Stimulation of Na,K-activated Adenosine Triphosphatase and Active Transport by Low External K$^+$ in a Rat Liver Cell Line

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ABSTRACT Exposure of ARL 15 cells, an established line from adult rat liver, to concentrations of external K$^+$ below 1 mM caused a rapid fall in intracellular K$^+$ and a corresponding rise in intracellular Na$^+$ that became maximal within 12 h. Upon continued exposure to low external K$^+$, these initial changes were followed by a striking recovery such that, by 24 h, intracellular Na$^+$ and K$^+$ concentrations approached their control values. Concomitant with this recovery, there was a substantial increase in Na,K-ATPase specific activity that was detectable at 12 h and maximal at 24 h. After restoration of the external K$^+$ concentration, the elevated level of enzyme activity showed little change for at least 24 h. In contrast, restoration of external K$^+$ resulted in a rapid rise in intracellular K$^+$ and a fall in Na$^+$ such that within 30 min the Na$^+$/K$^+$ ratio was lower than in control cells. This overshoot, together with a demonstrated increase in active ^{86}Rb$^+$ uptake under "V_{max}" conditions, confirms that the enhancement in Na,K-ATPase specific activity in response to low external K$^+$ represents an increase in functional Na,K pumping capacity.

INTRODUCTION

In all animal cells, the maintenance of cellular ionic composition and volume is mediated by the membrane-bound Na,K pump, which is often measured as the Na,K-activated adenosine triphosphatase (E.C. 3.6.1.3, Na,K-ATPase) (for reviews, see Glynn and Karlish, 1975; Cantley, 1981). In cultured cells, the pump is inhibited by exposure to digitalis glycosides or a reduction of external K$^+$ to critically low concentrations (Boardman et al., 1974). Because passive Na$^+$ and K$^+$ leaks continue, there is a net loss of intracellular K$^+$ and a net gain of Na$^+$ (Graves and Wheeler, 1982; Kim et al., 1984). The distortions in intracellular Na$^+$ and K$^+$ are followed in 12–24 h by an increase in Na,K-ATPase activity and transport capacity. That this "adaptive" response reflects an increased number of Na,K-ATPase molecules per cell is indicated by a coordinate increase in Na,K-ATPase activity and the number of ouabain-binding sites (Boardman et al., 1974; L. R. Pollack et al., 1981a).

To date, most studies on the adaptive responses to low external concentrations of K^+ in culture have used replicating cells (L. R. Pollack et al., 1981a; Graves and Wheeler, 1982). Although the findings of such studies are of considerable interest, they do not address the possibility that these responses may be cell-cycle dependent. Transient changes in transmembrane passive and active Na^+ and K^+ fluxes and increases in the intracellular Na^+ concentration occur during cell growth and replication. Increases in Na^+ influx and in active Na^+ transport have also been documented after exposure to mitogenic stimuli (Koch and Leffert, 1979; Vara et al., 1985). To avoid the complicating effects of cell-cycle heterogeneity, we have examined the effects of low external K^+ on an adult rat liver cell line (ARL 15) that displays strict density-dependent growth inhibition.

The main issues addressed in this study concern the response of quiescent cells to low external K^+ and the functional consequences of the associated increase in Na,K transport capacity. Exposure of confluent ARL 15 cells to low concentrations of K^+ was shown to produce a rapid increase in the intracellular Na^+ concentration and a complementary decrease in the intracellular K^+ concentration within hours. Upon continued exposure of these cells to low-K^+ medium, there was a striking restoration of the original transmembrane ionic gradients that was nearly complete except during the most extreme reductions in external K^+ concentration. Concomitant with this recovery, there was an increase in the functional Na,K pumping capacity, as reflected by both Na,K-ATPase activity and active monovalent cation transport.

METHODS

Cell Culture

The established rat liver cell line ARL 15 was a generous gift from the Naylor Dana Institute for Disease Prevention (Valhalla, NY) (San et al., 1979). The cells were grown in 60-mm culture plates by standard methods (Freshney, 1983) at 37°C and in a 5% CO_2 atmosphere using Williams' Medium E supplemented with 10% calf serum. Cells were allowed to reach confluence before use. In experiments performed at reduced concentrations of external K^+, we supplemented K^+-free Williams' Medium E with 5% calf serum and sufficient KCl to give the desired K^+ concentration. The reduced concentration of serum used in these experiments was necessary to achieve the desired low concentrations of K^+ in the final medium. In all experiments, the concentration of K^+ in the medium was confirmed by flame photometry (see below).

Determination of Intracellular Cation Content

Assays for cell Na^+ and K^+ were performed by flame photometry on cell lysates obtained after extensive washing to minimize contributions from the extracellular space. Immediately after removal of plates from the incubator, the medium on each plate was aspirated and then each plate was rapidly washed four times with 5 ml of ice-cold 100 mM $MgCl_2$ (Smith and Rozengurt, 1978). Preliminary experiments confirmed that this procedure resulted in an insignificant loss of cellular $^{22}Na^+$ or $^{86}Rb^+$ from cells preloaded with radioisotope. Monolayers were scraped with a rubber policeman in 1.0 ml of 15 mM $LiNO_3$ flame photometry standard (Instrumentation Laboratories, Boston, MA), and then transferred to microcentrifuge tubes with the aid of a subsequent rinse with an additional

0.5 ml of $LiNO_3$ standard. The tubes containing the resulting hypotonic lysates were capped and stored in the refrigerator until the time of assay (usually within 48 h). After centrifugation of the thawed lysates in a microcentrifuge (Beckman Instruments, Inc., Palo Alto, CA) to remove particulates, the supernatants were analyzed for Na^+ and K^+ in a flame photometer. Cellular Na^+ and K^+ were standardized against protein determined either directly on the $LiNO_3$ lysates before centrifugation, or on cell lysates in 0.1 M NaOH prepared from parallel plates (see below).

