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Reduction of K^+ efflux in cultured mouse fibroblasts, by mutation or by diuretics, permits growth in K^+-deficient medium

(furosemide/cotransport/mutant/bumetanide/ouabain-insensitive)

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ABSTRACT **The mouse fibroblastic cell line LM(TK^-) is unable to grow at external K^+ concentrations below a threshold value of 0.4 mM. At subthreshold K^+ concentrations, LM(TK^-) cells rapidly lose intracellular K^+ and eventually lyse. We have analyzed the pathway primarily responsible for K^+ efflux under these experimental conditions and report its specific inhibition by two diuretics, furosemide and bumetanide. Bumetanide, an analog of furosemide, was a more potent inhibitor (by several orders of magnitude) than was furosemide itself. The effects of ouabain and bumetanide were additive, suggesting independence of diuretic-sensitive K^+ efflux from Na^+/K^+ pump-mediated fluxes. Characterization of K^+ efflux in LTK-5, a mutant derived from LM(TK^-) and selected for its ability to grow at 0.2 mM K^+, indicated that the mutant had lost the diuretic-sensitive K^+ efflux pathway. Net cation fluxes, steady-state intracellular cation concentrations, and growth at reduced K^+ concentrations were comparable for LM(TK^-) cells maximally inhibited by diuretics and for the LTK-5 mutant grown either in the presence or absence of diuretics. Thus, reduction in K^+ efflux, either by diuretic addition or by genetic alteration, can permit the cell to maintain normal cation gradients and to grow at otherwise subthreshold external K^+ concentrations.**

Mammalian cells maintain a high steady-state K^+ concentration and a low steady-state Na^+ concentration relative to their surrounding physiological fluids. The transmembrane electrochemical gradients established by these two cations have been implicated in several physiological responses, including the regulation of cell volume (1, 2), nutrient cotransport (3–6), and membrane excitability (7–10). Because the steady-state K^+ concentration reflects the sum of influx and efflux components, both active and passive, an alteration in the activity of one component must be accompanied by a compensating alteration in another if the cationic steady state is to be maintained (11). Should a particular flux become altered so that antagonistic fluxes are unable to compensate, the resulting change in intracellular cation concentration may be lethal to the cell. Such considerations were the basis for the selection of two K^+ transport mutants that gained the ability to grow at a K^+ concentration below that minimally required for parental cell growth (12). Both mutants proved capable of maintaining high intracellular K^+ concentrations at reduced external K^+ through changes in ouabain-insensitive K^+ transport.

One of the mutants, LTK-5, exhibited altered activity of a Na^+-K^+ cotransport system (12, 13) similar to that described in human (14, 15) and avian (16) erythrocytes and in Ehrlich cells (17–21) and murine fibroblasts (22, 23). Specifically, in characterizing the mutant relative to its parent strain under conditions of K^+ depletion and high external K^+, Gargus and Slayman (13) noted that LTK-5 demonstrated enhanced furosemide-sensitive, Na^+-dependent K^+ influx. For two reasons, however, it seemed unlikely that the increase in K^+ influx was causally related to the ability of the mutant to survive selection. (*i*) When furosemide-sensitive K^+ influx was measured as a function of the extracellular K^+ concentration in both parent and mutant cells, it reached a half-maximal rate at 6 mM K^+, considerably higher than the K_m for the ouabain-sensitive Na^+/K^+ pump (1.3 mM). At 0.2 mM K^+, the concentration used in the selection, furosemide-sensitive influx was barely detectable, even in the LTK-5 mutant. (*ii*) If an enhancement in furosemide-sensitive influx were responsible for the ability of the mutant to maintain high intracellular K^+ under the selective conditions, then the addition of furosemide to cultures of LTK-5 growing at 0.2 mM K^+ should inhibit growth. In fact, however, furosemide at a concentration maximally inhibitory to Na^+-K^+ coinflux had no effect on the growth rate of the LTK-5 mutant, either at 0.2 mM K^+ or at 5 mM K^+ (13).

