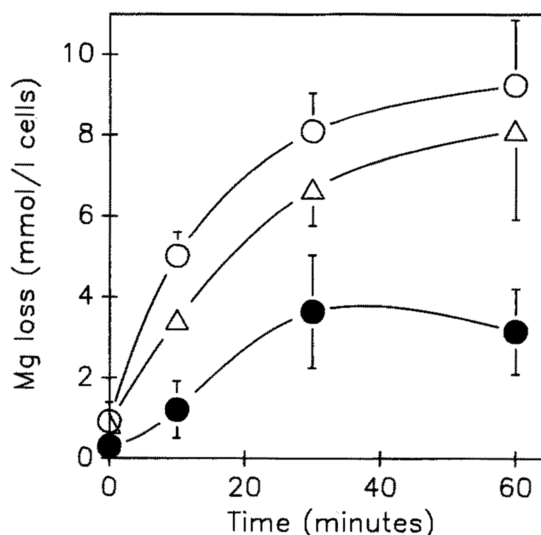

Fig. 5. Loss of Mg^{2+} from Mg-loaded guinea pig red cells into Mg-free medium. Cells were loaded with 10 mM Mg^{2+} as described in Fig. 4, washed and incubated in Mg-free medium. Medium was sampled at intervals and concentration of Mg determined by atomic absorption spectrophotometry. Open circles, control (medium same as in Fig. 4, without amiloride); filled circles, Na-free medium (with choline chloride replacing NaCl); triangles, 1 mM amiloride. Means ± SE of three experiments are shown.

was inhibited. Inhibition by amiloride was highly significant ($P < 0.01$, paired t-test) above Mg-loading concentrations of 4 mM. The response of Na influx to cytoplasmic Mg^{2+} was strongly sigmoidal with little increase below 4 mM Mg^{2+} and a half-maximal effect at about 6 mM (Fig. 4).

Mg Loss from Guinea Pig Red Cells. To confirm the presence of Na-Mg exchange by the more usual means, cells loaded in 10 mM Mg^{2+} were incubated in Mg-free medium and the time course of loss determined by assay of the incubation medium. The rate of loss was depressed from 34.5 to 5.5 mmol/liter/hr in Na-free medium (choline chloride replacing NaCl) (Fig. 5). Although the mean values of Mg^{2+} loss at various intervals appeared lower in the presence of amiloride, variation was large and the difference from control was not significant, statistically.

Amiloride-Sensitive Na Influx at 20°C

The results above appeared to indicate the presence of at least two amiloride-sensitive pathways of Na entry in guinea pig red cells. The hypothesis from the previous study (Zhou & Willis, 1989) that increase in amiloride-sensitive Na influx by cooling (Fig. 1) represented Na-H exchange now needed to be tested in two ways: finding features of Na influx at 20°C that accorded with this mechanism and finding features that ruled out Na-Mg exchange as the responsible path.

Absence of Na-Mg Exchange

As described above, there was no amiloride-sensitive Na influx apparent in cells depleted of Mg^{2+} by incubation in Mg-free medium with ionophore (Table 2). In hamster red cells, where the only amiloride-sensitive path apparent is the Na-Mg exchanger (Xu & Willis, 1991; Willis et al., 1992), Mg depletion also abolishes amiloride-sensitive Na influx. Thus, if increase in amiloride-sensitive Na influx by cooling is governed by Na-Mg exchange, it should be eliminated or at least strongly reduced by the Mg-leaching procedure.

When guinea pig red cells were Mg depleted by preincubation in Mg-free medium with ionophore, Na influx in 1 mM amiloride (1.7 ± 0.4 SE, four cases) was significantly ($P < 0.05$) less than in paired samples of Mg-depleted cells without amiloride (2.8 ± 0.6), and the AS Na influx (1.1 ± 0.3) was not

