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CONTROL OF GROWTH BY INTRACELLULAR POTASSIUM AND SODIUM CONCENTRATIONS IS RELAXED IN TRANSFORMED 3T3 CELLS

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SUMMARY. A decrease in intracellular K^+ concentration from 160-165 mM to about 120 mM, produced by the addition of ouabain to cultures of proliferating mouse 3T3 cells, increases the doubling time of the cell population from 1 day to 2 days. When cell K^+ is about 105-110 mM, and cell Na^+ is correspondingly increased from normally low levels to about 70 mM, cell proliferation ceases. Transformed 3T3 cells (SV3T3), in contrast, grow rapidly even when cell K^+ is as low as 50 mM, and cell Na^+ as high as 130 mM. This difference between the effect of the cell K^+/Na^+ ratio on growth of 3T3 and SV3T3 cells suggests that if these cations, or the (Na^+,K^+)ATPase pump which regulates their concentrations, are involved in normal growth control, then in SV3T3 cells, this control is relaxed.

Although it has long been known that loss of intracellular K^+ inhibits macromolecular synthesis and cell growth in a variety of cells (1,2,3,4), there has been doubt about the quantitative aspects of this connection. In animal cells, K^+ and Na^+ levels are regulated by the electrogenic (Na^+,K^+) ATPase pump, and perhaps also by an electrically silent, furosemide-sensitive, cotransport system (5,6). Inhibition of the pump produces opposite changes in cell K^+ and Na^+ concentrations; therefore the effects of decreased cell K^+, or of increased cell Na^+, can be equally attributed to a decrease in the ratio of K^+/Na^+. Hence no distinction is attempted here between the effects of changes in cell K^+ concentrations and in the K^+/Na^+ ratio.

It is reasonably certain that K^+ concentrations of cells in culture are not significantly altered by removing growth factors -- e.g., serum--from the culture medium (7). However, the lack of effect of growth factors on cell

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K^+ does not imply the converse--i.e., that cell K^+ can have no effect on the expression of growth factor activity. On the contrary, limitation of K^+ in the medium (and presumably in the cell) clearly inhibits the expression of growth factor activity (8). The details of this effect of cell K^+ on growth is the subject of this report. Two questions are to be considered: (a) how much of a decrease in the K^+/Na^+ ratio is needed to inhibit cell growth; and (b) is the sensitivity of growth in normal cells to decreasing cell K^+/Na^+ different than that in transformed cells.

MATERIALS AND METHODS

Cells and cell cultures: Mouse Balb/3T3 cells (American Type Culture Collection clone A-31) and SV3T3 cells (ATCC SV-T2, derived from clone A-31) were cultured in 30 cm^2 Falcon plastic tissue culture dishes in Dulbecco's modified Eagle medium with 20% fetal calf serum and antibiotics (penicillin, streptomycin, and gentamicin) in humidified 10% CO_2-air at 37°C. The use of high serum levels resulted in exponential growth of 3T3 cells one day longer than in 10% serum, and thus the experiments were deliberately designed so that neither serum factors nor an increase in cell density would limit growth.

All serum was dialyzed against two changes of 0.9% NaCl, using Spectra/Por tubing (Spectrum Medical Industries, Los Angeles, CA) of 10-12,000 molecular weight cutoff in early experiments, and 2,000 cutoff subsequently. 3T3 cultures were routinely inspected and transferred when no more than one-third confluent. New stocks were prepared from liquid N_2 every 4 to 6 weeks.

Measurement of growth rates: Cells were removed from culture dishes with 0.05% trypsin-0.02% EDTA and, after suitable dilution with isotonic buffer, counted in a Coulter Counter (Model ZBl). Selected measurements of size distribution were made with a Coulter Channelyzer.

