Low K⁺ Increases Na,K-ATPase Abundance in LLC-PK₁/Cl₄ Cells by Differentially Increasing β, and Not α, Subunit mRNA

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In this paper we establish the response of LLC-PK₁/Cl₁ cells, a pig kidney cell line, to incubation in medium containing 0.25 mM K⁺. The amounts of the Na,K-ATPase α and β subunits, determined by Western blot, increase coordinately to greater than 2-fold over control by 24 h in low K⁺ and remained elevated for the duration of the study period (48 h). Na,K-ATPase activity, measured enzymatically, increased 1.4-fold by 24 h and remained elevated. In order to determine if this response was initiated pretranslationally, α and β subunit mRNA levels were determined by Northern blot analysis. While there was no change in α-mRNA levels, β levels increased significantly, to 1.9-fold over control by 6 h of treatment and remained elevated. This selective increase in β-mRNA was accompanied by 1.6- and 3.1-fold increases in the respective rates of accumulation of newly synthesized α and β subunits, assessed by immunoprecipitating subunits from pulse-labeled cells. The degradation rates of mature Na,K-ATPase subunits did not change during 16 h of exposure to low K⁺, but after 16 h there was a selective decrease in the α degradation rate, relative to control. These results suggest that increased pretranslational regulation of the β subunit alone is sufficient to increase accumulation of both α and β subunits. These findings support the notion that in LLC-PK₁ cells newly synthesized β is rate-limiting and thus regulates, through αβ assembly, the number of pumps transported to the plasma membrane.

Na,K-ATPase, also known as the sodium pump, mediates active transport of sodium and potassium in eukaryotic cells. The enzyme is an integral plasma membrane protein composed of an α catalytic subunit (112–114 kDa) and a β glycosylated subunit (50–66 kDa). Exposure of cells to low extracellular potassium inhibits the activity of Na,K-ATPase because the activity is coupled to the forced exchange of sodium moving out of the cell to potassium moving into the cell (Skou, 1975; Jorgensen, 1980). This inhibition results in a disruption of transmembrane sodium and potassium gradients, as well as processes that depend on the gradients and membrane potential. Cells can compensate by increasing the number of sodium pumps in the membrane (reviewed by Pressley, 1988). This homeostatic response mechanism increases the amount of ions that can be pumped across the membrane, even though the amount of ions pumped by each Na,K-ATPase is inhibited in the low K⁺ environment.

Induction of Na,K-ATPase in response to low K⁺ has been studied in a number of cell lines (reviewed by Pressley, 1988). Sodium pump activity can be increased chronically by increasing the number of pumps, either by increasing pump synthesis, decreasing degradation, or both. The regulatory mechanisms appear to be different for different tissues. Pollack et al. (1981) found that changes in Na,K-ATPase abundance resulted from a decrease in degradation rate in HeLa cells. In cultured chick muscle cells, both a stimulation of synthesis and a decrease in the degradation rate of the sodium pump occur when the cells are challenged with the sodium channel activator verapamil (Wolitzky and Fambrough, 1986). Recently, Taormino and Fambrough (1990) demonstrated in the same system that the increase in sodium pump synthesis was driven by a transient 2.5-fold increase in β-mRNA followed by a more slowly increasing and modest (1.5-fold) but persistent increase in α-mRNA. In cultured liver cells, Pressley et al. (1986, 1988) also measured changes in α- and β-mRNA levels when cells were placed in low K⁺ medium; however, α-mRNA increased far more than β subunit mRNA. This laboratory (Bowen and McDonough, 1987) previously established that in renal MDCK cells there is a coordinate increase in α- and β-mRNA abundance which gives rise to an increase in α and β subunit synthesis and pump abundance. However, the increased pump abundance was not associated with an increased enzymatic activity or restoration of intracellular Na⁺ levels (Bowen and McDonough, 1987). Because of the ineffective nature of sodium pump up-regulation in MDCK cells, we began studies on another renal cell line LLC-PK₁, in which we were able to show that sodium pump up-regulation in response to low K⁺ was associated with increased sodium pump activity.

