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# Intracellular $K^+$ and the mitogenic response of 3T3 cells to peptide factors in serum-free medium

(epidermal growth factor/vasopressin/insulin/growth control/DNA synthesis)

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**ABSTRACT** **The stimulation of DNA synthesis in cultures of Swiss 3T3 cells by vasopressin, epidermal growth factor, and insulin added in serum-free medium is strikingly dependent on the intracellular $K^+$ content or concentration. The relationship between these parameters is sigmoid; DNA synthesis commences only when the intracellular $K^+$ increases above a certain threshold level (0.56 μmol/mg of protein; 90 mM). Addition of $K^+$ to $K^+$-depleted cultures reverses the block on DNA synthesis after a lag period of at least 8 hr. The sigmoid dependence of DNA synthesis on intracellular $K^+$ is generated in early $G_1$ phase rather than at the $G_1$/S boundary. The effects of $K^+$ on the $G_1$–S transition are, at least in part, exerted through its control of protein synthesis. In serum-free medium, the $K^+$ content is close to the threshold required for allowing a mitogenic response. The findings suggest that a small change in the intracellular $K^+$ level can influence the ability of these cells to initiate DNA synthesis in serum-free medium.**

Quiescent cultures of 3T3 cells can be stimulated to reinitiate DNA synthesis and cell division by addition of fresh serum or combinations of growth-promoting factors (1). Early changes in monovalent ion fluxes are implicated in the regulation of the mitogenic response of 3T3 cells to growth-promoting factors (1, 2). Addition of chemically diverse mitogenic agents, including serum (3), platelet-derived growth factor (PDGF) (4), fibroblast-derived growth factor (FDGF) (5), vasopressin (6, 7), phorbol esters (8), or melittin (9), rapidly increases $Na^+$ entry into quiescent 3T3 cells. This entry of $Na^+$ or the movement of other cations ($H^+$, $Ca^{2+}$) coupled to $Na^+$ entry, or both, could signal the initiation of cell proliferation (2). Increased $Na^+$ entry into mitogen-treated cells rapidly stimulates the activity of the $Na^+/K^+$ pump (10) and results in enhanced cellular levels of $K^+$ (10, 11). We have found that other sets of mitogenic agents increase the cellular level of cAMP (12) and stimulate $Na^+/K^+$ pump activity by a mechanism not involving a primary increase in $Na^+$ flux (13). Because mitogens that either stimulate the $Na^+$ influx across the membrane or elevate the intracellular level of cAMP enhance the activity of the $Na^+/K^+$ pump, uphill uptake of $K^+$ may be a point of necessary convergence in the action of diverse mitogenic agents.

These considerations prompted us to evaluate the precise relationship between the initiation of DNA synthesis and the level of cellular $K^+$ in 3T3 cells stimulated by insulin, epidermal growth factor (EGF), and vasopressin in serum-free medium. We found that the stimulation of DNA synthesis by these factors is steeply dependent on the intracellular $K^+$ content or $K^+$ concentration in a sigmoid manner. This sigmoid dependence is generated in early $G_1$ phase rather than at the $G_1$/S boundary. In serum-free medium, the cellular $K^+$ content is close to the threshold required to allow a mitogenic response; thus, fluctuations in this ion may play a role in regulating the mitogenic response of Swiss 3T3 cells to certain growth-promoting polypeptides.

## MATERIALS AND METHODS

**Cell Culture.** Stock cultures of Swiss 3T3 cells (14) were maintained in Dulbecco's modified Eagle's medium (DME medium) containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in humidified 10% $CO_2$/90% air at 37°C. Cells ($10^5$ per dish) were subcultured into 30-mm Nunc Petri dishes with medium containing 10% fetal bovine serum and grown to confluence in this medium. These cells were arrested in the $G_1/G_0$ phase of the cell cycle as judged by cytofluorometric analysis and by the fact that <1% of the cells were autoradiographically labeled after a 40-hr exposure to [$^3$H]thymidine.

**Intracellular Electrolyte Content.** Intracellular $K^+$ content was measured as described (3, 7) except that 5% trichloroacetic acid instead of toluene was used in the extraction solution. The cultures were washed rapidly six times with 0.1 M $MgCl_2$ at 4°C, and the dishes were allowed to dry. Then 0.4–0.7 ml of cold 15 mM LiCl/5% trichloroacetic acid was added to each dish. Cell $K^+$ was measured in a flame photometer (Corning EEL, model 430) with the LiCl serving as the internal standard.