Cation content: Cultures were rinsed rapidly with ice-cold unbuffered $MgCl_2$ (0.1M) six times (9), left to dry, covered with 2 ml of lithium nitrate diluent, and frozen and thawed twice. A portion was used for analysis of K^+ and Na^+ on a model 343 flame photometer (Instrumentation Laboratories, Waltham, MA). Plastic ware was used for storage of lithium diluent and for transfer of samples to plastic cups for analysis. The lot of $MgCl_2$ (Fisher Scientific Co.) was selected and tested for suitably low Na^+ content. Blank values for Na^+ due to contamination of $MgCl_2$ or plastic ware were negligible. Standard solutions of K^+ and Na^+ were supplied by Instrumentation Laboratories.

Cell water space: The method of 3-O-[methyl-^{14}C]-glucose distribution was used with minor modifications (10). Selected cultures were quickly rinsed once with warm medium identical to that used for growth, except that glucose was absent (KC Biologicals). 3-O-[methyl-^{14}C]-D- glucose (New England Nuclear or Amersham) and unlabeled 3-O-methyl-D-glucose (Sigma) at a final concentration of 0.5 mM were added, incubation continued for 5 minutes, and the cells quickly rinsed with $MgCl_2$ as described above. The addition of phloretin made no difference in the result, provided that the $MgCl_2$ solution was kept

ice-cold; hence phloretin was omitted. 3-O-[methyl-^{3}H]-D-glucose was used in initial experiments, but proved unsuitable because of the presence of radiolabeled impurities that were concentrated by the cell. The results of 3-O-[methyl-^{14}C]-D-glucose distribution were checked against parallel cultures incubated with [^{14}C]urea instead (11). These latter cultures were subjected in the cold room to six very rapid dips in large beakers of ice-cold $MgCl_2$; the results of both methods were identical. Cell contents of K^+ or Na^+ were converted to cation concentrations, without correction for activity coefficient Measurements of the rate of loss of $^{86}Rb^+$ from cells, and of the constancy of the measured K^+ and Na^+ values, as a function of the number of washes with $MgCl_2$, showed that 5 to 6 washes gave nearly the same results both for water space and cation content. If ice-cold PBS was used for rinsing, instead of $MgCl_2$, the measurements of water space were also unchanged. Radioactive samples of culture medium and cell extracts were counted in a Beckman LS-335 scintillation counter; errors due to quenching were negligible.

RESULTS AND DISCUSSION

Growth rates in the steady state: 3T3 cells were seeded in Dulbecco's medium with 20% fetal calf serum at low density. Cell growth was not apparent for 24 to 36 hours later; therefore ouabain (Sigma) was added, in a range of concentrations, usually not until the cells had been in culture for two full days. The results of only selected concentrations are shown in the Figures. Cell counts taken one and two days after ouabain addition showed that decreased steady state growth rates could be obtained, although single experiments did not always display the linearity shown for the averaged results of Figure 1. At one and two days, samples were processed for analysis of cell K^+ and Na^+ concentrations.

The results of three experiments with 3T3 cells were pooled and are given in Fig. 1. The corresponding data for the K^+/Na^+ ratio, in Fig. 3, shows that in untreated control cells, this ratio was about 10, and the doubling time about one day. After 0.07 mM ouabain, when the ratio of K^+/Na^+ had dropped to about two at 24 hours, and 2.7 at 48 hours, cell proliferation was partially inhibited with an increase in the doubling time to about 2 days. Total cation concentration of K^+ and Na^+ was about 170 to 180 mM.

Figs. 1 and 3 also show that 24 hours after 0.01 mM ouabain the cell K^+/Na^+ ratio in 3T3 cells dropped to about 1.5 (corresponding to K^+ and Na^+ concentrations of 108 and 72 mM). At these levels, growth effectively