These observations suggested that efflux pathways should be examined in an attempt to explain the growth phenotype of LTK-5. In this paper, we describe a significant diuretic-sensitive component of K^+ efflux in the parent LM(TK^-) cells which is markedly reduced in the LTK-5 mutant. This reduction permits the mutant to grow in K^+-deficient medium; a comparable reduction, brought about by addition of diuretics, permits the parent strain to grow under similar conditions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The cell lines used for this study were LM(TK^-), a thymidine kinase-deficient derivative of the mouse fibroblastic L-cell line (24), and a derived mutant, LTK-5, selected for its ability to grow at reduced K^+ concentration (12). Cell lines were routinely maintained at 37°C in a humidified 5% CO_2 atmosphere in monolayer culture flasks in α minimal essential medium (Sterile Systems, Logan, UT) supplemented to 5% (vol/vol) with fetal calf serum (Sterile Systems). Suspension cultures for use in growth and flux experiments were obtained by detaching the cells by incubation with trypsin/EDTA medium, washing, and resuspending in α minimal essential medium supplemented with 5% fetal calf serum in glass spinner bottles. Suspension cultures could be subcultured for several weeks without alteration of growth or transport properties. Many experiments required the use of a modified growth medium (β minimal essential medium) lacking K^+, permitting adjustment to the desired K^+ concentration by isosmotic substitution of NaCl for KCl. The experimental media were supplemented with 5% dialyzed fetal calf serum, and the final K^+ concentration was determined by flame photometry or atomic emission spectrometry. Ouabain was obtained from Sigma, and furosemide was purchased from Hoechst-Roussel (Somerville, NJ). Bumetanide was a generous gift from Peter Feit (Leo, Ballerup, Denmark) and was stored frozen in dimethyl sulfoxide.

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Growth and Plating Experiments. Cells in logarithmic phase of growth in suspension culture were centrifuged, washed in warmed, isosmotic, K^+-free medium, and suspended in the experimental media. Growth experiments were conducted in 100-ml glass spinner bottles inoculated at 1–2 × 10^5 cells per ml. Duplicate aliquots were withdrawn at zero time and at intervals for cell counting (Coulter, Hialeah, FL) until the culture achieved a stationary level. The efficiency of plating in media containing various K^+ concentrations was determined by harvesting and washing the cells as described above and inoculating 500–1000 cells per 10-cm culture dish in 10 ml of experimental medium. Colonies were allowed to grow for 8 days, after which they were washed once with phosphate-buffered saline (pH 7.4) and stained with 0.025% crystal violet in 1% acetic acid.

Flux Experiments. For determination of net K^+ efflux (and net Na^+ influx), cells in suspension culture were centrifuged and suspended in efflux medium containing the desired K^+ concentration supplemented to 5% with dialyzed fetal calf serum. The cell suspension (≈5 × 10^5 cells per ml) was decanted into a glass spinner bottle and returned to 37°C in the CO_2 incubator. Aliquots were removed at intervals, centrifuged, and suspended in 150 mM choline chloride. Samples of the choline chloride suspension were rapidly taken, and the mean cell volume and intracellular Na^+ and K^+ content were determined (12).

RESULTS

Effect of Diuretics on Cation Fluxes in LM(TK$^-$) Cells. Gargus and Slayman (13) have reported that LM(TK$^-$) cells contain a diuretic-sensitive Na^+-K^+ cotransport system, similar to that described in a variety of mammalian (14, 15, 17–23, 25–27) and avian (16, 28) cells and responsible for a significant portion of unidirectional K^+ influx at high external K^+ concentrations (5–20 mM). Current evidence suggests that K^+ and Na^+ movements (and possibly Cl^- movements) mediated by the cotransport system are nonconcentrative and that their direction and magnitude are dictated by the net electrochemical gradients of the ionic species involved (11, 21, 29, 30). It is therefore reasonable to expect that, at reduced extracellular K^+ concentrations, the cotransport system should mediate the net efflux of K^+.

An experiment to test this prediction in the parent LM(TK$^-$) cells is illustrated in Fig. 1. Cells grown in normal medium were resuspended in medium containing 0.2 mM K^+ (the selective concentration) and 1 mM ouabain (to block active K^+ uptake via the Na^+/K^+ pump), and the loss of intracellular K^+ was followed as a function of time in the presence and absence of diuretic. For this experiment, the diuretic bumetanide was used; it has been reported to be a highly specific inhibitor of Na^+-K^+ cotransport in avian erythrocytes (28) and, therefore, seemed preferable to furosemide, which is known to affect several other transport systems (28). Fig. 1 shows that, in the absence of bumetanide, net K^+ efflux was rapid, beginning at a rate of 1.49 mmol of K^+ per liter of cells per min and reaching completion within 3 hr. Addition of bumetanide at concentrations of 2.5–50 μM progressively inhibited efflux, with a maximal effect at 10 μM. This concentration was used in all subsequent studies.

