Intracellular Univalent Cations and the Regulation of the BALB/c-3T3 Cell Cycle

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ABSTRACT Addition of serum to density-arrested BALB/c-3T3 cells causes a rapid increase in uptake of Na^+ and K^+, followed 12 h later by the onset of DNA synthesis. We explored the role of intracellular univalent cation concentrations in the regulation of BALB/c-3T3 cell growth by serum growth factors. As cells grew to confluence, intracellular Na^+ and K^+ concentrations ($\{Na^+\}_i$ and $\{K^+\}_i$) fell from 40 and 180 to 15 and 90 mmol/liter, respectively. Stimulation of growth of density-inhibited cells by the addition of serum growth factors increased $\{Na\}_i$ by 30% and $\{K^+\}_i$ by 13–25% in early G_0/G_1, resulting in an increase in total univalent cation concentration. Addition of ouabain to stimulated cells resulted in a concentration-dependent steady decrease in $\{K^+\}_i$ and increase in $\{Na^+\}_i$. Ouabain (100 μM) decreased $\{K^+\}_i$ to ~60 mmol/liter by 12 h, and also prevented the serum-stimulated increase in $^{86}Rb^+$ uptake. However, 100 μM ouabain did not inhibit DNA synthesis. A time-course experiment was done to determine the effect of 100 μM ouabain on $\{K^+\}_i$ throughout G_0/G_1 and S phase. The addition of serum growth factors to density-inhibited cells stimulated equal rates of entry into the S phase in the presence or absence of 100 μM ouabain. However, in the presence of ouabain, there was a decrease in $\{K^+\}_i$. Therefore, an increase in $\{K^+\}_i$ is not required for entry into S phase; serum growth factors do not regulate cell growth by altering $\{K^+\}_i$. The significance of increased total univalent cation concentration is discussed.

Serum rapidly stimulates a pleiotypic program in quiescent 3T3 cells, followed by DNA synthesis at 12 h (5, 7). Perhaps the earliest regulatory event of this program induced by either serum or purified growth factors is the increased flux of the inorganic univalent cations Na^+ and K^+ into cells (12, 16, 20). The increased uptake of Na^+ causes an increased uptake of K^+, mediated by membrane-associated Na^+,K^+-ATPase. The intracellular K^+ concentration ($\{K^+\}_i$) increases by 10–30% (22). Inhibitors of Na^+,K^+-ATPase, such as ouabain, prevent this increase in K^+ uptake and $\{K^+\}_i$ and also inhibit cellular entry into S phase (10, 16, 22). Rozengurt and co-workers (12, 16, 20) have proposed that the increases in Na^+ influx and $\{K^+\}_i$ induced by growth factors play a crucial regulatory role in the cell cycle. According to this model, increased $\{K^+\}_i$ stimulates quiescent cells to pass through G_0/G_1 and synthesize DNA, perhaps by allowing increased protein synthesis. However, Moscatelli et al. (13) have shown that in chick embryo fibroblasts, DNA synthesis is inhibited only by drastic, perhaps unphysiologic, decreases in cellular K^+ content. We have studied the effect of ouabain on serum-stimulated BALB/c-3T3 cells, and now show that a low concentration of this Na^+,K^+-ATPase inhibitor prevents the increase in both K^+ uptake and $\{K^+\}_i$ without affecting DNA synthesis. Thus, the increase in $\{K^+\}_i$ does not control entry into the S phase.

MATERIALS AND METHODS

Cell Culture

BALB/c-3T3 (clone A31) cells were grown at 37°C in 10% CO_2 using the high glucose formation of Dulbecco's modification of Eagle's medium containing 10% bovine serum (Colorado Serum Co., Denver, Colo.) and supplemented with penicillin and streptomycin (Grand Island Biological Co., Grand Island, N. Y.). Stock cultures were split routinely before reaching confluence and were replaced from frozen stocks every 8–10 wk. Cells for experiments were seeded onto round glass coverslips (Rochester Scientific Co., Rochester, N. Y., area 1.1 cm^2) sitting in wells (Costar, Data Packaging, Cambridge, Mass., area 2.0 cm^2) at a cell density of ~2.5×10^4 cells/cm^2. The coverslips were treated overnight with 1.0 M HCl and 1.0 M H_2SO_4. After extensive washing with water, the coverslips were each transferred to 2.0-cm^2 wells before cell seeding. The cultures, which were not replenished with medium, were confluent and density-arrested within 5 d of planting; <3% of cells synthesized DNA in 24 h, and no increase in DNA synthesis was seen until 12 h after the addition of fresh serum. The cultures were used 5–7 d after seeding.