To identify which rates involved in the synthesis and turnover of sodium pump units are altered to account for Na,K-ATPase accumulation when kidney cells are placed in low potassium medium, we have examined both pre- and post-translational Na,K-ATPase compartments. In this paper, we establish that LLC-PK₁ cells, a clonal porcine kidney cell line, respond to low K⁺ incubation by differentially increasing the β subunit mRNA and synthesis which results in the accumulation of both α and β subunits of Na,K-ATPase to 2-fold over control levels. These observations suggest the working hypothesis that β subunits are rate-limiting for the assembly of αβ oligomers. A preliminary report of these findings...
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EXPERIMENTAL PROCEDURES

Cell Culture—The LLC-PK₁ clonal cell line 4 used in these experiments, obtained from E. Nord (University of California, Los Angeles), were originally isolated from pig kidney and exhibits characteristics found in the straight segment of proximal tubule cells as well as some distinctive characteristics (Hull et al., 1976; Rabito, 1986; Haggerty et al., 1988). Since LLC-PK₁ cells were maintained at 37°C in 5% CO₂, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum (7.5% horse serum + 2.5% fetal bovine serum). No antibiotics were used, and the cells were negative for mycoplasma and bacteria. Cells were trypsinized, plated in either 100-mm or 55-mm culture dishes, and grown to confluence prior to use. The low K⁺ medium for experiments was prepared from K⁺-free DMEM supplemented with 10% dialyzed serum. Control medium was prepared from K⁺-free DMEM supplemented to 5.5 mM K⁺. Experimental medium K⁺ concentrations were verified by flame photometry.

Intracellular Fluid—Cell water volume was determined from the uptake of 3-O-[14C]methylglucose volumetrically by Bowen and McDonough (1987), and extracellular volume was determined by measuring the equilibration space of freshly dialyzed [3H]inulin. Intracellular Na⁺ and K⁺ concentrations were determined using amiloride-labeled cell water.

Preparation of Subcellular Fractions—Subcellular fractions from each plate were rinsed three times with ice-cold phosphate-buffered saline, scraped, and centrifuged at 1600 X g for 3 min. The supernatant was aspirated, and the cell pellet was stored at -70°C prior to use. The cells were lysed by sonication in homogenization buffer (5% sorbitol, 5 mM histidine-imidazole buffer (pH 7.5), 0.5 mM Na₂EDTA, and 0.1 mM Na,K-ATPase assay EDTA) (Mannheim, 1987). Homogenate extracts were centrifuged for 3 min at 1600 X g, and the resulting supernatant was collected for use in Na,K-ATPase abundance and activity assays.

Immunoblot Analysis of Na,K-ATPase Subunit Abundance—Determinations of Na,K-ATPase subunit abundance in cell homogenates were performed by using a modification of the immunoblotting methods (1970) as previously described (McDonough et al., 1987; Smith and Schmitt, 1985).

Preparation of Antibody—Confluent monolayers of each plate were rinsed three times with ice-cold phosphate-buffered saline, scraped, and centrifuged at 1600 X g for 3 min. The supernatant was aspirated, and the cell pellet was stored at -70°C prior to use.

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incubated in low K+ medium ranging from 0.25 to 1 mM K+ for up to 24 h to determine the effect on Na,K-ATPase activity and abundance. 0.25 mM K+ produced the most consistent increase in Na,K-ATPase subunit abundance, and enzyme activity (data not shown), and therefore was selected for use in all experiments. Fig. 1A shows a typical Western blot autoradiogram of Na,K-ATPase a and b subunits from control and low K+-treated cells over a 48-h time course. The results were quantified by scanning densitometry and are summarized in Fig. 1B. Cells maintained for 24 h in medium containing 0.25 mM K+ showed a greater than 2-fold increase in the abundance of both the a and b subunits. This response was maintained for 48 h in low K+. The results of enzymatic Na,K-ATPase activity assays conducted on the same samples are also shown in Fig. 1B. Na,K-ATPase activity increased significantly by 16 h and after to a maximum induction at 24 h in low K+. The 1.4-fold increase in Na,K-ATPase activity observed at 24 h of low K+ treatment was significantly less than the 2-fold increase in subunit abundance. There was no further increase in activity by 48 h in low K+. Thus, during low K+ incubation, there is a progressive decrease in the ratio of enzymatic activity to enzyme subunit abundance suggesting accumulation of inactive or less active pumps.