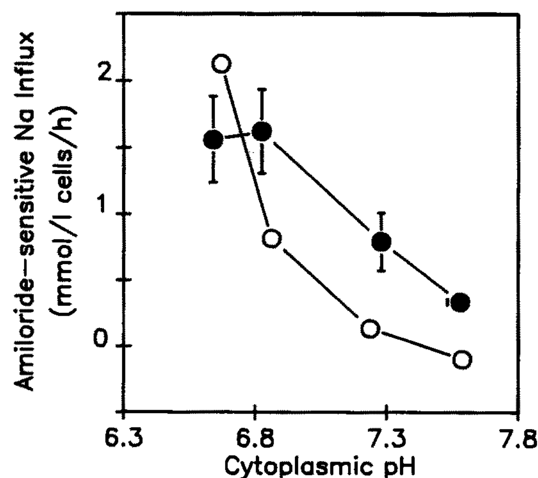

Fig. 6. Effect of cytoplasmic pH on amiloride-sensitive Na influx at 20°C. Cytoplasmic pH was set as described in Materials and Methods and in Fig. 2 and incubated at 20°C for measurement of Na influx. Other conditions and procedures as described in Fig. 2. Filled circles, 20°C; means ± SE of five experiments are shown, except at pH 6.8 where $N = 4$. Open circles, 37°C, same data as in Fig. 2 (SE omitted).

significantly less than that of untreated cells (1.7 ± 0.3) incubated in parallel. (In comparable experiments at 37°C there was no significant amiloride-sensitive Na influx in Mg-depleted cells, Fig. 4.) Thus, Mg depletion did not prevent cooling-induced AS Na influx.

Properties of Na-H^+ Exchanger at 20°C

Effect of Cytoplasmic pH on Na Influx at 20°C. While Na influx in red cells acidified to pH 6.8 was elevated over that of untreated cells, neither the increase nor the maximum rate (1.5 mmol/liter/hr) was as great as that at 37°C (Fig. 6).

Sensitivity of response to acidification was greater at 20°C than at 37°C. At 20°C the amiloride-sensitive Na influx in cells with "physiological" pH of 7.2 was significantly greater than 0 ($P < 0.05$), and it was fully activated at pH 6.8–7.0. By comparison, at 37°C amiloride-sensitive Na influx was not statistically significant ($P > 0.05$) at pH 7.2; it was significant at pH 6.8–7.0 but rose still further in cells with lower pH (Figs. 2 and 6).

Effect of Phorbol Ester and Hypertonic Incubation. Phorbol ester with Ca^{2+} in the medium produced no increase in amiloride-sensitive Na influx over that already caused by cooling alone (Table 1). Incubation of cells in hypertonic medium did cause a doubling of amiloride-sensitive Na infux at 20°C (Table 2), but this was much smaller than the nearly 30-fold increase produced by hypertonicity at 37°C.

Na Activation at 20°C. Increase in amiloride-sensitive Na influx in untreated cells at 20°C resembled that of osmotically activated cells at 37°C (Fig. 7 *inset* compared with Fig. 3 *inset*), with a plateau occurring between 50 and 100 mM and a further increase ("tail") between 120 and 150 mM Na, which was abolished by 5 μM amiloride, even though 5 μM amiloride had no inhibitory effect at lower concentrations of $[Na]_o$.

H^+ Loss at 20°C. As at 37°C, no H^+ loss was detectable in nonacidified cells at 20°C (Table 3). Acidified cells exhibited a significant loss of H^+ ($P < 0.01$), but hypertonically incubated, nonacidified cells did not.

Na Efflux vs. Na Influx. The last finding (absence of H^+ loss at 20°C) raised the question of whether there was any other counter ion for the increased amiloride-sensitive Na influx at 20°C. The most obvious possibility was external Na engaging in a Na-Na exchange. Accordingly, Na efflux was measured in parallel with Na influx (Fig. 8). Amiloride-sensitive Na efflux was only barely detectable at 20°C and was not detectable at 37°C (or at 5°C). Even at 20°C it was only a small fraction of Na influx (0.08 *vs.* 0.8 mmol/liter/hr).