**DNA Synthesis.** All determinations of DNA synthesis were performed in DME medium/Waymouth's medium (15), 1:1 (vol/vol). The cultures were washed twice with DME medium to remove residual serum immediately prior to assay. For determinations of DNA synthesis, the medium (2 ml) contained either 1.25 μM (0.25 μCi/ml; 1 Ci = $3.7 \times 10^{10}$ becquerels) or 0.2 μM (5 μCi/ml) [$^3$H]thymidine for measurement of acid-precipitable material or autoradiography, respectively (10).

**Adjustment of Intracellular $K^+$.** In the experiments comparing the effects of reducing intracellular $K^+$ early and late in $G_1$ phase, a procedure of "efflux and replacement" was used. The cells were washed three times with $K^+$-free DME medium and then fed with $K^+$-free 1:1 DME medium/Waymouth's medium containing insulin (10 μg/ml), EGF (5 ng/ml), and vasopressin (20 ng/ml). The efflux of $K^+$ was followed over a 3-hr period; at various times of efflux, the medium in different sets of dishes was replaced with similar medium containing a replacement level of $K^+$ found in parallel experiments to maintain the intracellular $K^+$ at approximately the efflux level.

**Protein Synthesis.** Protein synthesis was measured by the incorporation of [$^3$H]leucine (10 μCi/ml) into acid-precipitable

Abbreviations: EGF, epidermal growth factor; DME medium, Dulbecco's modified Eagle's medium.

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material. After pulse-labeling (1–2 hr), the medium was aspirated, and the cells were washed three times with phosphate-buffered saline and treated with 1 ml of ice-cold 5% trichloroacetic acid. The cells were kept in the cold for 20 min, washed once with cold 5% trichloroacetic acid and twice with ethanol, and then dissolved in 0.1 M NaOH/2% $Na_2CO_3$/0.1% $NaDodSO_4$ for measurement of radioactivity. Changes in the $K^+$ concentration of the medium do not alter the uptake of leucine into trichloroacetic acid-soluble pools.

**Intracellular Water Space.** Cell water was determined as described (16). The cultures were washed five times with glucose-free DME medium at 37°C and incubated for 10 min in 1 ml of the same medium. After this incubation, the medium was removed, and the cells were incubated with 1 ml of glucose-free DME medium containing 3-*O*-[$^{14}$C]methyl-D-glucose (0.1 mM, 1 $\mu$Ci/ml). After 30 min at 37°C, the medium was aspirated, and the cultures were washed five times with cold phosphate-buffered saline containing 0.5 mM phloretin. The radioactive material was extracted with 5% cold trichloroacetic acid, and samples were taken for protein determination (17).

**Materials.** [Arg$^8$]Vasopressin (390 units/mg) and bovine insulin (25.5 units/mg) were purchased from Sigma. Mouse EGF was from Collaborative Research, Waltham, MA. [$^3$H]Thymidine (20 Ci/mmol), [$^3$H]leucine (130 Ci/mmol), and 3-*O*-[$^{14}$C]methyl-D-glucose (329 mCi/mmol) were from the Radiochemical Centre. The serum used was fetal bovine (Flow Laboratories, Rockville, MD). All other material used was at reagent grade.

## RESULTS

**Dependence of DNA Synthesis Stimulation on Extracellular $K^+$.** To determine the influence of $K^+$ on the ability of 3T3 cells to initiate DNA synthesis, confluent and quiescent cultures of these cells were washed and transferred to medium containing vasopressin, EGF, insulin, [$^3$H]thymidine, and various concentrations of $K^+$. The cumulative incorporation of [$^3$H]thymidine into acid-insoluble material was measured after 20, 40, or 60 hr of incubation. The stimulation of DNA synthesis by the polypeptide growth factors markedly depended on the concentration of $K^+$ in the medium (Fig. 1). Half-maximal stimulations