Autoradiographic Determination of Entry into the S Phase

Cultures were continuously incubated with 5 μCi/ml [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) throughout each experiment. Entry into the S phase was determined by autoradiography. Cultures were fixed by treatment with methanol for 10 min (14). Wells were coated with photographic emulsion (NTB2, Eastman Kodak) and allowed to dry for at least 2 d, then developed with Microdol-X (Eastman Kodak) followed by Rapid Fixer with hardener (Eastman Kodak). At least 200 contiguous nuclei from a representative section were counted in each culture; all values given are the average of duplicate cultures.

Measurements of $\{K^+\}_i$

$\{K^+\}_i$ was determined by a modification of the technique used by Tupper and co-workers (21, 22). Coverslip cultures were rapidly rinsed three times in 100 mM $MgCl_2$ at 4°C and transferred to 15 mM $LiCl_2$; no loss of K^+ occurred with further rinsing. The coverslips were then frozen and thawed three times to lyse the cells, and the K^+ content per coverslip was measured by flame photometer. All points were the average of at least three cultures.

For each condition or time point tested, culture volume was determined in parallel cultures. Cultures were incubated in 0.25% trypsin-0.125 mM EDTA for 2 min, and the cells were suspended in Isoton (Coulter Electronics Inc., Hialeah, Fla.) containing 0.5% serum. The total cell number and the mean cell volume (MCV) were determined using an automated counter channelizer (Coulter Electronics Inc.). The channelizer was calibrated by comparing the packed BALB/c-3T3 cell volume obtained after centrifugation to the MCV times the total cell number obtained by the channelizer. Calibration with BALB/c-3T3 cells was identical to that obtained with human erythrocytes. Culture volume was the average of at least three determinations for each point.

In some experiments, cell water was determined as [^{14}C]- or [3-]^{3}H-*O*-methylglucose (3-O-MG) space (8). Cell water was determined to validate measurement of total cell volume by counter channelizer. 3-O-MG enters cells by facilitated diffusion and is not metabolized. At equilibrium, the intracellular concentration of 3-O-MG in cell water is approximately equal to the extracellular 3-O-MG concentration (8). Cultures were incubated at 37°C in glucose-free medium containing 1 mM 3-O-MG and 20 μCi/ml ^{14}C-3-O-MG (20 mCi/mmol) or ^{3}H-3-O-MG (60 Ci/mmol) (New England Nuclear). After 15–20 min, cultures were rinsed rapidly three times in glucose-free 110 mM $MgCl_2$, 1 mM HEPES, pH 7.4, 0.1 mM phloretin. Cells were solubilized in 1.0 M NaOH and radioactivity determined in Biofluor (New England Nuclear) in a scintillation counter. No loss of radioactivity per microgram protein resulted from further rinsing. Intracellular radioactivity reached a stable plateau by 15 min.

The $\{K^+\}_i$ was calculated as the K^+ per liter of cell volume as determined by counter channelizer (21); no correction was made for the percent of cell water.

Net $^{86}Rb^+$ Uptake

Coverslip cultures were transferred to medium containing 10 mM HEPES, pH 7.4, $NaHCO_3$ (4.4 mM), ^{86}RbCl (5 μCi/ml; 1.2 Ci/mmol) (New England Nuclear), 5.3 mM K^+, 1 mM furosemide (American Hoechst Corp., Somerville, N. J.), and serum growth factors and ouabain as in the prior medium. After a 6-min incubation at 37°C, cultures were rinsed three times in 110 mM $MgCl_2$ at 4°C, solubilized in 1.0 M NaOH; radioactivity was determined on a gamma counter. Under these conditions, net $^{86}Rb^+$ uptake was linear for at least 20 min. In either serum-stimulated or density-inhibited BALB/c-3T3 cells, ~30% of $^{86}Rb^+$ uptake is inhibitable by ouabain and ~50% is inhibitable by furosemide. The furosemide-inhibitable $^{86}Rb^+$ uptake may be caused by K^+ exchange across the cell membrane and does not contribute to net K^+ uptake in 3T3 cells (21). 1 mM furosemide does not alter entry into S phase of serum-stimulated BALB/c-3T3 cells, and incubation with 1 mM furosemide for 1 h does not alter $\{K^+\}_i$. Therefore, $^{86}Rb^+$ uptake was determined in the presence of furosemide in order to eliminate a portion of $^{86}Rb^+$ uptake which does not alter $\{K^+\}_i$.

Ouabain

Ouabain (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, Medford, Mass.) before being added to the cultures. In all experiments, the final concentration of DMSO was ≤0.2% (vol/vol); this concentration of DMSO has no effect on cellular entry into the S phase, cell volume, protein synthesis, $\{K^+\}_i$, or cellular replication.