Assay of Na,K-ATPase Activity

Assays for Na,K-ATPase were performed on whole-cell lysates, homogenates, or crude membrane preparations, depending on the experimental requirements. The homogenization medium contained 250 mM sucrose, 50 mM Tris-HCl, and 1.25 mM EGTA (pH 7.5 at 25°C). After two rinses with ice-cold medium, monolayers from individual plates were scraped into 0.5 ml of medium with a rubber policeman. All subsequent operations were performed at 4°C. The resulting cell suspensions were then lysed by rapid freezing in liquid nitrogen or by homogenization with 20 strokes of a Teflon pestle in a Potter-Elvehjem tissue grinder. Both lysates and homogenates contained ~1.5 mg protein/ml.

Crude membranes were prepared from homogenates after an initial centrifugation at 750 g for 15 min to remove nuclei and unlysed cells. The resulting supernatants were then centrifuged again at 100,000 g_{max} for 30 min to pellet the particulates. The lighter buffy coat was resuspended in homogenization medium at a concentration of ~100–200 μg protein/ml without disturbing the darker, underlying mitochondrial pellet. Lysates, homogenates, and membranes were stored at −80°C until assayed, usually within 1 wk. Preliminary experiments indicated that Na,K-ATPase activity in such preparations was unchanged when stored under these conditions for up to 2 wk.

The activity of the Na,K-ATPase was determined from the ouabain-sensitive rate of hydrolysis of radiolabeled ATP by a modification of a procedure for determining Ca-ATPase activity (Muallem and Karlish, 1980). To start the reaction, we added 20-μl aliquots of enzyme preparation to microcentrifuge tubes containing 200 μl of reaction medium that was prewarmed to 37°C. The resulting reaction mixtures contained 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 25 mM histidine, 0.20 mM EGTA (pH 7.5 at 25°C), 3 mM NaN_3, and tracer quantities of $[\gamma\text{-}^{32}P]ATP$ (triethylamine salt, Amersham Corp., Arlington Heights, IL), such that the final specific activity of the ATP was 200 μCi/mmol. Duplicate reactions were run for each sample in the absence and presence of 3 mM ouabain, a concentration of glycoside sufficient to completely inhibit Na,K-ATPase activity in ARL 15 cells. Concentrations of all substrates in the final reaction mixture were well above the half-saturating values required in the Na,K-ATPase reaction (Philipson and Edelman, 1977). All tubes were incubated at 37°C for 30–45 min, during which time <10% of the ATP initially present was hydrolyzed. Under such conditions, ATP hydrolysis was shown to be linearly proportional to both time and enzyme concentration.

The amount of inorganic phosphate released by the enzyme was determined by extraction of a phosphomolybdate complex into organic solvent. Reactions were terminated by the addition of 100 μl of ice-cold solution containing 10% (wt/vol) perchloric acid, 2 M H_2SO_4, 40 g/liter ammonium molybdate, and 2 mM NaH_2PO_4, and subsequent placement of the microcentrifuge tubes on ice. The released radiophosphate was recovered by shaking with 800 μl of ice-cold isobutanol for 20 s, the tubes were placed on ice for 4 min, and then the shaking and cooling were repeated three additional times. The tubes were centrifuged for 30 s in an Eppendorf table-top microcentrifuge (Brinkmann Instruments, Westbury, NY), and 500 μl of the upper (isobutanol) phase was transferred to a

scintillation vial containing 5 ml of Liquiscint (National Diagnostics, Somerville, NJ). Determinations of radioactivity were made in a Searle (Des Plaines, IL) Mark III liquid scintillation counter.

To standardize the assay and correct for nonenzymatic hydrolysis, we included three controls in each experiment. Standards were prepared containing 1/10 the total amount of ATP used in the assays (thereby approximating the amount of ATP hydrolyzed during the course of each incubation; see above). These standards, otherwise identical in composition to the standard reaction mixtures, were completely hydrolyzed by the addition of 50 μl of 20% (wt/vol) perchloric acid, followed by heating in a boiling water bath for 10 min. After subsequent cooling, 50 μl of a solution containing 4 M H_2SO_4, 80 g/liter ammonium molybdate, and 4 mM NaH_2PO_4 was added. This two-step addition avoided the formation of a blue reaction product during heating and resulted in a final mixture that was comparable in volume and composition to that of the experimental samples at the end of their incubation. These standards were extracted in isobutanol, radioactivity was determined as described above, and then the specific activity of the liberated phosphate complex was calculated. Additional standards containing 1/10 the concentration of ATP present in the experimental samples were not heated, but were extracted as usual to provide a background correction for the specific activity of the phosphate complex. Finally, each sample was corrected for nonenzymatic hydrolysis by subtracting the amount of radioactivity extracted from samples that were incubated with homogenization medium in the absence of added enzyme. The activity of the Na,K-ATPase was estimated from the difference between the rate of hydrolysis in the absence and presence of ouabain and was standardized against the amount of protein.