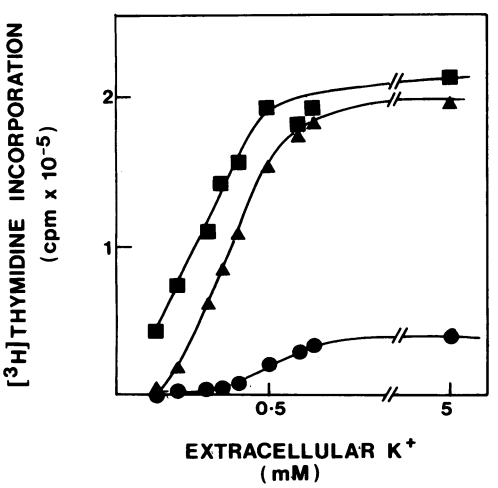


FIG. 1. Stimulation of DNA synthesis by pure growth factors in the presence of different concentrations of $K^+$ in the culture medium. Quiescent cultures of 3T3 cells were washed two times with $K^+$-free medium and then incubated in the presence of EGF (5 ng/ml), vasopressin (20 ng/ml), insulin (10 $\mu$g/ml), and different concentrations of KCl in the medium for 20 (●), 40 (▲), or 60 (■) hr. DNA synthesis was measured as the incorporation of [$^3$H]thymidine into acid-insoluble material.

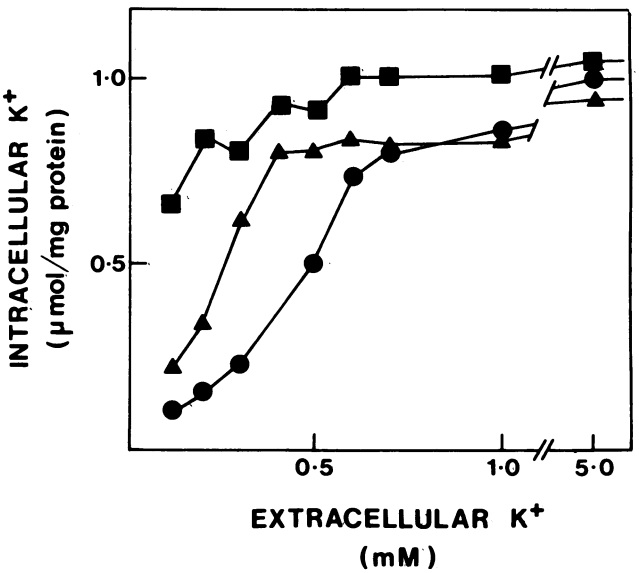


FIG. 2. Intracellular $K^+$ of 3T3 cells as a function of $K^+$ concentration in the medium, in the presence of pure growth factors. Quiescent cultures of 3T3 cells were washed twice with $K^+$-free DME medium and were incubated in 2 ml of DME medium/Waymouth's medium containing EGF (5 ng/ml), vasopressin (20 ng/ml), insulin (10 $\mu$g/ml), and different concentrations of KCl for 20 (●), 40 (▲), or 60 (■) hr at 37°C. At these times incubation was terminated and intracellular $K^+$ was measured. Protein content was measured in the same dish by the Lowry procedure (17) after the precipitated material was washed once with 5% cold trichloroacetic acid and twice with ethanol and the protein was dissolved in 0.1 M NaOH/2% $Na_2CO_3$/0.1% $NaDodSO_4$.

of DNA synthesis were achieved at 0.5, 0.36, and 0.27 mM $K^+$ when measured at 20, 40, and 60 hr of incubation, respectively.

**Time-Dependent Adaptation of 3T3 Cells to a Low-$K^+$ Medium.** The above findings suggest that 3T3 cells increase their ability to initiate DNA synthesis in a low-$K^+$ medium in a time-dependent fashion. It is known that prolonged exposure of rapidly growing HeLa cells to a medium deficient in $K^+$ increases the level of the $Na^+/K^+$ pump, and this reverses the changes in monovalent ion composition (18). A similar compensatory effect could account for the adaptation of 3T3 cells to reduced concentrations of $K^+$ in the medium. 3T3 cells are able to adapt to a low-$K^+$ medium and reaccumulate this ion after 40 or 60 hr of incubation as compared with the values seen in the cells incubated for 20 hr (Fig. 2). Time courses carried out with cultures transferred to medium containing 0.3 mM $K^+$ show that the intracellular $K^+$ reached its lowest level after 10 hr, stayed at that level for the subsequent 20 hr, and recovered after 40 hr of incubation (results not shown).