Calculated intracellular ion levels of confluent monolayers of control cells were 40 mM Na+, 80 mM K+. Measurement of intracellular cation levels in LLC-PK1 cells is complicated by the need to estimate the extracellular volume trapped by the domes and foldings typical of this cell line. The calculated concentrations of Na+ are higher than, and K+ lower than what we would expect in an intracellular compartment. This could result from an underestimate of the extracellular space, for example, as a result of a compartment of extracellular fluid on the basolateral side of the monolayer that does not equilibrate with the inulin added to the apical surface of the monolayer. Sodium levels of LLC-PK1 cells doubled by 12 h of incubation in 0.25 mM K+ medium, presumably as a consequence of reduced enzymatic turnover, and remained elevated after 24 h in low K+ (Fig. 2). Intracellular K+ fell to 37 mM by 4 h where it remained through 24 h. Thus, the increase in Na,K-ATPase activity and abundance is apparently not sufficient to offset the effect of the low K+ incubation on Na,K-ATPase catalytic turnover and K+ efflux. This ionic disruption could play a role in the decrease in the ratio of Na,K-ATPase activity to abundance, potentially by inhibiting intracellular transport, as has been observed in hepatocytes where K+ depletion inhibits intracellular transport of secretory proteins between the endoplasmic reticulum and the Golgi complex (Judah et al., 1989).

a and b Subunit mRNA Levels—To determine whether the change in Na,K-ATPase subunit abundance is a consequence of pretranslational up-regulation, we measured a and b subunit mRNA concentrations after 2-24-h incubation in low K+ medium relative to time paired controls. Fig. 3A is a typical Northern blot. The results are summarized in Fig. 3B. While there is no significant increase in a-mRNA abundance during the time course, the b subunit mRNA abundance increases to 1.88-fold by 6 h and remains elevated at this level over the 24-h time course.

The increase in the b subunit mRNA levels seen at 6 h was not inhibited by the protein synthesis inhibitor cycloheximide at a concentration of 1.5 µg/ml, which inhibited protein synthesis 91% (Fig. 4). These results indicate that the increase in b subunit mRNA levels is not dependent on the synthesis of an intermediary regulator. Increasing cycloheximide concentration to 5 µg/ml did not significantly increase the percentage of protein synthesis inhibition. Longer incubations in cycloheximide (8-16 h) inhibited basal levels of expression of the b subunit mRNA, especially the largest (upper band) b mRNA.

a and b Subunit Synthesis Rates—Although the data concerning abundance indicated a parallel change in a and b subunits in response to low K+, the mRNA data indicated that only b-mRNA levels were increased in response to low K+. In order to determine whether the a and b synthesis rates were coupled to the observed changes in mRNA levels or not, synthesis rates were measured in low K+-treated cells over the 24-h time course by immunoprecipitation of a and b subunits from pulse-labeled cells. A typical fluorogram is shown in Fig. 5A. Fig. 5, B and C, summarizes the change in synthesis rates of a and b relative to time-paired controls. The a subunit synthesis rate increased to 1.6-fold over control at 24 h. The b subunit synthesis rate increased to 3.1-fold.
**FIG. 3.** Effect of low K+ incubation on Na,K-ATPase α and β subunit mRNA abundance. A, Northern analysis of α and β subunit mRNA. 7 μg of total RNA from cells incubated in 0.25 mM K+ (K) or 5.5 mM K+ (C) probed with ^32P-labeled α and β subunit cDNA. α and β abundance of α and β subunit mRNA was determined by scanning densitometry of autoradiograms normalized to paired controls at each time point. Values are means ± S.E. (n values for each time point are shown in parentheses). When combining separate experiments, the following time points were pooled: 2 and 3 h, 4 and 5 h, 8 and 9 h, 16 and 18 h. * indicates significant difference between low K+-treated and control (p < 0.05).