Platelet-derived Growth Factor (PDGF)

Partially purified PDGF was prepared by heating frozen-thawed preparations of clinically outdated human platelets to 100°C for 10 min as previously described (14). The cellular debris was removed by centrifugation at 15,000 *g* for 30 min, dialyzed against 0.15 M NaCl, and passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.); aliquots were stored at −20°C. PDGF is a serum growth factor that is required for BALB/c-3T3 cell growth. Addition of both PDGF and fresh serum to density-inhibited cells insures rapid entry of most cells into S phase (14). PDGF alone stimulates influx of Na^+ and K^+ in quiescent 3T3 cells (12).

RESULTS

Effect of Density Inhibition of Cell Growth on $\{K^+\}_i$

Subconfluent 3T3 cells replicating at different rates in different serum concentrations have a similar $\{K^+\}_i$ of ~160–180 mmol/liter (23). To learn whether density-dependent inhibition of growth is associated with a decrease in $\{K^+\}_i$, we plated BALB/c-3T3 (clone A31) cells sparsely in medium containing 10% (vol/vol) bovine serum, and the total cell number and $\{K^+\}_i$ were determined at intervals. The $\{K^+\}_i$ of the subconfluent exponentially replicating cultures was ~180 mmol/liter. When the cells reached confluence, the $\{K^+\}_i$ decreased to 90 mmol/liter (Fig. 1). Thus, in BALB/c-3T3 cells, density-dependent inhibition of growth is associated with a 50% decrease of $\{K^+\}_i$.

Most of the decrease in $\{K^+\}_i$ resulted from a decrease in K^+ content per cell. Between days 2 and 5, mean cell volume decreased by 37% from 4,300 to 2,870 μm^3; however, the K^+ content per cell decreased 65% from 0.83 to 0.26 pmol/cell. During the same interval, K^+ content per gram protein decreased by <10% because the protein per cell decreased about as much as K^+ content per cell (Table I). Thus, as the BALB/c-3T3 cells grew to confluence, the decrease in cell size was less than the decrease in K^+ content, and the $\{K^+\}_i$ fell from 180 to 100 mmol/liter. Postconfluent cultures between days 5 and 7 increased in mean cell volume to 3,400 μm^3, and appear to have lost only a small amount of K^+ content per gram protein (Table I). Thus the further decrease in $\{K^+\}_i$ from 100 mmol/liter in confluent to 90 mmol/liter in postconfluent cultures

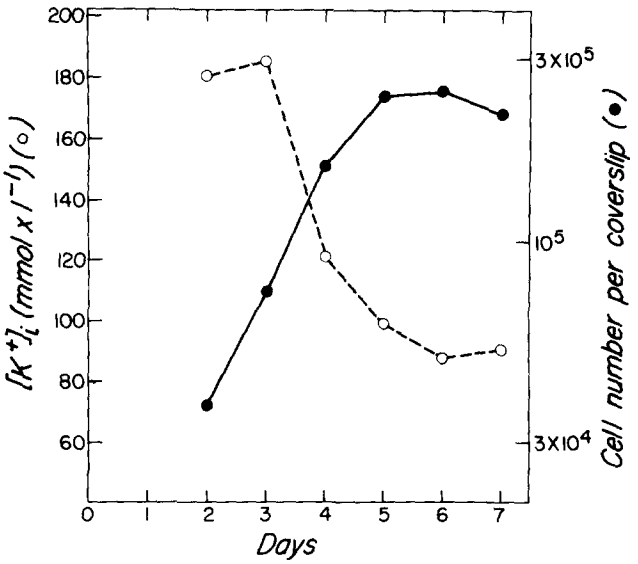

FIGURE 1 Density-dependent inhibition of BALB/c-3T3 cell growth is associated with a decrease in $\{K^+\}_i$. BALB/c-3T3 cells were planted on acid-treated glass coverslips (area 1.1 cm^2) in Dulbecco's modified Eagle's medium containing 10% bovine serum. The cell number (●) and $\{K^+\}_i$ (○) were determined at intervals beginning 48 h after planting, as described in Materials and Methods.