**Relationship Between DNA Synthesis Stimulation and Intracellular Level of $K^+$.** To evaluate the effect of intracellular $K^+$ on the ability of the cells to initiate DNA synthesis, cultures of 3T3 cells were exposed to vasopressin, EGF, insulin, and various concentrations of $K^+$ in the medium. In view of the adaptation phenomenon described above (Fig. 2), we measured both intracellular $K^+$ content and cumulative [$^3$H]thymidine incorporation after a 40-hr incubation. The striking feature of the combined results of 11 experiments (Fig. 3) is the steep dependence of DNA synthesis on intracellular $K^+$. Similar results were obtained when the cultures were incubated for 60 hr instead of 40 hr. These findings suggest that an increase in the intracellular $K^+$ above a certain threshold is necessary for the stimulation of DNA synthesis by the growth-promoting factors.

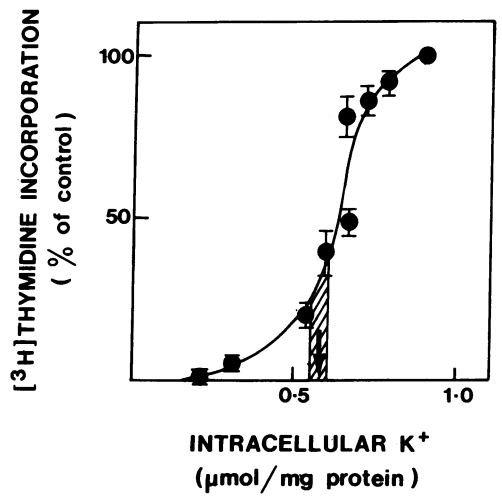


FIG. 3. DNA synthesis as a function of the internal concentration of $K^+$. The growth factors were added to confluent and quiescent cultures of 3T3 cells in DME medium/Waymouth's medium containing different concentrations of KCl. Intracellular $K^+$ was measured in a flame photometer at 40 hr. At the same time, DNA synthesis was assessed by incorporation of [$^3$H]thymidine into acid-insoluble material after continuous exposure to the tracer. Each point is the mean ± SEM of 11 experiments. The arrow and the hatched area represent the mean ± SEM (nine experiments) of the intracellular $K^+$ in cultures incubated in 5 mM $K^+$ medium for 40 hr in the absence of growth factors.

When the cultures of quiescent 3T3 cells were washed and transferred to serum-free medium containing the normal concentration of $K^+$ (5.2 mM) but without the growth factors (Fig. 3, arrow) or to medium supplemented with 5% plasma (results not shown), the $K^+$ content (μmol/mg of protein) decreases from 0.68 ± 0.03 ($n$ = 8) to 0.58 ± 0.03 ($n$ = 9), which falls in the ascending part of the curve relating DNA synthesis and intracellular $K^+$. These findings indicate that the increase in $K^+$ content induced by the growth-promoting factors (from 0.58 ± 0.03 to 0.88 ± 0.06; $P < 0.001$) is required for an optimal rate of initiation of DNA synthesis in serum-free medium.

The strikingly steep dependence of DNA synthesis on the cellular $K^+$ content was observed also when the incorporation of [$^3$H]thymidine was assessed by autoradiography of labeled nuclei instead of by total incorporation (Fig. 4*A*). These findings indicate that the level of intracellular $K^+$ primarily affects the ability of the cells to enter DNA synthesis rather than the uptake of [$^3$H]thymidine or the rate of DNA chain elongation.

In other experiments we measured cell water and expressed our results in terms of intracellular $K^+$ concentration instead of as a function of cell protein. We also found a strikingly steep dependence of DNA synthesis on cellular $K^+$ in the 90–130 mM range (Fig. 4*B*).

To determine the point in $G_1$ at which cells are blocked by reducing intracellular $K^+$, 3T3 cells were incubated with vasopressin, EGF, and insulin in medium containing 0.3 mM $K^+$ and [$^3$H]thymidine for 28 hr. Less than 6% of the cells entered S phase under these conditions. The $K^+$ concentration was then raised to the normal level of 5.0 mM, and incubation with [$^3$H]thymidine was continued. The intracellular level of $K^+$ rose rapidly (60 min), whereas DNA synthesis increased only after a lag period that exceeded 8 hr (data not shown). Because control cells began DNA synthesis 12–13 hr after stimulation, we conclude that cells exposed to reduced levels of $K^+$ are blocked early in $G_1$.