**FIG. 4.** Effect of cycloheximide on control and K-stimulated Na,K-ATPase α and β subunit mRNA levels. LLC-PK1 cells were pretreated with or without cycloheximide (1.5 μg/ml) for 30 min, then incubated in 0.25 mM K+ (K) or 5.5 mM K+ (Control) for 6 h either in the absence (−) or presence (+) of cycloheximide (1.5 μg/ml). Total RNA (7 μg) from plates of cells were analyzed by Northern blots for Na,K-ATPase α and β subunit mRNAs (autoradiogram is representative of two similar experiments).

Over control by 24 h of incubation in low K+. It should be pointed out that this synthesis rate was measured after a 30-min pulse of ^[35]S)methionine. Thus, increased stability of subunits labeled during this period will be manifest as an increase in the rate of accumulation of newly synthesized subunits.

At 16 and 24 h, the increases in the α and β subunit synthesis rates exceed the changes in the α and β subunit mRNA levels (shown in Fig. 3) by 1.5- to 1.8-fold. These results suggest an additional layer of regulation of the two subunits, in this case, coordinate. The results can be explained by either increased translatability of the messages, or, alternatively, without invoking increased translatability, by increased stability of newly synthesized α and β peptides which would be reflected as an increase in the rate of accumulation of newly synthesized subunits.

There was not a change in overall protein synthesis rate of the LLC-PK1 cells used in this study (measured as methionine incorporation into trichloroacetic acid-precipitable protein) in response to incubation in low K+. In contrast, increased cell growth occurs in the cultured African Green Monkey renal cell line BSC-1 incubated in medium K+ reduced to 3.2 mM (Walsh-Reitz and Toback, 1983). In addition, systemic hypokalemia is known to cause significant renal hypertrophy localized to the collecting tubules (reviewed by Fine and Norman, 1989). We conclude that the response of LLC-PK1 cell Na,K-ATPase subunit synthesis to incubation in low K+ is specific and not the result of a growth response to low K+ typical of that found in collecting tubules.

**FIG. 5.** Effect of low K+ incubation on Na,K-ATPase α and β subunit synthesis rate from cells incubated for indicated times in 0.25 mM K+ (K) or 5.5 mM K+ (C). During the last 30 min of incubation, the cells were labeled with 0.5 mCi/ml ^[35]S)methionine. Equal amounts of incorporated radioactivity from each sample were subject to immunoprecipitation. B, synthesis rates of α subunit, determined by scanning densitometry of autoradiograms, control values are normalized to the mean 4-h control value. C, synthesis rates of β subunit, determined by scanning densitometry of autoradiograms, control values are normalized to the mean 4-h control value. Values are means ± S.E. of three experiments. * indicates significant difference between low K+-treated and control (p < 0.05).
increase in labeled subunit in low K⁺ relative to control indicates an increase in the stability (decreased degradation rate) of that Na,K-ATPase subunit. No changes in the degradation rate of mature Na,K-ATPase subunits were seen during the first 16 h of exposure to low K⁺. Thus, we conclude that changes in abundance during this time are due to changes in α and β synthesis rates. After 16 h in low K⁺, there is a marked and significant increase in the amount of labeled α remaining relative to that remaining in the control cells, indicating a decrease in the degradation rate of α subunit in low K⁺ during this period. The β subunit degradation rate does not change relative to control during the 32 h of low K⁺ incubation. These unexpected results indicate that α and β degradation rates are uncoupled as a consequence of low K⁺ incubation. As can be recognized in Fig. 6A, the rates appear to be coupled in the control state. In one experiment in which the control samples were normalized to a constant amount of protein, 27% of the labeled β subunit and 29% of the α subunit autoradiographic signal remained at 16 h relative to the initial signal (autoradiogram not shown). Thus, α and β appear to have very similar turnover times. Low K⁺ incubation appears to selectively increase the turnover time of the α subunit. 