TABLE I

Effect of Density Inhibition on Cell K^+, Na^+, and Volume

	Cell No.	Mean cell volume	K^+ content	K^+ per cell	$\{K^+\}_i$	$\{Na^+\}_i$	$\{Na^+ + K^+\}_i$
	No. × 10^{-4}	*μm^3*	*mmol K^+/g protein*	*pmol K^+/cell*	*mmol/liter cell volume*		
Day 2	3.8 ± 1.0*	4,300 ± 150*	0.79 ± 0.07*	0.83 ± 0.16‡	180 ± 36‡	40 ± 12‡	220
Day 5 (confluent)	23.6 ± 0.2	2,870 ± 60	0.73 ± 0.09	0.26 ± 0.02	100 ± 3	11.7 ± 0.7	112
Day 7 (post confluent)	21.4 ± 0.2	3,400 ± 300	0.65 ± 0.04	0.30 ± 0.03	90 ± 3	14.7 ± 0.6	105

BALB/c-3T3 cells were planted on coverslips (area 1.1 cm^2) in Dulbecco's modified Eagle's medium containing 10% bovine serum. At 2, 5, and 7 d after planting, Na^+, K^+, and total protein were determined on some coverslips and cell number and mean cell volume on others as described in Materials and Methods.

* Standard deviation.

‡ Approximate standard deviation calculated from random paired samples.

was mainly because of an increase in mean cell volume. Thus, measurement of K^+ content per gram protein or K^+ content per cell does not accurately reflect $\{K^+\}_i$, because the relationship between cell protein and cell volume varies at different cell densities.

We also measured $\{Na^+\}_i$ as cells grew to confluence. $\{Na^+\}_i$ decreased from 40 mmol/liter in subconfluent logarithmically growing cells to 15 mmol/liter in confluent cells (Table I). Thus, the total univalent cation concentration ($\{Na^+ + K^+\}_i$) fell from 220 mmol/liter to 112 mmol/liter as cells became confluent. Because cell volume determination is critical to measurement of $\{K^+\}_i$ and $\{Na^+\}_i$, cell water was also measured in adherent cells on each day. The 3-O-MG water space varied little in relation to cell volume, averaging 74 ± 13% of total cell volume throughout the experiment. Thus, two independent measurements, cell volume and cell water, demonstrated a marked decrease in $\{K^+\}_i$ and $\{Na^+\}_i$ as BALB/c-3T3 cells grew to confluence. The decrease in both $\{Na^+\}_i$ and $\{K^+\}_i$ at confluence is consistent with the hypothesis that cells stop growing when these cations decrease and initiate growth when both increase.

Inhibition of Increased K^+ Uptake and Increased $\{K^+\}_i$ by Ouabain

To learn whether increased K^+ uptake and increased $\{K^+\}_i$ are closely associated with growth stimulation of confluent cells, the inhibitory effects of ouabain on K^+ uptake, $\{K^+\}_i$, and DNA synthesis were explored. Density-inhibited BALB/c-3T3 cells were maximally stimulated to synthesize DNA by the addition of both calf serum and partially purified human PDGF (14); varying concentrations of ouabain were also present to inhibit K^+ uptake. To quantify DNA synthesis, we added tritiated thymidine ([^{3}H]TdR) to some cultures; the cells were fixed 24 h later and processed for autoradiography to determine the percentage of cells that entered S phase. Ouabain at 500 μM completely prevented entry into S phase. However, ouabain at 100 μM did not inhibit DNA synthesis (Fig. 2 *a*).

To quantify K^+ uptake, we determined the rate of $^{86}Rb^+$ uptake 1 h after serum addition. $^{86}Rb^+$ uptake was determined in the presence of furosemide, which partially prevents K^+-K^+ exchange across the membrane. K^+-K^+ exchange does not contribute to K^+ uptake which could alter $\{K^+\}_i$ (21). In the presence of furosemide, 70% of $^{86}Rb^+$ uptake was inhibitable by ouabain at concentrations ≥1 mM. $^{86}Rb^+$ uptake was measured 1 h after serum stimulation because, as shown for nutrients (1), uptake measured sooner gives inconsistent results because of mechanical effects. In the absence of ouabain, $^{86}Rb^+$ uptake increased by ~50% above the level in quiescent cells. Ouabain blocked this increase in $^{86}Rb^+$ transport as a function of concentration. In the presence of 100 μM ouabain, the serum factor-induced increase in $^{86}Rb^+$ uptake was prevented (Fig. 2 *b*), but the cells synthesized DNA (Fig. 2 *a*).