**Relationship Between DNA Synthesis Stimulation and the Level of Intracellular $K^+$ in Late $G_1$.** When quiescent 3T3 cells were stimulated by vasopressin, EGF, and insulin for 24 hr in the presence of 1 mM hydroxyurea, they accumulated at a point near the $G_1$/S boundary; when the hydroxyurea was removed, the cells began DNA synthesis after a lag period of about 60 min (results not shown). Does the steep dependence of S-entry on intracellular $K^+$ persist in cells that have progressed into late $G_1$? To answer this question, cells were stimulated with the polypeptide growth factors in the presence of 5 mM $K^+$ and 1 mM hydroxyurea for 24 hr, and the intracellular $K^+$ was ad-

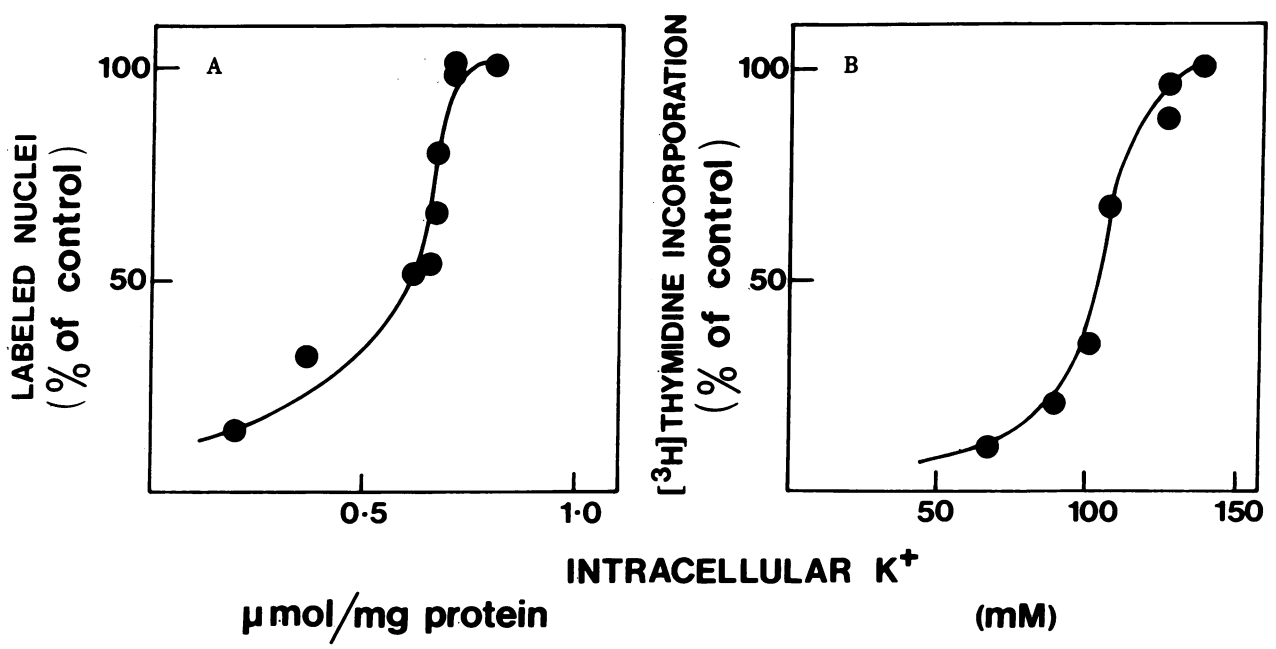


FIG. 4. (*A*) Autoradiographic labeling of nuclei of 3T3 cells stimulated by EGF, vasopressin, and insulin as a function of the intracellular content of $K^+$. Cultures were exposed to mitogens and [$^3$H]thymidine in the presence of different concentrations of $K^+$ in the medium, for 40 hr. The incorporation of [$^3$H]thymidine into DNA was assessed by autoradiography. There was 57% of the labeled nuclei in the cultures stimulated in 5 mM $K^+$. (*B*) DNA synthesis as a function of the internal concentration of $K^+$. Intracellular $K^+$ and DNA synthesis were determined as described in Fig. 3. Intracellular water was measured in separate dishes.