**DISCUSSION**

In this paper, we have determined that pre- and post-translational mechanisms are responsible for regulating Na,K-ATPase in kidney cells placed in potassium-deplete medium. As demonstrated in Figs. 3 and 5, LLC-PK₁ cells respond to a low K⁺ challenge by differentially up-regulating the Na,K-ATPase β subunit mRNA and synthesis, which results in a coordinate increase in both the α and β subunit abundance and enzyme activity (Fig. 1). These changes are followed by a decrease in the degradation rate of α after 16 h in low K⁺ that would also favor accumulation of pumps. The differences in the level of expression of α and β subunit mRNAs suggest that translational or post-translational control mechanisms serve to maintain equimolar amounts of Na,K-ATPase α and β subunits observed in renal tissue (Cantley, 1981). Our findings of non-coordinate regulation of α and β subunits are consistent with the observation that Na,K-ATPase α and β subunits are coded by separate unlinked genes (Kent et al., 1987; Yang-Feng et al., 1988). Our results show that increased pretranslational regulation of the β subunit alone is sufficient to increase accumulation of both α and β subunits. A report of a similar mechanism has been suggested by Taormino and Fambrough (1990) for up-regulation of Na,K-ATPase in cultured chick skeletal muscle where β mRNA is increased transiently, without concomitant increase in α mRNA, leading to an increase in Na,K-ATPase biosynthesis. Thus, β synthesis appears to be rate-limiting in both LLC-PK₁ cells and cultured chick skeletal muscle. Our working hypothesis is that the β subunit regulates, through assembly of αβ heterodimers, the number of sodium pumps transported to the plasma membrane.

Results of transfection studies in oocytes and cell lines support the dependence of the α subunit on the β subunit for transport to the plasma membrane (Noguchi et al., 1987; Takeyasu et al., 1988a, 1988b; Geering et al., 1989). Preliminary reports from Caplan et al. (1987) and Renaud et al. (1989) have suggested that a regulatory protein, such as the immunoglobulin heavy chain binding protein (Bole et al., 1986), assists the assembly of α and β Na,K-ATPase subunits.

If newly synthesized β subunit is rate-limiting for assembly of αβ complexes and the α subunit is synthesized in relative excess, what happens to the excess α? Studies of multimer assembly of other membrane proteins such as the T-cell receptor (Minami et al., 1987; Bonifacino et al., 1989) have revealed that unassembled subunits are degraded in a pre-Golgi compartment. By this mechanism, cells can prevent accumulation of incomplete complexes and ensure that only proteins containing their full complement of subunits reach the cell surface (Lippincott-Schwartz et al., 1988; Rose and Doms, 1988; Hurtley and Helenius, 1989). Thus, the fraction of α not complexed with the β subunit is presumably targeted for degradation from a pre-Golgi reticulum compartment. Upon incubation in low K⁺, there is an increase in the amount of α subunit protein in the absence of a change in α subunit mRNA or a change in mature α degradation rate. The mechanism responsible for the increase remains to be determined. Our findings predict that the degradation rate of α in the pre-Golgi compartment will be decreased in low K⁺. It remains possible that this model may not hold for cells where β is apparently not rate-limiting such as cultured liver cells (Pressley et al., 1988) and MDCK cells (Bowen and McDonough, 1987) where both α- and β-mRNA increase in response to low K⁺ incubation.