Na Efflux into Na-Free Medium. Comparison of results in Table 3 with those in Fig. 5 suggests an apparent paradox: Na infux at 20°C was so much more sensitive to cytoplasmic acidification than at 37°C that activation occurred even in nonacidified cells, yet no measurable Na-dependent H^+ loss was occurring. This paradox is easily resolved by reference to a common view of the Na-H exchanger (Aronson, Nee & Suhm, 1982; Grinstein & Rothstein, 1986) in which it is envisioned to possess a regulatory site for H^+ on the cytoplasmic face. Thus, increased activity of the system could relate to a regulatory site rather than to the transport-specific binding of H^+.

The existence of the regulatory site was first postulated by Aronson et al. (1982) in kidney cell membranes, and was confirmed by demonstrating the activating effect of cytoplasmic H^+ on Na *efflux* into *Na-free* medium. Under these conditions a stimulatory effect of H^+ on Na efflux could not be interpreted as representing a stimulation of the carrier by its transported ligand, H^+ (which could only result in increased influx).

Accordingly, we investigated the effect of acidification on Na efflux into Na-free medium to detect whether a similar regulatory site was demonstrable in guinea pig red cells, and if so, whether it showed increased sensitivity with cooling. The results (Fig. 9) showed that at 37°C between pH_i 7.3 and 6.8 there was an increase in AS Na efflux from nil (0.2 ± 0.2) to 1.4 ± 0.3 mmol/liter/hr. At 20°C, as with AS Na

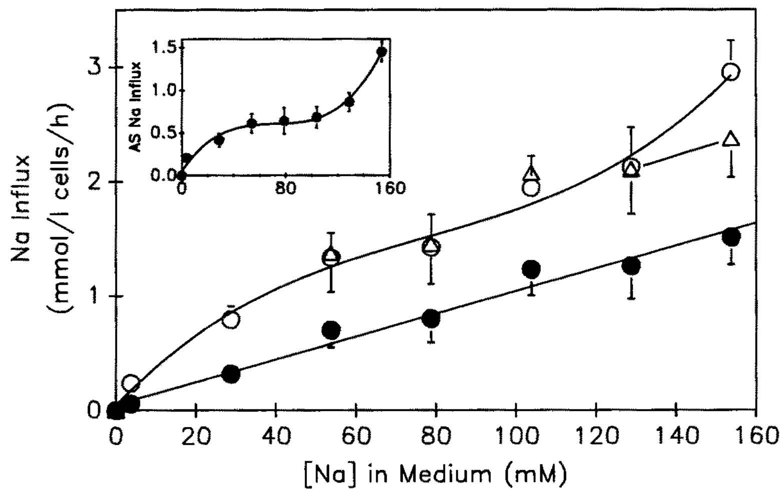

Fig. 7. Na activation of amiloride-sensitive influx at 20°C in guinea pig red cells. Cells were incubated in media of various Na concentrations (choline chloride replacing NaCl) without (open circles) and with 1 mM amiloride (filled circles) or 5 μM amiloride (triangles). Lines drawn for values in 1 mM amiloride and 5 μM amiloride represent linear regressions. All other curves were smoothly drawn to connect points. Means ± SE of eight experiments are shown, except those for 5 μM amiloride where N = 2–5. *Inset:* Amiloride-sensitive Na influx based on difference with and without 1 mM amiloride.

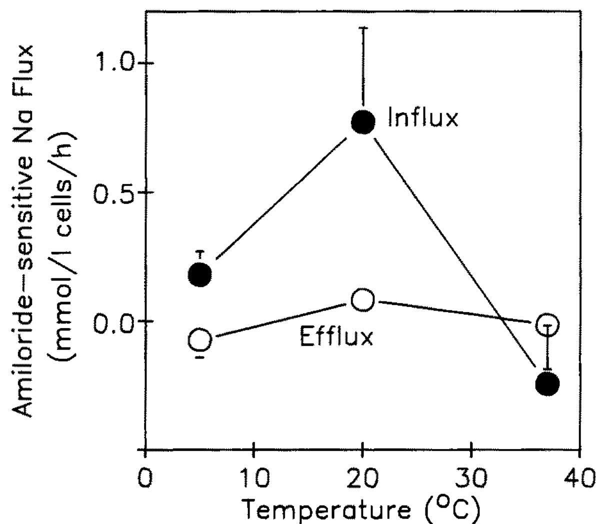

Fig. 8. Comparison of amiloride-sensitive Na efflux with amiloride-sensitive Na influx. For Na efflux determinations cells were preincubated with ^{24}Na for 2 hr at room temperature, as described in Materials and Methods. They were then washed and incubated in nonradioactive medium with and without 1 mM amiloride and the medium sampled at intervals. Parallel sets of cells were treated in the identical manner except ^{24}Na was substituted with nonradioactive Na in the preincubation. Na influx was determined in these cells close in real time to the estimation of efflux in the companion set. Results shown represent the mean ± SE of 3–5 cases.