The $\{K^+\}_i$ was determined in other cultures in late G_1, 12 h after serum and PDGF addition. In the absence of ouabain, the $\{K^+\}_i$ increased from 90 to 117 mmol/liter. Ouabain decreased the $\{K^+\}_i$ as a function of concentration; in 100 μM ouabain, the $\{K^+\}_i$ was 61 mmol/liter and in 500 μM ouabain, the $\{K^+\}_i$ was 10 mmol/liter (Fig. 2 *c*). Determination of $\{K^+\}_i$ 6 h after serum addition gave similar results, but $\{K^+\}_i$ had decreased less by 6 h than by 12 h at each concentration of ouabain (Table II). Thus, ouabain at 100 μM reduced net K^+ uptake to below the rate in unstimulated cells, resulting in a 40% fall in $\{K^+\}_i$ by 12 h, the lag phase for DNA synthesis. However, it did not inhibit entry into S phase. Cultures with decreased K^+ uptake during early G_0/G_1 and decreased $\{K^+\}_i$ during mid and late G_1 were able to enter the S phase. Apparently, 100 μM ouabain sufficiently inhibits Na^+,K^+-ATPase such that $\{K^+\}_i$ gradually decreases during G_0/G_1 in spite of possible synthesis of new cell membrane Na^+,K^+ pumps (3). Therefore, an increase in $\{K^+\}_i$ might not be required for serum-stimulated quiescent BALB/c-3T3 cells to enter S phase. However, an undetected transient increase in $\{K^+\}_i$ may possibly have occurred.

Effect of 100 μM Ouabain on $\{K^+\}_i$, $\{Na^+\}_i$, and Entry into S Phase

To determine whether serum-stimulated cells could enter the S phase without undergoing an increase in $\{K^+\}_i$, we monitored the effect of the continuous presence of 100 μM ouabain on $\{K^+\}_i$, $\{Na^+\}_i$, and entry into S phase as a function of time for 24 h. Density-inhibited BALB/c-3T3 cultures were transferred to medium containing calf serum supplemented with partially purified PDGF with or without 100 μM ouabain; the $\{K^+\}_i$ and the $\{Na^+\}_i$ were measured at intervals throughout the cell cycle. Cultures were also fixed periodically and processed for autoradiography to determine the rate that cells entered the S phase. The stimulated cells entered the S phase, after a lag of 12 h, at the same rate in the presence or absence of ouabain (Fig. 3 *a*). In the absence of ouabain, $\{K^+\}_i$ increased from 92 to 104 mmol/liter within 3 h, stayed at this level until 15 h, and then decreased to its initial value as has been described (22). In the presence of ouabain, the $\{K^+\}_i$ decreased throughout the period of the experiment (Fig. 3 *b*); at no time did it rise above the level present in quiescent cells in spite of presumed synthesis of new Na^+, K^+-ATPase molecules (3). Thus, although the $\{K^+\}_i$ rises rapidly after the addition of serum growth factors to density-inhibited cells, this increase is not required for DNA synthesis.