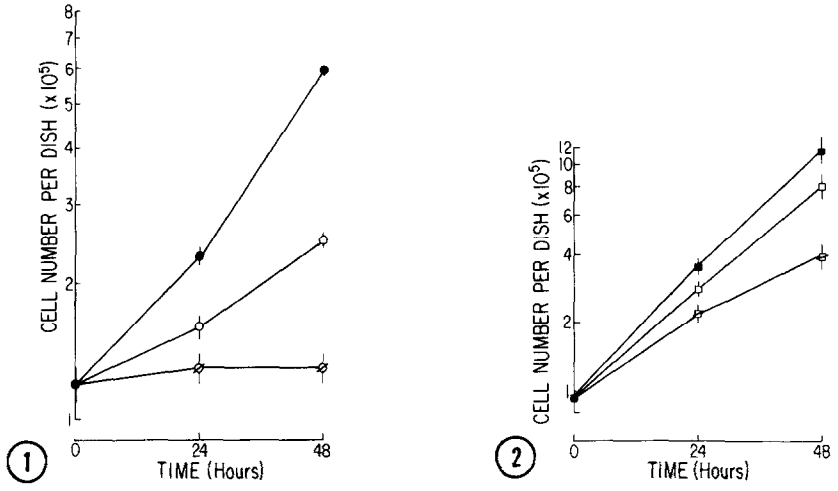

Fig. 1. Dependence of growth rate of 3T3 cells on ouabain concentration. 3T3 cells were seeded at an initial density of about 0.7 x 10^5/30 cm^2 dish and, after two days, cell counts were made on sample dishes. Other dishes received ouabain at a final concentration of 0 (●), 0.07 (○), or 0.1 mM (⌀), and cell counts were made on the two subsequent days. In this Figure and in Figs. 2 and 3, results were averaged for three experiments, in each of which samples were taken in duplicate; the error bars indicate ± s.e. of the mean.

Fig. 2. Dependence of growth rate of SV3T3 cells on ouabain concentration. SV3T3 cells were seeded at an initial density of about 0.7 x 10^5/30 cm^2 dish and, after one day, cell counts were made on sample dishes. Other dishes received ouabain at a final concentration of 0 (■), 0.1 (□), or 0.2 mM (⌀), and cell counts were made on the two subsequent days.

ceased. The ratio of 1.9 at 48 hours is sufficiently high so that one might expect cells to grow; their failure to do so may be explained either by realizing that the observations were inexact, since the standard error of the mean is substantial, or by assuming that if measurements of cell number had been made a day or two later, some recovery of growth rate might have been seen. The important point is that even when cell K^+ levels are only modestly reduced, inhibition of cell proliferation can be clearly seen.

For ouabain-treated cells, the total cation concentration was about the same as that in untreated cells, but the measurements were not sufficiently exact to put probable limits on this statement. Preliminary data suggest a 10 to 20 % increase in total K^+ and Na^+ in ouabain-treated cells. Loss of cell K^+ was always accompanied by an increase in cell Na^+. Selected

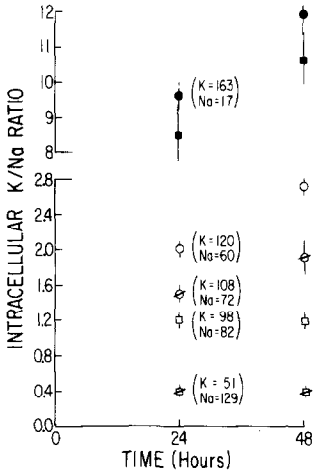

Fig. 3. Intracellular K^+/Na^+ ratios at 24 and 48 hours after addition of ouabain to growing cultures. The sampling of cultures of 3T3 and SV3T3 cells was made from the same experiments shown in Figs. 1 and 2: 3T3 cells after addition of ouabain at concentrations of 0 (●), 0.07 (○), or 0.1 mM (Ø); SV3T3 cells after addition of ouabain at concentrations of 0 (■), 0.1 (□), or 0.2 mM (Ø).

measurements with a Coulter Channelyzer showed a small decrease in cell volume after partial inhibition with ouabain -- less than 10 percent -- over the course of the experiment; in untreated cells, cell volume decreased slightly with increasing cell density (data not shown).