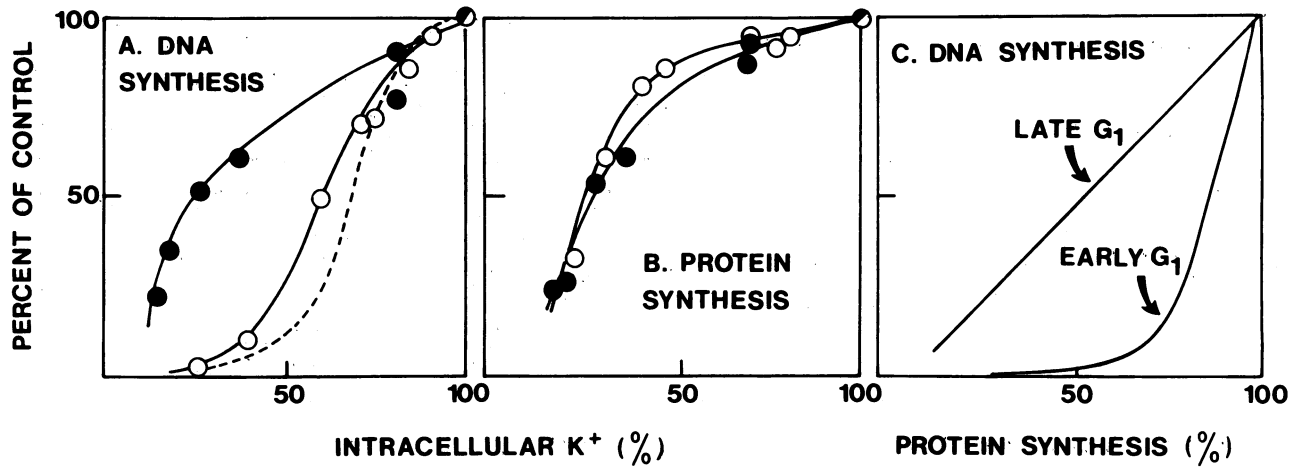


FIG. 5. DNA synthesis (*A*) and protein synthesis (*B*) as a function of intracellular $K^+$. Quiescent cultures of 3T3 cells were stimulated with insulin, EGF, and vasopressin in the presence of 1 mM hydroxyurea. Intracellular $K^+$ was adjusted in different sets of dishes to a series of different levels, either at time zero ("early K shift") (○) or at 24 hr ("late K shift") (●), by using the efflux-plus-replacement procedure. At 27 hr, the hydroxyurea was removed by three washes with DME medium containing the replacement level of $K^+$, and the cells were fed with fresh medium containing the replacement level of $K^+$ plus either [$^3$H]thymidine or [$^3$H]leucine. Control cultures contained 5.0 mM $K^+$ at all times. Six dishes in each set were given [$^3$H]thymidine, and duplicate dishes were removed at 4, 6, and 8 hr after hydroxyurea removal for measurement of [$^3$H]thymidine incorporation ("DNA synthesis"). Other dishes in each set were used for pulses of [$^3$H]leucine ("protein synthesis"). Intracellular $K^+$ and total protein were determined at the end of the efflux period, at the end of each [$^3$H]leucine pulse, and at the time of each [$^3$H]thymidine measurement. For each level of extracellular $K^+$, an average value for intracellular $K^+$ over the entire incubation period was determined by dividing the time-course into appropriate segments and calculating a weighted average of the mean values for each segment. (*A*) Rate of [$^3$H]thymidine incorporation (as a percentage of the control rate) versus the average intracellular $K^+$ (as a percentage of the control value). The rates were determined by measuring the slopes of the curves between 4 and 8 hr, when the data were plotted as cpm per mg of protein against time; the curves were linear during this period. ---, Tracing of the curve in Fig. 3, where intracellular $K^+$ was adjusted more slowly in the absence of hydroxyurea, starting at time zero, and was measured only at 40 hr. (*B*) Rate of protein synthesis (as a percentage of the control rate) versus average intracellular $K^+$ (as a percentage of the control value). The rate of protein synthesis was taken as the cpm per mg of protein at the end of a 1- or 2-hr pulse of [$^3$H]leucine, added 1 hr after adjustment of the internal $K^+$. (*C*) DNA synthesis as a function of protein synthesis. The curves were drawn by taking data from *A* and *B*.

justed to various concentrations by the efflux-and-replacement procedure; the hydroxyurea was then removed by washing, and the intracellular $K^+$, protein content, and incorporation of [$^3$H]thymidine were monitored over an 8-hr period. For comparison, the intracellular $K^+$ levels of parallel cultures were adjusted (by efflux and replacement) at the start of $G_1$, rather than 24 hr later. The results (Fig. 5*A*) indicate that cells exposed to low levels of $K^+$ in early $G_1$ show the same steep dependence of S-entry on intracellular $K^+$ as was seen in the earlier experiments done without hydroxyurea. (The dotted line is a tracing of the curve in Fig. 3). In contrast, when cells were allowed to complete $G_1$ and then were subjected to a reduction in intracellular $K^+$, the dependence curve was markedly shifted to the left (i.e., S-entry became less sensitive to a reduction in intracellular $K^+$). Thus, the sigmoidicity of the response to intracellular $K^+$ disappears in late $G_1$.