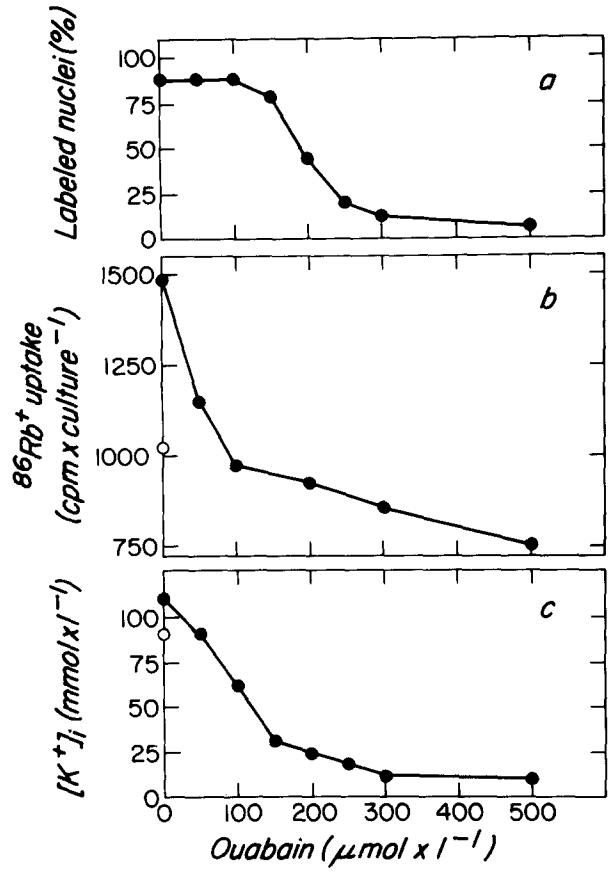

FIGURE 2 Effect of varying concentrations of ouabain on serum-induced DNA synthesis, $^{86}Rb^+$ uptake, and $\{K^+\}_i$ in density-inhibited BALB/c-3T3 cells. BALB/c-3T3 cells were grown to confluence on acid-treated glass coverslips. 5 d after planting, the cultures were transferred to fresh medium containing 10% (vol/vol) bovine serum and platelet extract (133 μg/ml) containing partially purified PDGF; ouabain was added to the cultures to give the final concentrations shown. (*a*) [^{3}H]TdR (final concentration 5 μCi/ml) was added to some cultures, which were fixed 24 h after serum addition. The percent of cells synthesizing DNA was determined by autoradiography. (*b*) Net $^{86}Rb^+$ uptake was determined 1 h after stimulation (●) or in unstimulated cultures (○). Cultures were incubated 6 min at 37°C in medium containing 10 mM HEPES, pH 7.4, 4.4 mM $NaHCO_3$, $^{86}Rb^+$ (5 μCi/ml), 1 mM furosemide, and serum, PDGF, and ouabain, as above. Furosemide was added to inhibit K^+-K^+ exchange which does not contribute to net K^+ uptake. (*c*) $\{K^+\}_i$ was determined 12 h after growth stimulation (●) or in unstimulated cultures (○).

The increase in $\{K^+\}_i$ was caused by an increase in K^+/cell. Mean cell volume increased by 27% at 12 h and by 39% at 24 h after serum and PDGF addition to cells; however, K^+/cell increased more dramatically than $\{K^+\}_i$, as previously described (22). Cells in 100 μM ouabain transiently underwent a decrease in cell volume associated with a marked fall in K^+/cell at 1 h, but subsequently mean cell volume increased by 18% at 12 h and by 21% at 24 h. Thus, in the presence of 100 μM ouabain, the K^+/cell decreased markedly and mean cell volume eventually increased less dramatically to result in the progressive decrease in $\{K^+\}_i$.

The addition of serum to growth-arrested 3T3 cells causes the rate of Na^+ entry into the cells to increase (12, 20). We found that the $\{Na^+\}_i$ in cells stimulated with serum and PDGF increased from a basal value of 14.1 ± 1.8 to 19.1 ± 1.2 mmol/liter (± = approximate standard deviation) within 1 h of serum and PDGF addition (Fig. 3 *c*). The $\{Na^+\}_i$ remained at this new higher level until 9 h, dropped below basal values at 15 h, and returned at 24 h. Thus, serum growth factors induce rapid elevation in $\{Na^+\}_i$ during G_0/G_1 phase. In the presence of ouabain, $\{Na^+\}_i$ increased throughout the cell cycle (Fig. 3 *c*).

The total intracellular univalent cation concentration ($\{Na^+$

TABLE II

Effect of Ouabain on $\{K^+\}_i$

	$\{K^+\}_i$ Time after stimulation	
Ouabain concn	6 h	12 h
μM	mmol/liter	
0	117	97
50	116	91
100	84	61
150	56	35
200	58	26
500	20	10

Density-inhibited BALB/c-3T3 cells were transferred to fresh medium containing 10% bovine serum, platelet extract (133 μg/ml) containing partially purified PDGF, and ouabain at the concentrations shown. $\{K^+\}_i$ was determined 6 or 12 h later as described in Materials and Methods.