An experiment to explore the bumetanide sensitivity of efflux in greater detail is illustrated in Fig. 2*A*. LM(TK$^-$) cells were suspended in 0.2 mM K^+ medium with and without 10 μM bumetanide and 1 mM ouabain. In the absence of drugs, there was a conspicuous net efflux of K^+, with the intracellular concentration falling from 130 to 30 mmol/liter of cells in 3 hr. Ouabain accelerated the loss, presumably by preventing the recapture of K^+ by the Na^+/K^+ pump. As in the preceding

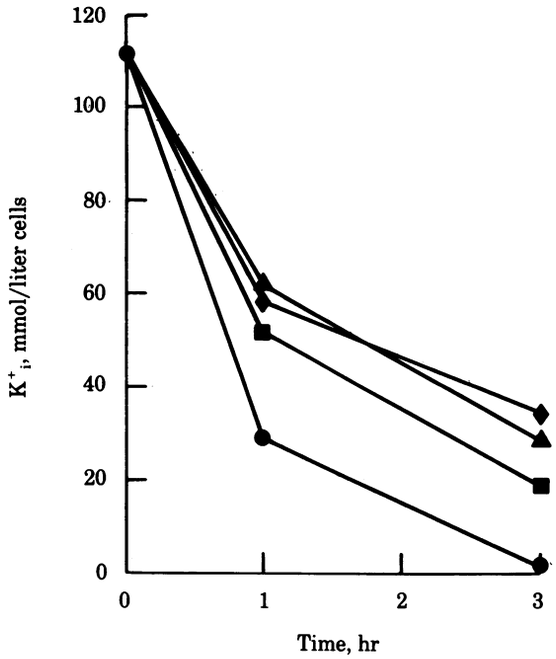

FIG. 1. Effect of bumetanide on net K^+ efflux in LM(TK$^-$) cells. Cells were suspended at 5 × 10^5/ml in β minimal essential medium containing 0.2 mM KCl, 1 mM ouabain, 5% dialyzed fetal calf serum, and 0 μM (●), 2.5 μM (■), 10 μM (▲), or 50 μM (◆) bumetanide. Samples were taken at zero time and after 1 and 3 hr of incubation and assayed for intracellular K^+ content.

experiment, bumetanide acted to retard efflux, and its effect was the same in the presence or absence of ouabain. Thus, a bumetanide-sensitive component of net K^+ efflux, independent of the ouabain-sensitive Na^+/K^+ pump, can be defined in LM(TK$^-$) cells.

Fig. 2*B* illustrates the behavior of intracellular Na^+ during the same experiment. In the absence of drugs, there was a net influx of Na^+ until, after 3 hr, the intracellular Na^+ concentration (140–155 mM) was close to the extracellular concentration (150 mM). Ouabain accelerated Na^+ influx slightly, and bumetanide inhibited it, both in the presence and absence of ouabain. In all cases, the net inward movement of Na^+ approximately balanced the net outward movement of K^+.

Effect of Diuretics on Cation Fluxes in the LTK-5 Mutant. The next step was to examine cation fluxes in the LTK-5 mutant under the same conditions to see whether a reduction in K^+ efflux might be observed which would underlie the ability of the mutant to grow and to maintain a high intracellular K^+ concentration at reduced external K^+. The results of such an experiment are given in Fig. 3. When LTK-5 was suspended in 0.2 mM K^+ medium in the absence of drugs, there was an initial net efflux of K^+ but—in contrast to the results seen with LM(TK$^-$)—efflux slowed with time, and the intracellular K^+ concentration leveled off at a new steady-state value of 70–80 mmol/liter of cell water. In the presence of ouabain concentrations sufficient to inhibit the Na^+/K^+ pump, this new steady state could not be maintained, and intracellular K^+ continued to fall (though somewhat more slowly than in the parent cells). The most striking result was that bumetanide had no detectable effect on K^+ efflux either in the presence or absence of ouabain, indicating that the diuretic-sensitive component of efflux was greatly reduced in the LTK-5 mutant.