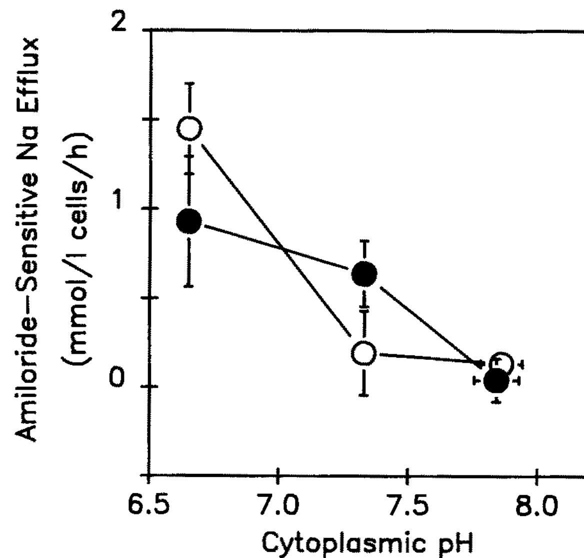

Fig. 9. Effect of cytoplasmic pH on Na efflux into Na-free medium. Cells were loaded with ^{24}Na as in Fig. 9, and their pH was adjusted as in Figs. 2 and 8. They were washed and incubated in Na-free medium: 150 mM choline chloride, 7.5 mM KCl, 10 mM glucose, 5 mM adenosine, 10 mM MOPS (pH 7.4), 0.1 mM ouabain. Open circles, 37°C; filled circles, 20°C. Results shown represent the mean ± SE of 5–7 cases.

influx, sensitivity of AS Na efflux to pH_i was shifted right. Thus, while none was measurable at pH_i 7.8, a statistically significant ($P < 0.05$) AS Na efflux was observed at pH_i 7.3 (0.6 ± 0.2, Fig. 9) and this was not less, statistically, than the value at pH_i 6.7 (0.9 ± 0.4, Fig. 9).

Discussion

The questions posed in the Introduction can be simplified to: Is a Na-H exchange mechanism present in guinea pig red cells? Does it account for the remarkable increase in amiloride-sensitive Na influx (and net uptake) with cooling? The answer to both questions, based on the results of this study, appears to be "yes." Even though at least one other amiloride-sensitive path can be identified (Na-Mg ex-

change), it does not appear to play a role in cooling-augmented Na entry. These issues will be discussed separately below.

Presence of a Na-H Exchanger

The first conclusion, that a Na-H exchanger is present, is the more straightforward of the two. It was already known that cytoplasmic acidification increased Na influx and that the effect was entirely abolished by amiloride (Zhou & Willis, 1989). We now add to this that osmotic shrinkage and phorbol ester (with Ca^{2+} present in the medium) also powerfully enhanced amiloride-sensitive Na influx. Osmotic shrinkage has frequently been used as an activator of this mechanism in red cells (Parker & Castranova, 1984; Jennings, Douglas & McAndrew, 1986), but phorbol ester has only been shown in one previous study to affect Na flux in mammalian (i.e., human) red cells (Postnov et al., 1987) and in one other study to activate Na-H exchange in fish red cells (Motais et al., 1992).

In this study the action of phorbol ester required Ca^{2+} in the medium. Phorbol esters operate through protein kinase C whose phosphorylation of membrane constituents requires Ca^{2+} (Cohen & Foley, 1986). Since Ca^{2+} is normally very low in red cells (less than 10 nM), its presence in the medium is presumably necessary to permit a sufficient cytoplasmic level for this activation. Whether phorbol ester actually increases Ca^{2+} concentration by increasing its uptake in guinea pig red cells is an interesting possibility.