The ratio of Na,K-ATPase activity to α and β subunit abundance decreases during the low K⁺ treatment, suggesting that either there is a population of immunoreactive subunits that are inactive or that the pumps induced by low K⁺ are less enzymatically active than the control pumps. Ismail-Beigi et al. (1988) studied the kinetics of sodium pumps induced by low K⁺ incubation in a cultured rat liver cell line and concluded that the induced pumps were indistinguishable from control pumps. In their study, the increase in Na,K-ATPase activity was equivalent to the increase in functional Na,K-ATPase abundance estimated by ouabain-stabilized phosphorylation, an assay that would not detect inactive pumps.
or subunits. In contrast to our results in LLC-PK1 cells, preliminary studies from this laboratory in primary cultures of renal proximal tubule cells indicate that low K+ incubation provokes an equivalent increase in Na,K-ATPase activity and α and β protein abundance (Tang and McDonough, 1990).

In this study we observed a marked decrease in the degradation rate of mature α, but not β, subunit after 16 h in low K+. Since little is known about the degradation routes, or the potential for recycling β, it is difficult to speculate on a rationale for this difference. Wolitzky and Fambrough (1986) described a similar decrease in degradation rate of Na,K-ATPase subunits also occurring late in the up-regulation response of chicken skeletal muscle treated with veratridine. The results of Pollack et al. (1981) in ouabain-treated HeLa cells indicated that all of the up-regulation of Na,K-ATPase abundance could be accounted for by a decreased turnover rate. The mechanism and regulation of degradation of Na,K-ATPase subunits remain to be described in order to account for this potentially significant regulatory mechanism.

Finally, do the changes in biosynthetic rates observed in this study account for the observed increase in abundance of Na,K-ATPase? Change in abundance of Na,K-ATPase depends on changes in synthesis rate and changes in degradation rates of the subunits. The analysis can be simplified up to 16 h of low K+ treatment, where there is no change in degradation rates of α or β. However, the analysis is complicated by the assumption that the newly synthesized subunits are subject to degradation from the pre-Golgi pool, and that the degradation rates of nascent subunits are apparently subject to regulation, depending on the rate of αβ heterodimer formation. Ideally, we would like a measure of synthesis rate of αβ heterodimers. Without this, we can determine the predicted effect of the change in synthesis rates of the separate subunits on the abundance of those subunits. The following equation relates change in synthesis rate to change in abundance:

$$\frac{dN(t)}{dt} = S(t) - k_d N(t)$$

Where N(t) is the abundance of either α or β subunit (as quantitated in Fig. 1 by immunoblots), S(t) is the synthesis rate as a function of time (as quantitated in Fig. 5 by immunoprecipitation from pulse-labeled cells), and K_d is the degradation rate (which, as shown in Fig. 6, was unchanged during the first 16 h of low K+ treatment). Upon calculation of the change in abundance predicted by the change in synthesis rate, the solution of the differential equation (1) was normalized to control, for which we assume the steady state condition:

$$\frac{dN(t)}{dt} = 0$$

What is the change in abundance of α, N(t), at 16 h predicted by the change in the rate of accumulation of newly synthesized α subunits (equated with synthesis rate, S(t) in this analysis)? We assume that S(t), increases linearly from control, defined as 1, to 1.4 at 16 h. We have measured the half-life (t) of α and β in untreated cells to be 10 h (data not shown). Knowing t, one can determine K_d = ln(2)/t. Equation 1 predicts that α abundance at 16 h will be 1.6-fold of control, identical with the abundance measured experimentally at 16 h. Similarly, for the case of β subunit, if we assume that the synthesis rate increases linearly from control, defined as 1, to 2.6-fold over control at 16 h, Equation 1 predicts that β abundance at 16 h will be 2.0-fold over control. This is higher than the abundance measured experimentally (1.6-fold) but still within the experimental range. These calculations indicate that the increased rate of accumulation of newly synthesized α and β subunits can entirely account for the observed increase in the subunits’ abundance. Measurements of the true synthesis and degradation rates of the nascent α and β proteins should further refine our quantitative analysis of the data to accomplish our goal of determining the steps in Na,K-ATPase biosynthesis affected by low K+.

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