As in LM(TK$^-$), net Na^+ influx mirrored K^+ efflux quite closely in LTK-5 (Fig. 3*B*). In the absence of drugs, Na^+ en-

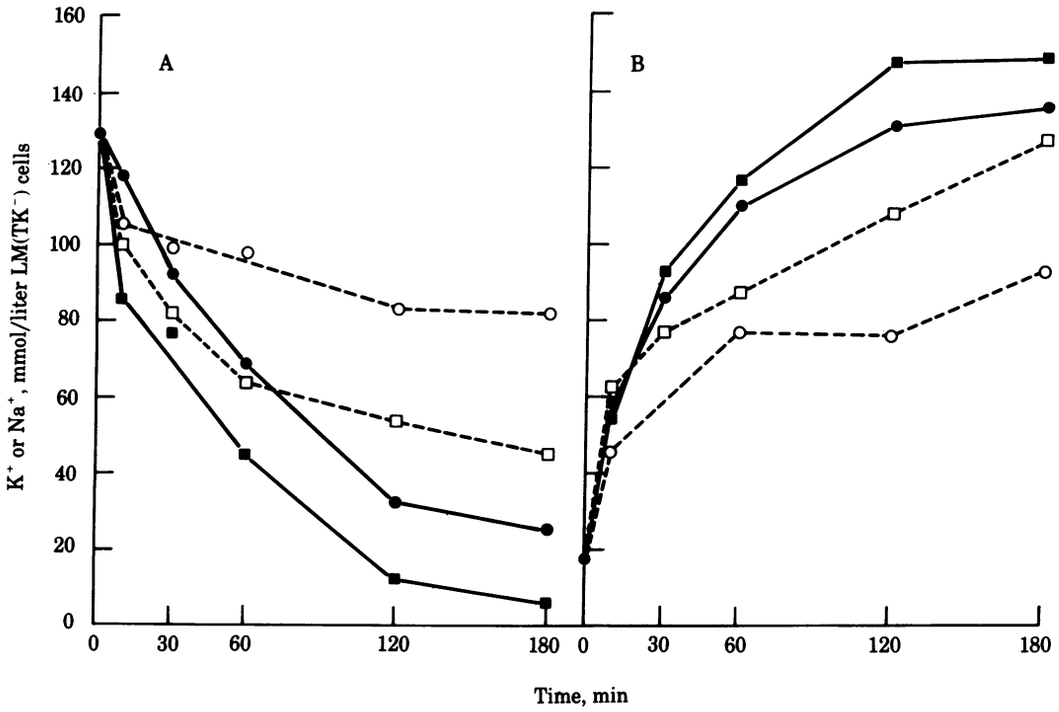

FIG. 2. Effects of bumetanide and ouabain on net K^+ efflux (*A*) and net Na^+ influx (*B*) in LM(TK^-) cells. Cells were suspended at 5×10^5/ml in β minimal essential medium containing 0.2 mM KCl, 5% dialyzed fetal calf serum, and the following drugs: ●, none (control); ○, 10 μM bumetanide; ■, 1 mM ouabain; or □, 10 μM bumetanide plus 1 mM ouabain. Samples were analyzed for intracellular K^+ (*A*) and, with a correction for trapped extracellular medium (13), for intracellular Na^+ (*B*). Data represent averages from six experiments.

tered the cells rapidly over the first hour and then leveled off at a new steady-state concentration of 80–90 mmol/liter of cells. In the presence of ouabain, Na^+ entry continued, and the intracellular concentration exceeded 120 mmol/liter of cells by the end of the experiment. Once again, bumetanide had no detectable effect, either in the presence or absence of ouabain.

Effect of Diuretics on Growth in Low K^+. The existence of a diuretic-sensitive pathway of net K^+ efflux in LM(TK^-) cells and its reduction in the LTK-5 mutant now make it possible to understand an initially surprising result concerning the effect of diuretics on growth. Gargus and Slayman (13) observed that 1 mM furosemide did not alter the growth rate of LTK-5 cells, either in normal medium (containing 5 mM K^+) or in selective medium (containing 0.2 mM K^+). Furosemide did, however, permit the parental LM(TK^-) cells to grow at 0.2 mM K^+, even though they were unable to do so in the absence of drug. Fig.