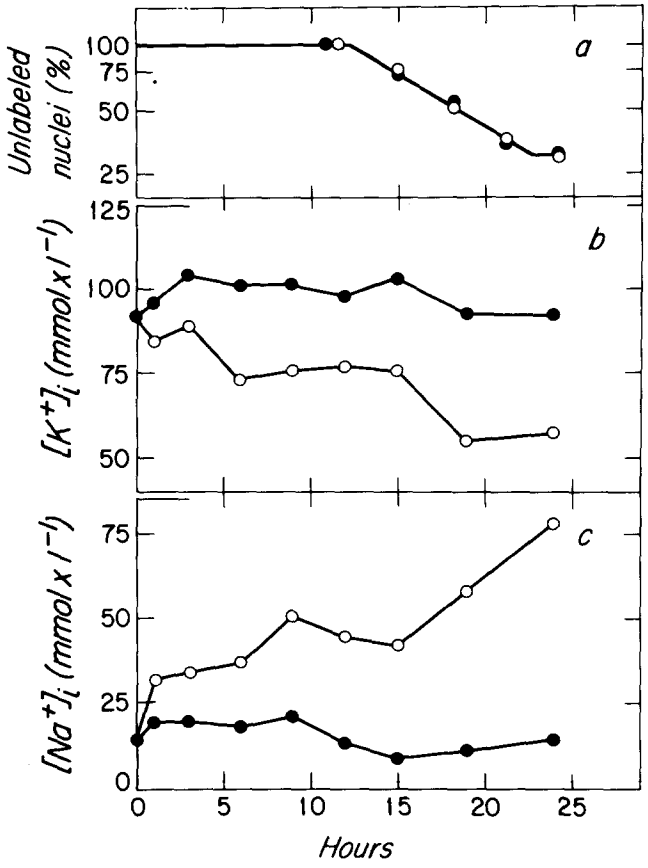

FIGURE 3 Serum-stimulated cells with decreased $\{K^+\}_i$ enter the S phase at a normal rate. Density-inhibited BALB/c-3T3 cells were stimulated to synthesize DNA by the addition of serum and partially purified PDGF in the presence (○) or absence (●) of 100 μM ouabain. (*a*) Cultures were fixed at intervals and DNA synthesis was determined by autoradiography. The data are plotted according to the fashion of Smith and Martin (19). (*b*) The $\{K^+\}_i$ was determined at intervals. (*c*) The $\{Na^+\}_i$ was determined by flame photometry at intervals.

TABLE III

Effect of Serum and PDGF Stimulation on $\{Na^+ + K^+\}_i$

Time after stimulation	$\{Na^+ + K^+\}_i$	
	Without ouabain	With ouabain
h	*mmol/liter*	
0	106	106
1	116	116
3	123	123
6	119	110
9	123	112
12	112	118
15	111	119
19	104	119
24	119	126

$+ K^+\}_i$) increased in cells stimulated with PDGF and serum. In resting cells, $\{Na^+ + K^+\}_i$ was 106 mmol/liter and increased by 16% to 123 mmol/liter 3 h after addition of serum and PGDF in the presence or absence of ouabain (Table III). Thus, the ouabain-induced decrease in $\{K^+\}_i$ was matched by an increase in $\{Na^+\}_i$.

DISCUSSION

It has been proposed that univalent cations play a critical role in regulating the growth of several types of mammalian cells including cultures of fibroblast-like cells such as 3T3 (7, 11, 16, 20, 22), lymphocytes (6, 18), or hepatocytes (9). Induction of DNA synthesis can be prevented by either the Na^+ influx inhibitor amiloride (*N*-amidino-3,5-diamino-*b*-chloropyrazine) (9, 20) or ouabain (10, 12, 16, 22), implicating both Na^+ and K^+ in cellular growth control. Rozengurt and co-workers (16, 20) have presented a unifying hypothesis in which both $\{Na^+\}_i$ and $\{K^+\}_i$ play a critical role in regulating cellular replication. According to this model, polypeptide growth factors stimulate growth by inducing the rapid entry of Na^+ into the cell. The increased $\{Na^+\}_i$ stimulates an Na^+,K^+-ATPase mediated increase in K^+ uptake and ultimately in $\{K^+\}_i$, which in turn induces cellular entry into the S phase (12, 20). In accord with this model, we find that density-inhibited nongrowing cells have a lower $\{K^+\}_i$ than subconfluent growing cells. We also find that the addition of serum growth factors to density-inhibited BALB/c-3T3 cells causes the $\{K^+\}_i$ to rise, as has previously been demonstrated by Tupper et al. (22). However, this increase in $\{K^+\}_i$ does not control entry into the S phase, because when the increase in $\{K^+\}_i$ is prevented by 100 μM ouabain, cells enter S phase at a normal rate (Fig. 3).

Addition of serum growth factors to density-inhibited BALB/c-3T3 cells also caused the $\{Na^+\}_i$ to rise (Fig. 3), contributing to an increase in total univalent cation concentration. Rozengurt and his colleagues have recently found an increase in Na^+ content per mg of protein 20 min after the addition of serum to density arrested BALB/c-3T3 cells (12). The present data supports the hypothesis that serum stimulation of quiescent cells results in an influx of Na^+ which stimulates an Na^+,K^+-ATPase-mediated influx of K^+. In BALB/c-3T3 cells these influxes result in an increase in both $\{Na^+\}_i$ and $\{K^+\}_i$ (12). However, the serum-mediated increase in Na^+ influx and $\{Na^+\}_i$ does not appear to solely control entry into the S phase because addition of either the Na^+ ionophore monensin (20) or the Na^+,K^+-ATPase inhibitor ouabain to quiescent cells increases both the uptake of Na^+ and the $\{Na^+\}_i$, but does not stimulate (20; data not shown) or enhance serum-induced DNA synthesis (Figs. 2 and 3). Thus, an increase in either $\{Na^+\}_i$ or $\{K^+\}_i$ alone does not stimulate DNA synthesis.