Correlated with amiloride-sensitive Na influx was a Na-dependent H^+ loss in acidified cells and (at 37°C) hypertonically incubated cells. For the purpose of this study that simple demonstration is sufficient to confirm the conclusion. The experiments described here were not designed to estimate a stoichiometry between Na and H^+, and, as further results showed (discussed below), stoichiometry of coupling may be a variable quantity in any case.

In particular, the results obtained with acidified cells, in which net H^+ loss over short intervals is tracked by continual alkalinization of the medium (thus maintaining the gradient) cannot be meaningfully compared with tracer fluxes of Na over 20 min in medium buffered to pH 7.4 (while the imposed gradient is dissipating). The results obtained in hypertonically incubated cells, however, are carried out under less disparate conditions, though they were still not ideal for determining a coupling ratio. For what it is worth, the average value of 7 for amiloride-sensitive Na influx (Table 2) and 11 (with large variation, Table 3) for average value of net Na-dependent H^+ loss in shrunken cells gives a ratio near enough to the expected value of 1 (Montrose & Murer, 1988; Semplicini, Spalvins & Canessa, 1989), considering that the amiloride inhibition of Na influx may have been less effective as a discriminator than complete Na removal was for H^+ loss.

Na-Mg Exchange

The finding of a large increase in Na influx in Mg-loaded cells and, conversely, a large Na dependence of Mg^{2+} loss from Mg-loaded cells, seems to show that red cells of guinea pig, like those of rat and hamster (Gunther, Vormann & Hollriegl, 1990; Xu & Willis, 1991), possess an unusually abundant Na-Mg exchange. It differs from that in rat, hamster and other red cells investigated, however, in being more strongly sigmoid (i.e., little effect below 4 mM, a grossly elevated cytoplasmic concentration) and less easily inhibited by amiloride. Whatever may be the explanation for these peculiarities, they help to allay suspicion of this pathway as the culprit in cooling-activated increase in amiloride-sensitive Na influx.

Cytoplasmic Mg^{2+}, incidentally, has been shown to be an activator of volume sensitive Na-H exchange in dog red cells (Parker, Gitelman & McManus, 1989). This effect, however, has a Km of about 0.6 mM, far too low to account for the increase in Na influx observed in this study.

Source of Cold-Induced Increase in Amiloride-Sensitive Na Influx

The conclusion that Na-H exchange accounts for the increase in amiloride-sensitive Na influx with cooling rests upon two arguments: it cannot be accounted for by the other identified pathway (Na-Mg exchange) and it exhibits properties expected of an activated Na-H exchange.

In human red blood cells incubation of cells in Mg-free medium with A23187 ought to have lowered Mg^{2+} to less than 10^{-7} M (Flatman & Lew, 1977). In hamster red cells, which appear to possess no Na-H exchange mechanism, the same treatment abolishes amiloride-sensitive Na influx and indeed all saturable Na influx completely (Xu & Willis, 1991). In the present study, however, the same treatment had no such effect on the cooling-induced increase in amiloride-sensitive Na influx of guinea pig red cells. Assuming that the method is as effective in guinea pig cells as in human and hamster red blood cells, this result appears to indicate that Mg-Na exchange cannot be involved in the cooling-induced increase in amiloride-sensitive Na influx. Furthermore, as

pointed out above, the low sensitivity of the path to amiloride and to Mg^{2+} make Na-Mg exchange an unlikely candidate.

The argument that amiloride-sensitive Na influx exhibits properties expected of an activated Na-H exchange has both a negative and a positive aspect. The "negative" aspect is that at 20°C activators of Na-H exchange either have no effect (phorbol ester in this study, Table 1, cytoplasmic Ca^{2+}, Zhou & Willis, 1989) or have much less effect (acidification, Fig. 6, hypertonic incubation, Table 2) than at 37°C. On the assumption that lowering temperature activates this pathway of Na entry, this is what would be expected. That is, in the limit, activators of a common pathway should not have additive effects.