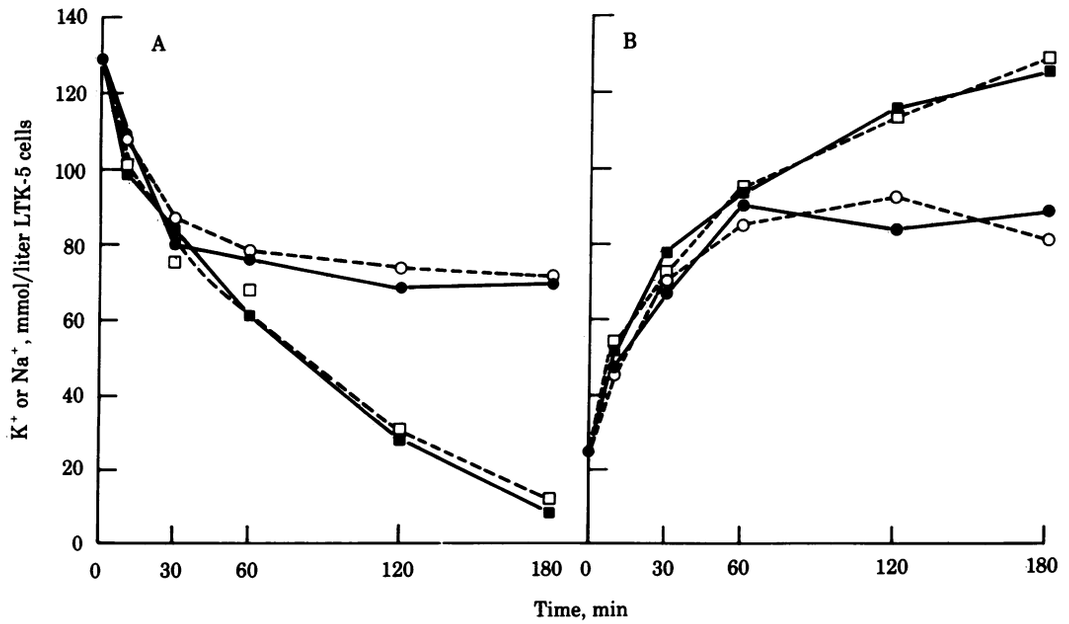

FIG. 3. Effects of bumetanide and ouabain on net K^+ efflux (*A*) and net Na^+ influx (*B*) in the LTK-5 mutant. Conditions are as described in the legend to Fig. 2. Data are averages from four experiments. ●, No drugs (control); ○, 10 μM bumetanide; ■, 1 mM ouabain; □, 10 μM bumetanide plus 1 mM ouabain.

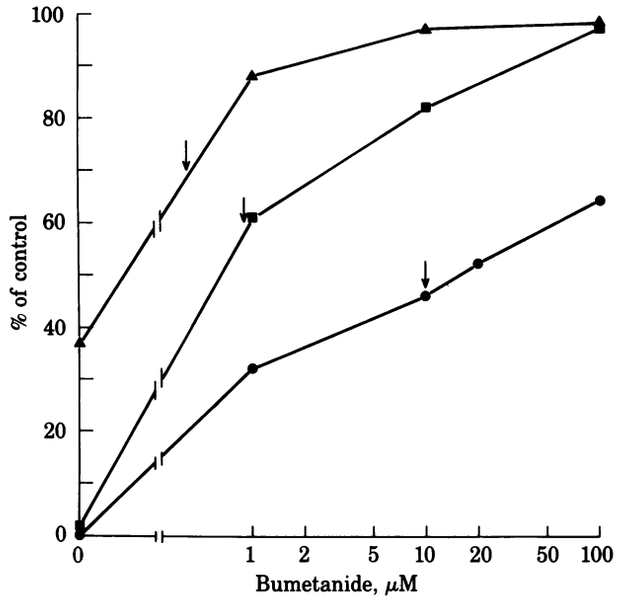

FIG. 4. Effect of diuretics on the growth rate and plating efficiency of LM(TK$^-$) cells at low K^+ concentrations. ●, Growth rate in suspension culture was measured in β minimal essential medium containing 0.2 mM KCl, 5% dialyzed fetal calf serum, and increasing concentrations of bumetanide. Cell number was determined during the logarithmic phase of growth, and the growth rate was normalized to the rate in α minimal essential medium containing 5.4 mM K^+ (1.00 generation per day; ref. 12). Plating efficiency was measured by inoculating cells at low density in β minimal essential medium containing 0.6 mM KCl (■) or 0.8 mM KCl (▲) in addition to dialyzed serum and increasing concentrations of bumetanide. Colony formation was compared to that of control plates in α minimal essential medium with 5.4 mM K^+. Data are averages from two experiments. Arrows represent the effect of 1 mM furosemide in simultaneous experiments for purposes of comparison of the relative potency of the two diuretics.