For translation of the K^+/Na^+ ratios shown in Fig. 3 into approximate intracellular concentrations, the assumption has been made that K^+ and Na^+ add up to 180 mM throughout. This is probably correct within 10 or 15%. Fig. 1 shows that a ratio of K^+/Na^+ of between two and three, although considerably less than that of ten in the controls, nevertheless represents a substantial concentration of cell K^+; a ratio of two corresponds to cell K^+ of 120 mM, well within the plausible range of physiological concentrations in vivo. It is of interest that at least for one cell type, mouse vaginal epithelial cells, the reported ratio of intracellular K^+/Na^+, as measured by electron microprobe analysis, is less than one, rising above this within a day after stimulation by estradiol (12). For quiescent lymphocytes, however, the cell K^+/Na^+ ratio is already high, and does not change significantly

after stimulation (13). It remains to be seen whether other cell types will be found to have relatively low K^+/Na^+ ratios and whether such a decreased ratio exerts some control in these cells on the expression of growth factors.

In contrast, control over growth rate by the K^+/Na^+ ratio in transformed cells (SV3T3) is considerably relaxed. In the more extreme example shown(Fig. 2), after inhibition of SV3T3 cells by 0.2 mM ouabain, the rate of cell multiplication was fully 50% of that of untreated control cells. Yet the K^+/Na^+ ratio was as low as 0.4, corresponding to a cell K^+ of about 50 mM and an Na^+ of about 130 mM. Increased concentrations of ouabain, as expected, fully inhibited growth of SV3T3 cells (data not shown).

How can these results be explained? Our earlier work showed that in intact cells and in cell-free extracts -- both for bacteria and mammalian cells -- a sufficient decrease in the K^+/Na^+ ratio inhibited protein synthesis (4,14). In the intact reticulocyte, a decrease in cell K^+ below 50 mM, but in human fibroblasts, a decrease below 110 or 120 mM, was needed before inhibition of protein synthesis became noticeable (4,15). Inhibition was not due to failure of amino acid transport but rather to a direct effect on intracellular polypeptide synthesis (4). Measurement of the rate of decline of protein synthesis in 3T3 cells treated with ouabain, and analyzed for cell K^+ and Na^+ over intervals, showed that the decrease in cell K^+/Na^+ required to inhibit incorporation of [^{3}H]-leucine into polypeptide is too large to account for the present results (data not shown). Hence several possibilities exist: (a) the effect of a decreased K^+/Na^+ ratio on protein synthesis is delayed, and therefore would not be fully observed after brief decreases in K^+/Na^+; (b) small decreases in protein synthesis may be sufficient to inhibit proliferation; support for this possibility comes from observations that such small degrees of inhibition by cycloheximide inhibit the G_0 to S transit (16,17), and that cycloheximide can induce the formation of a G_1 period in mutant cells lacking G_1 (18); (c) protein synthesis may not be the mechanism by which decreased cell K^+/Na^+ inhibits growth; inhibition

may be mediated by an, as yet, unknown mechanism, perhaps involving other intracellular ions, effects on the plasma membrane, or some function of the Na^+/K^+ ATPase pump beyond that of transporting ions (19).

The results reported here show that changes in cell K^+ and Na^+ concentrations have the potential to regulate cell growth in non-tumor cells but that, at least for transformed cells, the regulatory process is insensitive or impaired. We have found results similar to those for 3T3 cells with normal human diploid IMR-90 cells, but have not yet studied transformed human cells (20).

For a number of restrictive conditions that control the growth of 3T3 cells -- e.g., low serum, amino acid restriction, decreased calcium concentrations -- it has been shown that tumor cells escape these controls (21,22, 23). It is not known if some of these restrictive conditions, including decreased cell K^+/Na^+, have a common pathway of action, or at least a common mechanism for escape by tumor cells. The interest in changes in cell K^+/Na^+ as a possible mechanism for control, in contrast to some of the others listed, is that the (Na^+,K^+)ATPase pump is located on the cell surface, and may be subject to regulation both of the activity and the number of pump sites, by mechanisms yet to be uncovered.

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