Measurement of Active $^{86}Rb^+$ Uptake

Active transport was assayed by measuring the ouabain-sensitive uptake of the K^+ congener $^{86}Rb^+$. 30 min before the assay, the low-K^+–treated cells were returned to normal concentrations of external K^+, either by a medium change or by the addition of sufficient concentrated KCl solution to adjust the K^+ concentration to normal. As a result, the intracellular cation concentrations in the low-K^+–treated cells were adjusted to near-normal values, facilitating comparison with the control cells (see Results). To initiate isotopic uptake, we added 1 ml of fresh medium (warmed to 37°C and equilibrated with 5% CO_2) containing 10 μCi of $^{86}Rb^+$ (100–800 mCi/meq; New England Nuclear, Boston, MA) to each 4-ml plate with gentle mixing, and then returned the plates to the incubator. Duplicate assays were run in the absence or presence of 3 mM ouabain added with the $^{86}Rb^+$. Preliminary experiments demonstrated that preincubation with ouabain for 0–20 min did not increase the extent of inhibition of $^{86}Rb^+$ uptake over a subsequent 5-min period, which suggests that inhibition of active transport occurs rapidly. The uptake of isotope was allowed to proceed for 5 min at 37°C. During this period of incubation, the rate of uptake was approximately linear. The uptake was terminated by washing with ice-cold 100 mM $MgCl_2$, and lysates were prepared using the procedures described for intracellular cation analysis (see above). The radioactivity of the lysates and aliquots of the labeled medium was determined in a scintillation spectrometer (5360 Autogamma, Packard Instrument Co., Downers Grove, IL). Preliminary studies showed that a brief exposure to $^{86}Rb^+$ (1–2 s), followed by the usual washing, resulted in negligible binding or uptake of the isotope. After gamma scintillation counting, we analyzed aliquots of the lysate for protein content as described below. Uptake was expressed as the volume of medium cleared of $^{86}Rb^+$ per unit time and was standardized against protein content. The rate of transport mediated by the Na,K pump was estimated from the difference in $^{86}Rb^+$ uptake rates in the absence and presence of ouabain.

In some experiments, the Na^+ ionophore monensin was used to raise the intracellular Na^+ concentration (Smith and Rozengurt, 1978). The uptake of $^{86}Rb^+$ was measured as described above, except that 5 min before the assay, monensin was added to the medium to a final concentration of 30 $\mu g/ml$. This concentration was sufficient for maximal stimulation of $^{86}Rb^+$ uptake in ARL 15 cells.

Determination of Protein and DNA

Cellular protein and DNA were determined on alkaline lysates of cell monolayers. After two rinses with 4 ml of 0.9% NaCl, plates were treated with 2 ml of 0.1 M NaOH. The resulting lysates were transferred to individual tubes by means of a rubber policeman, and rinsed with an additional 2 ml of 0.1 M NaOH to ensure complete recovery of the viscous DNA released by the alkali. The pooled lysates from each plate were stored at $-20°C$ until the time of assay (usually within 1 wk). Total protein in the alkaline lysates was measured by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Proteins were also determined on the lysates prepared for enzyme or cation analysis after dilution in alkaline medium. The DNA in the alkaline lysates was extracted by a minor modification of the method of Burton (1956), and measured by the procedure of Giles and Myers (1965), using calf thymus DNA as the standard.

Materials

Culture medium and serum were purchased from Grand Island Biologicals (Grand Island, NY) and plastic culture dishes and flasks were purchased from Corning Glass Works (Medfield, MA). Ouabain octahydrate, bovine serum albumin, calf thymus DNA, EGTA, Tris-ATP, and Tris-HCl were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from Fisher Scientific Co. (Pittsburgh, PA).

Statistical Analysis

All data are expressed as means ± SEM with the number of samples shown in parentheses. Differences between groups were examined by Student's t test and were considered significant for $P < 0.05$.

RESULTS

Effect of Low K^+ on Intracellular Na^+ and K^+

To assess the effects of low external K^+ concentrations on intracellular Na^+ and K^+, we exposed confluent ARL 15 cells for 24 h to medium in which the K^+ concentration ranged from 5.4 (normal) to 0.4 mM. Intracellular Na^+ and K^+ contents were not altered significantly by lowering the external K^+ concentration to ~1.0 mM (Fig. 1). Below 1.0 mM external K^+, however, intracellular K^+ content decreased and intracellular Na^+ content increased, such that at 0.4 mM external K^+ (the lowest concentration tested), intracellular contents of Na^+ and K^+ were approximately reversed with respect to their initial values.