4 gives added information on this phenomenon. In the experiment described by the lowest curve, LM(TK$^-$) cells were transferred to 0.2 mM K^+ medium containing increasing concentrations of bumetanide, from 0 to 100 μM. In the absence of drug, the cells were unable to grow at this K^+ concentration, as had been reported earlier. Addition of bumetanide permitted growth and, at the highest drug concentration tested (100 μM), the growth rate reached 60% of the control rate observed in normal medium.

The upper two curves illustrate the results of comparable plating experiments. At 0.8 mM K^+, the plating efficiency of LM(TK$^-$) cells dropped to 37% of the control and, at 0.6 mM K^+, the cells failed to plate altogether. In both cases, 100 μM bumetanide restored the plating efficiency to greater than 90% of the control value. For comparison, the arrows in Fig. 4 indicate the effect of 1 mM furosemide in the same experiments.

These results confirm the experiments of Gargus and Slayman (13) with furosemide and show that the more potent diuretic drug, bumetanide, also confers the ability to grow at subthreshold concentrations of K^+.

DISCUSSION

Causal Relationship Between Reduction of K^+ Efflux and Ability to Grow at Reduced External K^+. In order to maintain their characteristic cation asymmetry, mammalian cells spend 20–40% of their metabolic energy on K^+ uptake and Na^+ extrusion (11). This energy is used to drive the ATP-dependent Na^+/K^+ pump which, at normal physiological K^+ concentrations (5 mM), is externally saturated and capable of maintaining high internal K^+ against the existing pathways of K^+ efflux. When the extracellular K^+ concentration is lowered, however, the pump operates more slowly and a threshold K^+ concentration can be identified (0.4 mM in the parental cells used in this study; ref. 13) below which the cells are unable to retain adequate K^+ (or perhaps extrude sufficient Na^+) to permit growth.

Analysis of cation movements at a subthreshold K^+ concentration (0.2 mM) has revealed a major bumetanide-sensitive component of net K^+ efflux in LM(TK$^-$) cells. Further work will be required to establish the relationship of this flux to the diuretic-sensitive Na^+-K^+ cotransport system that has been described in other cell types (11, 17, 21, 27, 28), particularly in view of the fact that K^+ efflux is accompanied in the present case by a net bumetanide-sensitive movement of Na^+ in the opposite direction (into the cells). Whatever the details of the mechanism, it is clear that inhibition of bumetanide-sensitive K^+ efflux permits the cells to maintain a relatively high steady-state K^+ concentration (≈80 mmol/liter of cells) in the face of a low K^+ medium and, consequently, to grow. The bumetanide concentration that produces maximal stimulation of growth (100 μM) is higher than the concentration giving maximal inhibition of net K^+ efflux (10 μM), but this apparent discrepancy can presumably be accounted for by the different time scales of the two kinds of experiments. Growth measurements were made over 6 days (suspension cultures) or 8 days (plating experiments), allowing time for inactivation or breakdown of drug, whereas flux measurements were complete within 3 hr.

An alternative explanation for the higher concentrations of bumetanide needed to inhibit growth emerges from preliminary experiments that show that bumetanide-sensitive K^+ efflux is significantly increased in cells that have been grown for 24 hr in the presence of bumetanide (unpublished data). These results indicate that bumetanide-treated cells adjust to the new conditions by increasing the number or activity of diuretic-sensitive efflux sites. Such a regulatory compensation, expressed over the long time period of the growth experiments, could lead to a requirement for higher concentrations of bumetanide to maximally inhibit efflux and thus to stimulate growth.

Properties of the LTK-5 Mutant. Just as the diuretic-sensitive component of K^+ efflux can be inhibited by addition of drug, it can also be reduced by mutation, as in LTK-5, once again allowing the maintenance of steady-state intracellular cation concentrations that are compatible with growth. The results of Gargus and Slayman (13) indicate that the LTK-5 mutant has not lost diuretic-sensitive transport altogether because, at high extracellular K^+ concentrations, significant (even enhanced) furosemide-sensitive K^+ influx was seen. Further work will be required to pinpoint the nature of the LTK-5 mutation. In the meantime, it is of interest that another mutant strain (LTK-1), selected in the same way for the ability to grow at 0.2 mM K^+, retains bumetanide-sensitive K^+ efflux but shows a reduction in a separate, drug-insensitive component of efflux at 0.2 mM K^+ (unpublished results). Thus, it appears that an appropriate mutational change in any component of the overall cation flux can bring influx and efflux into balance and thereby permit growth.

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