The present data demonstrate that the increase in K^+ uptake and in $\{K^+\}_i$ do not have a regulatory role in the cell cycle; the addition of 100 μM ouabain to serum-stimulated density-inhibited cells prevents an increase in K^+ uptake and in $\{K^+\}_i$, but does not affect S phase entry. Higher concentrations of ouabain (500 μM) prevent the initiation of DNA synthesis. One possibility is that high (500 μM) ouabain concentration results in a severely decreased $\{K^+\}_i$ which prevents S phase entry by directly interfering with protein synthesis (4, 10). Ouabain appears to inhibit protein synthesis and cell growth only when $\{K^+\}_i$ is drastically reduced (4, 10, 13). However, Sanui and Rubin have recently found that protein synthesis is not inhibited at 1 h but is inhibited at 3 h after addition of ouabain, resulting in drastic reduction of K^+ content, and suggest that decreased intracellular magnesium may participate in the inhibition of protein synthesis by ouabain (17).

Other investigations have also suggested that neither Na^+ nor K^+ are involved in fibroblast growth regulation. Tupper and Zografos have demonstrated that $\{K^+\}_i$ does not decrease when the growth rate of exponentially replicating populations is slowed by serum derprivation (23). However, these investigators did not study the relationship of $\{K^+\}_i$ to density-dependent inhibition of growth. The present study demonstrates that the $\{K^+\}_i$ and $\{Na^+\}_i$ fall as exponentially replicating populations become density inhibited. Furthermore, the addition of serum to density-arrested cells causes an increase in both $\{K^+\}_i$ and $\{Na^+\}_i$. Rubin and his colleagues have shown that a decrease in cellular Na^+ or K^+ content per milligram of protein does not necessarily prevent DNA synthesis in chick embryo fibroblasts (13). However, $\{Na^+\}_i$ and $\{K^+\}_i$ were not measured, and as shown above, large changes in cell volume associated with growth can significantly alter $\{K^+\}_i$ without affecting K^+ content per milligram of protein. In the present study, both K^+ per milligram of protein and $\{K^+\}_i$ were measured. The results are expressed as $\{K^+\}_i$ because the regulatory activity of K^+ is probably proportional to its concentration. In in vitro systems the concentration of K^+, rather than the content per milligram of protein, regulates protein synthesis (10).

Although an increase in neither $\{Na^+\}_i$ nor $\{K^+\}_i$ per se appears to be required for cell growth, an increase in total univalent cation concentration, manifested as an increase in $\{K^+\}_i$, $\{Na^+\}_i$, or $\{Na^+ + K^+\}_i$, could be required. The data demonstrate that serum stimulation of density-inhibited cells results in a 15–30% increase in total univalent cation concentration ($\{Na^+ + K^+\}_i$) within 3 h, whether or not 100 μM ouabain is present (Table III). In the presence of ouabain, the increase in univalent cations is entirely caused by an increase in $\{Na^+\}_i$, while in the absence of ouabain, the increase was mostly caused by an increase in $\{K^+\}_i$. Whether the increase in univalent cations was due to Na^+ or K^+, the stimulated cells entered the S phase. Thus, an increase in $\{Na^+ + K^+\}_i$ may be required for entry into S phase, and the increase may occur in either Na^+ or K^+, or both. In addition, the total univalent cation concentration in subconfluent growing cells was more than twice that found in density-inhibited cells. Recently, a study of subconfluent and confluent BALB/-3T3 cells by an entirely different technique, energy-dispersive x-ray microanalysis, has confirmed that subconfluent growing 3T3 cells have higher intracellular concentrations of both Na^+ and K^+

than density-inhibited cells (15). Thus, intracellular univalent cation concentration is higher in either subconfluent or confluent growing cells than in density-arrested cells.

Related changes appear to occur in lymphocytes. Exposure of lymphocytes to the mitogen phytohemagglutinin results in a small increase in $\{Na^+\}_i$ from 15 to 20 mmol/liter (18). Unlike BALB/c-3T3 cells, the lymphocytes transiently decrease cell volume without increasing K^+ content per milligram of protein, resulting in a transient 30% increase in $\{K^+\}_i$ (6). Measurements of K^+ content per milligram of protein would not have detected this change in K^+ concentration, Thus, total univalent cation concentration transiently increases in lymphocytes stimulated with phytohemagglutinin.

The significance of variations in intracellular univalent cation concentration with the cellular growth state is unknown. Carrasco and Smith (2) have shown that variations in total intracellular univalent cation concentration may result in the preferential translation of certain mRNAs. An increase in total univalent cation concentration in G_0/G_1 could possibly be required for optimal synthesis of proteins required for BALB/c-3T3 cell growth. To determine whether or not an increase in total intracellular univalent cation concentration is required for cell growth, it will be necessary to manipulate the total cation concentration during cell growth stimulation. It remains possible that the increase in total univalent cation concentration, manifested mostly as an increase in $\{K^+\}_i$, may be an epiphenomenon resulting from changes in membrane Na^+ permeability (20), and may not be required for cell growth.

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