These dramatic changes in intracellular Na^+ and K^+ contents took place with only a small increase in the total of the two, which indicates that a net exchange of K^+ for Na^+ had occurred (Fig. 1). The minimal changes in the sum of Na^+ + K^+ imply small changes in cell volume after prolonged exposure to low external K^+. Our calculations suggest that at 24 h, cell swelling was maximal in 0.65 mM external K^+, and was never greater than 12%.
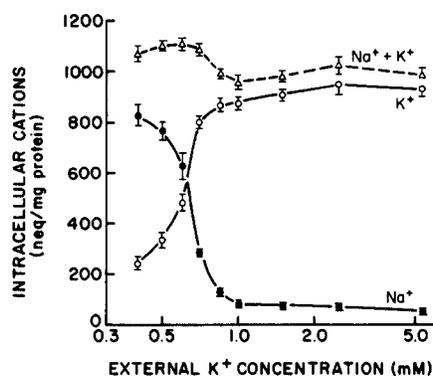
FIGURE 1.   Effects of external K^+ concentration on the intracellular content of Na^+ and K^+ in ARL 15 cells after 24 h exposure. Note the logarithmic scale of the abscissa. Bars indicate SEMs ($n = 4$ from two independent experiments).

Time Course of Low-K^+ Effect on Intracellular Cations

The time course of the effect on intracellular Na^+ and K^+ contents was defined by incubating confluent cells at external K^+ concentrations of 5.4, 1.0, 0.85, or 0.65 mM for up to 48 h (Fig. 2). If the cells remained isosmotic with their environment, then the sum, $Na^+ + K^+$, should be proportional to cell volume, and intracellular concentrations of Na^+ and K^+ should be approximately proportional to $Na^+/(Na^+ + K^+)$ and $K^+/(Na^+ + K^+)$, respectively. As expected, the ratio $Na^+/(Na^+ + K^+)$ showed the greatest increase at the lowest concentrations of external K^+. Despite continued exposure of the cells to low-K^+ medium, a remarkable degree of correction followed the initial increases in intracellular Na^+, such that recovery was nearly complete, except at the lowest concentration
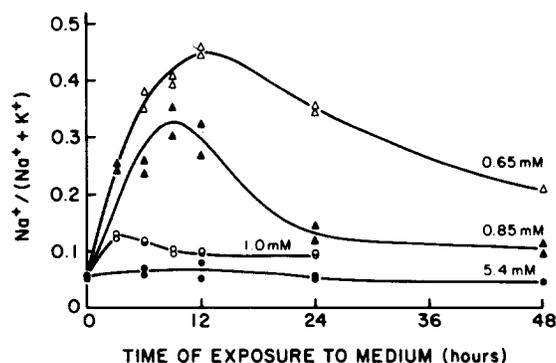

FIGURE 2.   Representative time course of intracellular Na^+ in ARL 15 cells at various external K^+ concentrations. The ordinate shows the ratio $Na^+/(Na^+ + K^+)$, which is approximately proportional to the intracellular Na^+ concentration (see text). Although the absolute values varied between experiments, the shapes and relative positions of the curves were similar in three additional experiments.

of external K$^+$ tested (0.65 mM). The time required to attain the maximal rise in internal Na$^+$, as well as the time required for subsequent correction, increased with progressively decreasing concentrations of external K$^+$. By 24 h, the time point chosen for the experiments shown in Fig. 1, recovery of the intracellular Na$^+$ was nearly complete at 1.0 mM external K$^+$—hence the apparent absence of an effect on internal Na$^+$ and K$^+$ at relatively late time points (cf. Fig. 1).

Activity of the Na,K-ATPase: Response to Low K$^+$

The striking recovery of intracellular Na$^+$ and K$^+$ concentrations upon continued exposure to low-K$^+$ medium was more impressive in magnitude than what has been described previously in other cell lines (Graves and Wheeler, 1982; Kim et al., 1984). Although a number of mechanisms might contribute to this recovery, earlier studies in other cell lines indicate that exposure to low external K$^+$ results in substantial increases in Na,K-pump number (L. R. Pollack et al., 1981a; Kim et al., 1984). To examine such a possibility in ARL 15 cells, we exposed confluent

TABLE I

Effect of Low-K$^+$ Treatment on Na,K-ATPase Activity in Various Preparations of Rat ARL 15 Cells

	Na,K-ATPase specific activity		Percent change
	Control	Low-K$^+$–treated	
	μmol P$_i$ released/h·mg protein		
Lysate	1.36±0.04	2.62±0.03[‡]	+93
Homogenate	1.53±0.06	2.90±0.08[‡]	+90
Membranes	2.46±0.20	5.43±0.05[‡]	+121

* Cell monolayers were exposed to control (5.4 mM) or low (0.65 mM) concentrations of external K$^+$ for 24 h and then harvested. Preparations were obtained as described in the text. Values are reported as means ± SEM; $n = 4$.

[‡] $P < 0.05$ for low-K$^+$–treated vs. control.

cultures for 24 h to medium containing either 0.65 or 5.4 mM K$^+$ and then assayed for Na,K-ATPase activity. Three methods of preparing the enzyme were tested: (a) lysis of the cells by a freeze-thaw cycle, (b) homogenization of the cell suspension in a Potter-Elvehjem tube, and (c) isolation of a crude membrane suspension from that homogenate. All three methods showed an approximately twofold increase in the specific activity of Na,K-ATPase after exposure of the cells to 0.65 mM external K$^+$ (Table I). In freeze-thaw lysates, homogenates, and crude membranes from control cells, Na,K-ATPase activity accounted for ~55% of the total ATPase activity. In low-K$^+$–treated cells, Na,K-ATPase activity accounted for 63–66%. Because the lysates and homogenates gave similar increases in Na,K-ATPase specific activity, we used the simpler lysis procedure in all the subsequent experiments.