**Relationship Between Protein Synthesis and the Level of Intracellular $K^+$ in Early and Late $G_1$.** Protein synthesis, particularly polypeptide chain elongation, is dependent on intracellular $K^+$ (19, 20). Because entry into S phase in 3T3 cells depends on the accumulation of one or more specific proteins (21–23), we asked whether the effect of reduced $K^+$ on the $G_1$–S

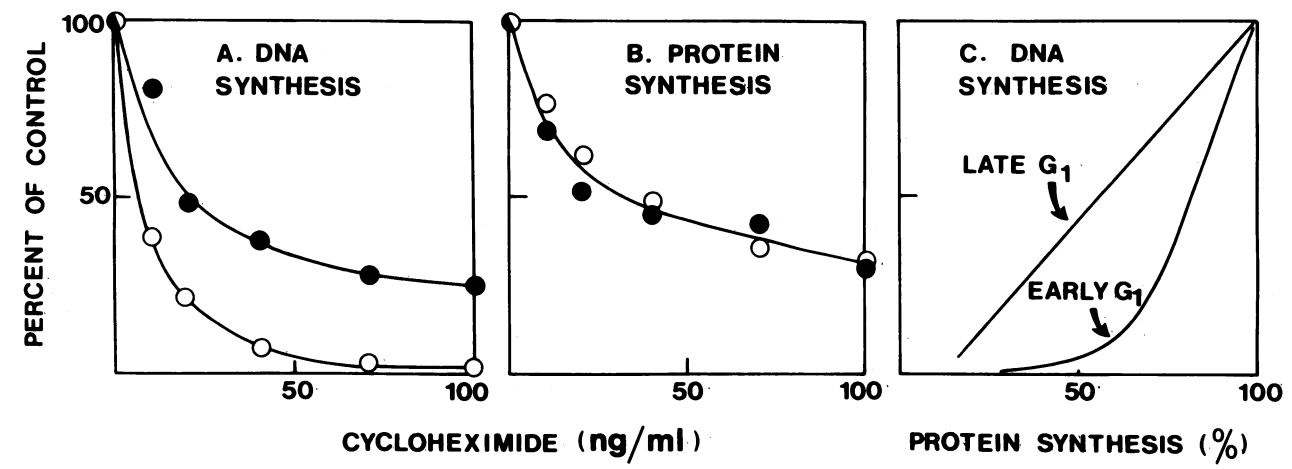


FIG. 6. Effect of cycloheximide on the rates of DNA (*A*) and protein synthesis (*B*). Quiescent cells were stimulated at time zero with insulin, EGF, and vasopressin in the presence of 1 mM hydroxyurea and 5 mM $K^+$. The hydroxyurea was removed by washing at 24 hr. Cycloheximide was added at time zero, and protein synthesis was taken as the average of three 1-hr pulses of [$^3$H]leucine, starting at 1, 7, and 24.5 hr (○); or cycloheximide was added at 24 hr, and protein synthesis was determined by a single 1-hr pulse starting at 24.5 hr (●). (*C*) DNA synthesis as a function of cycloheximide-limited protein synthesis. The data are taken from *A* and *B*.