Of course, this "non-additiveness" by itself might only mean that the Na-H pathway is completely *inhibited* by cooling and that some other pathway has been turned on. That is why it is necessary both to eliminate other possible candidates and also to find features that positively connect the elevated influx with the Na-H pathway. Two such "positive" connections were observed in this study. First, cytoplasmic alkalinization abolishes amiloride-sensitive Na efflux into Na-free medium at 20°C, which, like AS Na influx at 20°C and unlike AS Na efflux into Na-free medium at 37°C, is significant at physiological cytoplasmic pH (Fig. 9). Secondly, amiloride-sensitive Na influx at 20°C shows the same kind of saturating dependence on external Na concentration (Fig. 7) as hypertonically activated amiloride-sensitive Na influx (presumably Na-H) exchange at 37°C (Fig. 3), including the "tail" above 120 mM Na that is sensitive to low amiloride concentrations.

(This "tail" is a bit of a mystery; either it represents a kinetic peculiarity of the Na-H exchange system or else a completely separate pathway. If it is a separate pathway it would have to be a very similar one [i.e., activated by hypertonic shrinkage, abolished by alkalinization, sensitive to amiloride]. A separate form of Na-H exchange, highly sensitive to amiloride and with low sensitivity to Na, has previously been postulated as a "housekeeping" system in the basolateral membranes of epithelial cells [Haggerty et al. 1988; Casavola, Helmle-Kolb & Murer, 1989]. Presence of a more exotic system, such as shrinkage-activated Na-taurine cotransport [fish red cells, Fincham, Wolowyk & Young, 1987] or a shrinkage-activated Na-K-Cl cotransport [avian red cells, Haas, Schmidt & McManus, 1982] seems unlikely since these have not been demonstrated in mammalian cells. In any case, the "tail" did not contribute more than 40% of the rise in amiloride-sensitive Na influx observed in this study.)

Arguments of identity based on parallels, such as we have presented here, have a respectable history in biology, in having demonstrated, as examples, that genes are on chromosomes and that Na-K ATPase is truly the Na-K pump. While we have perhaps not yet exhausted the potential of this approach (e.g., further comparative studies with analogues of amiloride and with other inhibitors of amiloride-sensitive channels, such as benzamil, or with specific, inhibitory antibodies; investigations in other cell types, etc.), a definitive test of whether the mechanism is intrinsically cold-driven will only be possible with purified Na-H exchanger reconstituted into a standard lipid bilayer.

"Slippage"

The finding of no increase in Na-dependent H^+ loss at 20°C in unacidified cells when amiloride-sensitive Na influx was greatly elevated does not accord with an activation of Na-H exchange, *per se*. There is, however, no paradox if it is possible for the Na-H exchange mechanism to operate in a noncoupled ("slippage") mode. That this is possible is also suggested by the fact that there was no Na-dependent H^+ loss in cells further activated by osmotic shrinkage at 20°C (Table 3). A Na-for-nothing mode of the Na-H exchanger has also been postulated in intestinal epithelial cells (Post & Dawson, 1991). Such loose coupling has also been found for Ca-H exchange in dog red cells (Milanick, 1990).

Sensitivity of Regulatory Site to H^+

Appearance of a slippage behavior in this pathway suggests that affinity for H^+ as a transported ligand has decreased with cooling, yet activation by cytoplasmic H^+ indicated a greater affinity (Fig. 6). Using the same approach as Aronson et al., 1982, we have demonstrated the presence of a regulatory effect of cytoplasmic H^+ on amiloride-sensitive Na efflux into Na-free medium (Fig. 9) as was also apparently observed by Jennings et al. (1986) in rabbit red cells. It appears that the increased sensitivity to H^+ which accounts for this pathway being turned on at 20°C is at this cytoplasmic modifier site.

Whether this change in sensitivity is a result of alteration of the cytoplasmic milieu (ATP, Mg^{2+}, Na, Ca^{2+}, other modifiers) or in the molecule itself as a direct result of cooling is a matter for conjecture and for further investigation of this important phenomenon.

This research was supported by NIH Grant, GM11494.

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Received 7 February 1992; revised 22 June 1992