To ensure that this effect of low external K$^+$ on the activity of Na,K-ATPase was not dependent on a known hormonal regulator of Na,K-ATPase content, we also performed experiments using thyroidectomized calf serum. In a parallel

study, exposure to 0.65 mM external K^+ in 10% thyroidectomized calf serum also increased the Na,K-ATPase activity of lysates as compared with controls. Additional experiments using other commonly available culture media have given similar results (data not shown).

Concentration Dependence of Effects on Na,K-ATPase Activity

Additional experiments were performed to define in more detail the dependence of the increase in Na,K-ATPase specific activity on the K^+ concentration of the medium. Reduction of the external K^+ concentration from 5.4 to 1.5 mM had
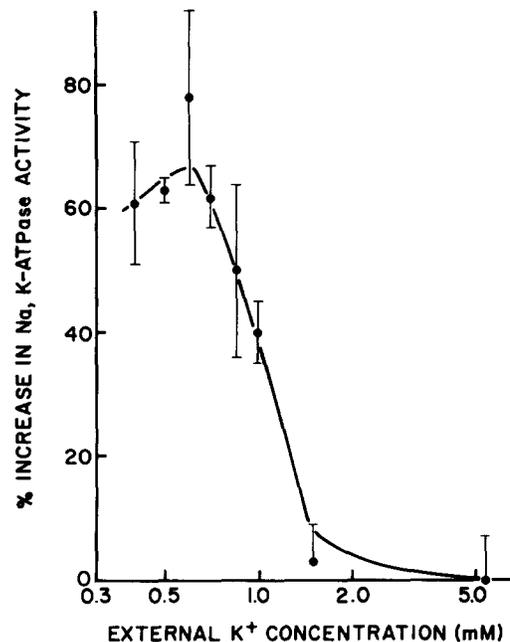

FIGURE 3. Effects of external K^+ concentration on the specific activity of the Na,K-ATPase in ARL 15 cells after 24 h exposure. Note the logarithmic scale of the abscissa. The data are expressed as percent increases over the control values. The control (5.4 mM) specific activity was 0.70 ± 0.05 μmol/h·mg protein ($n = 4$). Bars indicate SEMs ($n = 4$ from two independent experiments).

no detectable effect on the specific activity of Na,K-ATPase after 24 h (Fig. 3). Further reductions in external K^+, however, produced a progressive increase in activity. The maximal increase at 0.6 mM K^+ was ~65% above the value for control cells in 5.4 mM K^+. Below 0.6 mM external K^+, the response may have declined slightly. The concentrations of external K^+ that elicited an increase in the specific activity of the enzyme were also those that perturbed intracellular Na^+ and K^+ concentrations (cf. Fig. 1).

In contrast to the substantial increase in Na,K-ATPase activity at low external K^+ concentrations, there was little, if any, change in total protein content per
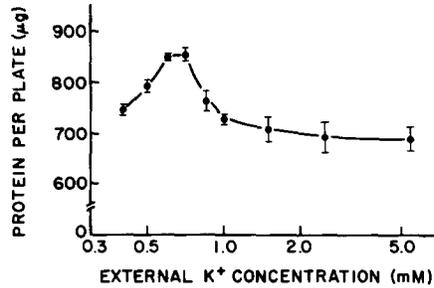
FIGURE 4.   Effects of external K^+ concentration on cell protein in ARL 15 cells after 24 h exposure. Note the logarithmic scale of the abscissa. Bars indicate SEMs ($n = 4$ from two independent experiments).

plate in these confluent cultures after 24 h (Fig. 4). Exposure to even very low concentrations of external K^+ did not result in a loss of cell protein. Although exposure to concentrations of K^+ between 0.6 and 1.0 mM resulted in a slight increase in protein per plate after 24 h when compared with control cells, no effect was observed on the protein/DNA ratio in response to low-K^+ treatment. After 24 h in 0.65 or normal 5.4 mM external K^+, the protein/DNA ratios were 14.5 ± 0.5 ($n = 8$) and 14.2 ± 0.6 ($n = 8$), respectively.

Time Course of Effects on Na,K-ATPase Activity

Three concentrations of external K^+, 0.65, 1.0, and 5.4 mM, were used to define the time course of the increase in Na,K-ATPase specific activity. Low-K^+-dependent increases in enzyme specific activity were statistically significant at 12 h (Fig. 5). By this time, the changes in intracellular Na^+ and K^+ had reached their respective maxima and minima (cf. Fig. 2). There was no detectable difference in Na,K-ATPase specific activity between control and low-K^+-treated cells at 6 h, but this may have resulted from a transient increase in the controls.
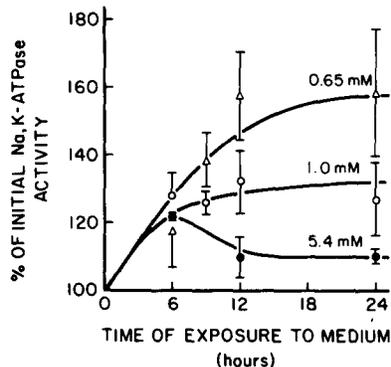

FIGURE 5.   Time course of Na,K-ATPase specific activity in ARL 15 cells at various external K^+ concentrations. The data are expressed as percent increases over the control values. The initial specific activity was 0.73 ± 0.04 μmol/h·mg protein ($n = 8$). Bars indicate SEMs ($n = 4$ except at 24 h, where $n = 6$).