transition could be accounted for entirely by its effect on protein synthesis. If so, doses of $K^+$ and of cycloheximide giving identical reductions in protein synthesis should reduce DNA synthesis to an equivalent extent. To test this possibility, the intracellular $K^+$ levels were adjusted (by efflux and replacement) in either early $G_1$ or late $G_1$, and the rate of protein synthesis was determined as the incorporation of [$^3$H]leucine into acid-precipitable material (Fig. 5*B*). In addition, we also determined the effect of limiting concentrations of cycloheximide on both protein synthesis and DNA synthesis (Fig. 6 *A* and *B*). Curves relating DNA synthesis to protein synthesis, when the latter was limited by either $K^+$ or cycloheximide, are seen in Figs. 5*C* and 6*C*, respectively. These curves show that DNA synthesis (S-entry) is less affected by an equivalent reduction in protein synthesis in late $G_1$ than in early $G_1$ regardless of whether protein synthesis is inhibited by lowering cellular $K^+$ or by adding cycloheximide. Indeed, Figs. 5*C* and 6*C* indicate that a 50% inhibition of DNA synthesis requires a 50% reduction in protein synthesis in late $G_1$ but only a 10–20% inhibition in early $G_1$. The similarity between Figs. 5*C* and 6*C* argues that, at least in part, the effects of $K^+$ on the $G_1$–S transition are exerted through its control of protein synthesis.

## DISCUSSION

Ion fluxes and redistributions may, it has been suggested, play a role in regulating the proliferative response of quiescent cells to growth-promoting factors (1, 2). Entry of $Na^+$ ions is one of the earliest responses known to mitogenic agents (3–9), and the movement of other ions such as $H^+$ and $Ca^{2+}$ coupled to $Na^+$ entry could signal the initiation of proliferation (2). Increased $Na^+$ influx stimulates $Na^+/K^+$ pump activity and increases the $K^+$ in the cell (10, 11). The findings presented in this paper demonstrate that the initiation of DNA synthesis in cultures of 3T3 cells stimulated by vasopressin, EGF, and insulin is strikingly dependent on cellular $K^+$ content or concentration. The relationship between these parameters is sigmoid and, therefore, DNA synthesis is stimulated only when the intracellular $K^+$ has been increased above a certain threshold level. The threshold intracellular $K^+$ concentration for initiation of DNA synthesis was about 90 mM; maximal DNA synthesis occurs at 130 mM. We found that in serum-free medium, the intracellular $K^+$ of 3T3 cells falls to a level close to the threshold (see Fig. 3); thus, an increase in the cellular concentration of this cation may play a role in regulating the proliferative response of quiescent fibroblasts to polypeptide growth factors. Because $K^+$ influx through the $Na^+/K^+$ pump is obligatorily linked to $Na^+$ extrusion (24), the loss of $K^+$ from cells exposed to a low-$K^+$ medium is compensated by an increase in cell $Na^+$. Thus, although we interpreted our findings in terms of fluctuation in intracellular $K^+$, an influence of cellular $Na^+$ on the effects described here remains formally open.

Recently, Frantz *et al*, (25) reported that an increase in intracellular $K^+$ is not required for stimulation of DNA synthesis by a combination of serum and platelet-derived growth factor in 3T3 cells. These apparently contrasting findings can be understood on the basis of our recent observation indicating that the sensitivity of 3T3 cells to internal $K^+$ is modulated by the growth factors; 3T3 cells stimulated by a combination of serum and platelet-derived growth factor are much less sensitive to a reduction in cellular $K^+$ than cultures stimulated by the polypeptides in serum-free medium (unpublished data). This suggestion is consistent with the recent observation that the growth of untransformed 3T3 cells is more inhibited by a reduction in cellular $K^+$ than that of simian virus 40-transformed cells (26), which are known to produce potent growth-promoting factors (5, 27).

The sigmoid dependence of DNA synthesis on intracellular $K^+$ concentration is generated in early $G_1$ rather than at the $G_1$/S boundary, as shown by experiments in which the intracellular $K^+$ was adjusted at various levels either at the time when the mitogens were added (early $G_1$) or after accumulation of the cell population at the $G_1$/S boundary by hydroxyurea. These results clearly indicate that $K^+$ ions control a process prior to the initiation of DNA synthesis.

There is evidence indicating that the accumulation of one or various labile proteins in $G_1$ is necessary for accomplishing the initiation of DNA synthesis (21–23), and it is known that $K^+$ is required for protein synthesis in many cell types (19, 20, 28), including 3T3 cells. Furthermore, as shown with $K^+$, inhibition of polypeptide chain elongation by cycloheximide prevents initiation of DNA synthesis more potently when added in early $G_1$ than when added in late $G_1$ (Fig. 5; refs. 21–23). Therefore, it is plausible that the accumulation of $K^+$ ions promoted by vasopressin, insulin, and EGF in serum-free medium in early $G_1$ is required to sustain protein synthesis at a rate that is required for an optimal rate of entry into S phase.


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