This small increase may reflect the replacement of the culture medium with fresh medium at the beginning of the experiment. The rise in Na,K-ATPase activity persisted for 24 h, by which time the recovery of intracellular Na^+ and K^+ concentrations was nearly complete, except in cells exposed to 0.65 mM K^+.

Effects of External K^+ Repletion: Na,K-ATPase Activity

To document the reversibility of the low-K^+ effect on Na,K-ATPase activity, we exposed ARL 15 cells to 0.60 mM K^+ for 24 h and then added sufficient KCl to restore the external K^+ concentration to 5.4 mM. Despite restoration, there was no detectable decrease in the specific activity of the Na,K-ATPase for at least 24 h (Fig. 6). By 48 h, the specific activity of both the low-K^+-treated and control cells had declined, although the difference remained significant. This decrease
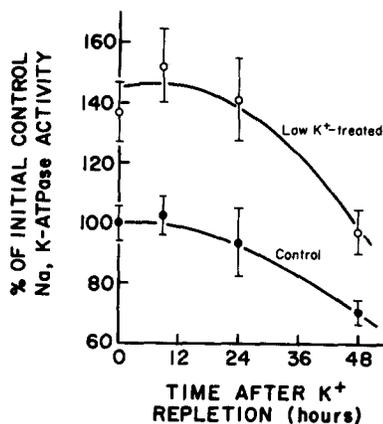

FIGURE 6. Time course of Na,K-ATPase specific activity in control and low-K^+-treated ARL 15 cells after external K^+ repletion. Cells were exposed to either 0.65 or 5.4 mM (control) external K^+ for 24 h before repletion. The data are expressed as percentages of the control immediately before repletion. The initial specific activity of the control was 0.64 ± 0.06 μmol/h·mg protein ($n = 6$). Bars indicate SEMs ($n = 6$ from two independent experiments).

in Na,K-ATPase specific activity may have been caused by depletion of nutrients or growth factors from the culture medium after incubation for 3 d or by prolonged maintenance in the confluent state.

Effects of External K^+ Repletion: Intracellular Na^+ and K^+

The close correlation of the time course and concentration dependence of the effects of low K^+ on Na,K-ATPase activity and intracellular Na^+ and K^+ concentrations suggested a causal relationship between increases in enzyme activity and subsequent correction of the altered intracellular ions. To examine a functional correlate of enhanced levels of Na,K-ATPase, we exposed cells to 0.65 mM K^+ for 24 h, a period sufficient to produce an increase in enzyme specific activity (see above). The subsequent restoration of the external K^+ concentration resulted in a decrease in intracellular Na^+ and an increase in K^+ within 30 min (Table

TABLE II

Effect of Acute External K⁺ Repletion on Intracellular Na⁺
and K⁺ in Rat ARL 15 Cells Treated with Low External K⁺*

	Control	Low-K⁺–treated	Repleted
Intracellular Na⁺	55±2	370±140	60±5
Intracellular K⁺	1,057±28	720±68	1,359±84‡
Total Na⁺ + K⁺	1,112±30	1,090±58	1,419±89‡
Na⁺/(Na⁺ + K⁺)	0.049±0.002	0.342±0.043	0.042±0.001‡

* Cell monolayers were exposed to control (5.4 mM) or low (0.65 mM) concentrations of external K⁺ for 24 h, and then half of the low-K⁺–treated plates were returned to 5.4 mM for 30 min. Intracellular ion contents are expressed as nanoequivalents per milligram protein. Values are reported as means ± SEM; $n = 9$ for control cells, $n = 7$ for low-K⁺–treated and repleted cells.

‡ $P < 0.05$ for repleted vs. control cells.

II). Indeed, the decrease in the $Na^+/(Na^+ + K^+)$ ratio demonstrates that an overshoot occurred, such that the intracellular Na^+ concentration became transiently lower and the intracellular K^+ concentration became transiently higher than the corresponding values in control cells.

Active Rb⁺ Uptake

We assessed that the increase in Na,K-ATPase specific activity after exposure to low external K^+ reflects a true increase in pump-mediated transport capacity by measuring the rate of active ⁸⁶Rb⁺ uptake in control cells and in cells that had previously been exposed for 24 h to medium containing 0.65 mM K^+. To examine active transport rates under conditions of comparable intracellular Na^+ concentrations, we exposed the low-K⁺–treated cells to 5.4 mM external K^+ for 30 min before the assay, a period sufficient to reduce intracellular Na^+ concentrations to control levels (or slightly lower) without reducing Na,K-ATPase specific activity (see above). Under these conditions, the rate of ouabain-sensitive ⁸⁶Rb⁺ uptake was enhanced by 48% in the cells previously exposed to low external K^+ (Table III).

TABLE III

Effect of Low-K⁺ Treatment on ⁸⁶Rb⁺ Uptake in Rat ARL 15 Cells*

	⁸⁶Rb⁺ clearance		Percent change
	Control	Low-K⁺–treated	
	μl medium/min·mg protein		
Ouabain-sensitive	1.96±0.16	2.90±0.26‡	+48
Ouabain-insensitive	1.09±0.02	1.22±0.05	+12

* Cell monolayers were exposed to control (5.4 mM) or low (0.6 mM) concentrations of external K⁺ for 24 h, and then the low-K⁺–treated cells were returned to 5.4 mM for 30 min before the measurement of ⁸⁶Rb⁺ uptake in the absence or presence of 3 mM ouabain. Uptake is expressed as microliters medium cleared of ⁸⁶Rb⁺ per minute per milligram protein. Values are reported as means ± SEM; $n = 5$ from two independent experiments.

‡ $P < 0.05$ for low-K⁺–treated vs. control.

It was likely, in the preceding experiment, that the rates of active monovalent cation transport in both the control and the low-K^+–treated cells were limited by the low concentrations of intracellular Na^+ (Smith and Rozengurt, 1978; Soltoff and Mandel, 1984). To assess the transport enzyme under V_{max} conditions, we increased the intracellular concentrations of Na^+ in control and low-K^+–treated cells by brief preincubation with the Na^+ ionophore monensin (Smith and Rozengurt, 1978), and determined the rate of ouabain-sensitive $^{86}Rb^+$ uptake. In the presence of monensin, the rate of $^{86}Rb^+$ uptake increased by two- to threefold in both control and low-K^+–treated cells. Moreover, the ouabain-sensitive uptake rate was markedly increased in the cells that had previously been exposed to low external K^+ (Table IV). The fractional increase in transport activity (+53%) was similar to the observed fractional increase in Na,K-ATPase specific activity (+67%), which suggests that the latter reflects an increase in functional pumping capacity.

TABLE IV

*Effect of Low-K^+ Treatment on Monensin-stimulated Active $^{86}Rb^+$ Uptake and Na,K-ATPase Activity in Rat ARL 15 Cells**

	Control	Low-K^+–treated	Percent change
Ouabain-sensitive $^{86}Rb^+$ uptake	5.35±0.27	8.21±0.26‡	+53
Na,K-ATPase specific activity	0.60±0.05	1.00±0.15‡	+67

* Cell monolayers were treated as described in Table III, except that monensin (30 μg/ml) was added 5 min before measurement of $^{86}Rb^+$ uptake. Uptake is expressed as microliters medium cleared of $^{86}Rb^+$ per minute per milligram protein. Specific activity of the Na,K-ATPase is expressed as micromoles P_i released per hour per milligram protein. Values are reported as means ± SEM; $n = 5$ from two independent experiments.
‡ $P < 0.05$ for low-K^+–treated vs. control.

DISCUSSION

The present studies were prompted by the desire to identify a cell culture system in which the response to low external concentrations of K^+ could be analyzed in the absence of cell-cycle heterogeneity. The response of quiescent monolayers of the rat liver cell line ARL 15 to low external K^+ proved to be of sufficient magnitude to allow a comparison of both the K^+ concentration dependence and the time course of changes in intracellular cations and Na,K-ATPase specific activity. Moreover, a comparison of the enzyme activity with direct measurements of active transport under V_{max} conditions demonstrated the functional competence of the adaptive increase in Na,K-ATPase activity.

Exposure of ARL 15 rat liver cells to concentrations of external K^+ below 1.0 mM caused a rapid fall in the concentration of intracellular K^+ and a corresponding increase in the concentration of intracellular Na^+. After 24 h, reductions of external K^+ from 5.4 mM to, but not below, 1.0 mM did not perturb intracellular Na^+ and K^+ significantly. This lack of a response above 1.0 mM external K^+ is

consistent with the observation that the Na,K pump is fully saturated with respect to external K^+ at concentrations in this range (Haber and Loeb, 1983; Soltoff and Mandel, 1984). In contrast, intracellular concentrations of Na^+ and K^+ changed markedly after decreases in external K^+ below 1.0 mM, which is consistent with previously reported $K_{1/2}$ values of the Na,K-ATPase for K^+ and the well-known steepness of the external K^+ activation curve for active Na,K transport (Sachs and Welt, 1967; Haber and Loeb, 1983).

The sequence of events controlling the initial low-K^+-stimulated changes in intracellular ion concentrations probably corresponds to the short-term regulation of the Na,K pump outlined by L. R. Pollack et al. (1981a). At external K^+ concentrations below that needed to saturate the external cation site, external K^+ would limit catalytic turnover, leading to a reduction in the pumping rate. Active transport of Na^+ and K^+ would no longer balance their rates of passive leak; therefore, the intracellular concentration of K^+ would begin to decline and Na^+ would begin to increase. Despite the low external K^+ concentration, the resulting increase in the internal Na^+ concentration would stimulate the transport rate. Thus, the tendency of the cells to gain intracellular Na^+ through the passive leak would be partially counterbalanced by Na^+ stimulation of the pump, which eventually would establish a new, but altered, steady state for the concentrations of intracellular Na^+ and K^+. As L. R. Pollack et al. (1981a) have suggested, this sequence of events may be sufficient to maintain cell viability over the short term, but at the cost of a higher-than-normal intracellular concentration of Na^+ and a limited ability to respond to further changes in the environment.

Short-term regulation, however, cannot explain the remarkable recovery of the intracellular Na^+ and K^+ concentrations observed in these experiments. Even at the lowest concentrations of external K^+ examined, the perturbations in intracellular Na^+ and K^+ reached their respective maxima and minima within 12 h of the initial exposure and were approaching control values by 24 h. Indeed, at external K^+ concentrations as low as 0.85 mM, recovery was nearly complete. Both the initial perturbations in cation concentrations and the recovery itself occurred in the absence of any marked changes in cell volume, as estimated by the total intracellular content of $Na^+ + K^+$. Although there have been previous reports of some degree of correction of the perturbations in intracellular cation concentrations upon continued exposure to low external K^+, the recovery has not been as dramatic as that described here (Graves and Wheeler, 1982; Kim et al., 1984).

Consistent with the results of previous studies in cultured Chinese hamster ovary cells (Graves and Wheeler, 1982), long-term exposure of ARL 15 cells to reduced external K^+ concentrations resulted in significant increases in Na,K-ATPase specific activity in whole-cell lysates, homogenates, and crude membrane preparations. Augmentation of enzymatic activity was produced by exposure to the same concentrations of external K^+ that produced significant effects on the intracellular concentrations of Na^+ and K^+. Comparisons of the time course of recovery of intracellular Na^+ and K^+ concentrations with that of the increase in Na,K-ATPase specific activity demonstrate that the maximal increase in enzyme activity was present at ~12 h and correlated with the time at which recovery of

intracellular Na^+ and K^+ concentrations was taking place. These observations suggest that the return of intracellular Na^+ and K^+ concentrations toward their respective normal values was mediated, at least in part, by the observed increase in Na,K-ATPase activity and that this increase reflects functional Na,K pumps.

Additional observations strongly support the inference of an increase in functional Na,K transport capacity in ARL 15 cells after prolonged exposure to low-K^+ medium. The intracellular K^+ content in low-K^+–treated cells was driven above the level in control cells within 30 min of external K^+ repletion, as might be expected if pumping capacity was enhanced. Also consistent with this conclusion is the observation that the increase in Na,K-ATPase activity was accompanied by an increase in active $^{86}Rb^+$ uptake, both at normal intracellular Na^+ concentrations and under V_{max} conditions, in which cells were loaded with Na^+ by exposure to monensin. Whether the enhanced Na,K-ATPase activity occurred by induction of new Na,K pumps or by activation of pre-existing pumps remains to be determined. Using cultured HeLa cells and chick ventricular cells, previous workers have measured an increase in the number of pumps after exposure to low-K^+ medium by the specific binding of ouabain (L. R. Pollack et al., 1981a; Kim et al., 1984).

After restoration of the external K^+ to normal values, the enhanced levels of Na,K-ATPase activity produced by low-K^+ treatment showed no detectable fall over the first 24 h. This lack of a decline may reflect slow turnover of the intracellular signal that initiates and maintains the increase in enzyme activity or slow turnover of the enzyme itself. The latter hypothesis is consistent with the slow degradation rate for the subunits of the Na,K-ATPase in rat renal cortex (Lo and Edelman, 1976; Lo and Lo, 1980), but not with the rapid turnover in cultured HeLa cells (L. R. Pollack et al., 1981b). Direct measurement of the synthesis and degradation rates of the enzyme in ARL 15 cells is needed to clarify this issue.

Another distinctive feature of the ARL 15 liver cells was their tolerance to low external K^+. Prolonged exposure of confluent cells to concentrations of K^+ as low as 0.65 mM resulted in no loss of cell protein and possibly even a slight increase in total cell protein and DNA per plate after 24 h. In mouse fibroblasts and dog kidney cells, low concentrations of external K^+ inhibit growth and protein synthesis (M. Pollack and Fisher, 1976; McRoberts et al., 1983). The small changes in protein and DNA in ARL 15 cells argue against selection of a low-K^+–tolerant subpopulation of cells as an alternative explanation for the recovery of intracellular Na^+ and K^+, as well as for the increase in Na,K-ATPase activity. A time course for development of the increase in Na,K-ATPase activity that is more protracted than the mean generation time for these cells also argues against this possibility.

The nature of the signal for the increase in Na,K-ATPase activity remains elusive and was not addressed in the present study. Potential candidates include changes in intracellular Na^+, H^+, and Ca^{++} concentrations and in the transmembrane electrical potential. For example, Boardman et al. (1974) have implicated an increase in intracellular Na^+ as a signal for the low-K^+ response in HeLa cells. It is also possible that the intracellular signal may affect other parameters

contributing to the adaptive response such as changes in membrane cation permeability (Graves and Wheeler, 1982). Further elucidation of the signal and its cellular targets should provide useful insights into the links between short-term regulation of the Na,K pump and eventual "adaptation" of the cell to an altered ionic environment.

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