

CALCIUM, MAGNESIUM, AND GROWTH CONTROL IN THE WI-38 HUMAN FIBROBLAST CELL

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ABSTRACT

WI-38 and SV40WI-38 cells have been synchronized using centrifugal elutriation. This technique allows for the rapid harvesting of early G_1 phase cells from exponentially growing populations of both the normal and transformed cell. Using these cells, as well as WI-38 cells synchronized by serum deprivation, we have examined the effects of extracellular Ca and Mg levels on the progression of cells through G_1 phase. A differential sensitivity to both Ca and Mg deprivation is observed between normal and transformed cells. The WI-38 cell requires higher levels of both ions for traversal of G_1 phase and for continued proliferation as compared to the transformed cell. The temporal nature of the Ca and Mg requirements for the WI-38 cell has been examined during G_1 phase. Ca is strictly required during early and late G_1 phase, but not necessarily throughout mid- G_1 . An early as well as a late G_1 Ca requirement is also found in serum-stimulated WI-38 cells. In contrast, the Mg requirement of WI-38 cells does not appear to be temporally well-defined. Mg appears to be a permissive factor, required throughout G_1 phase rather than at certain prescribed intervals. On the basis of these data, it seems unlikely that these two cations exert their effects on cell growth entirely through a common competitive mechanism. Ca would appear to be involved in early serum or growth factor-mediated G_1 events and later pre-S-phase events, as suggested in previous studies on other cell lines

KEY WORDS calcium · magnesium · human fibroblast · growth

The divalent cations Ca and Mg have been implicated as growth-regulating substances in a variety of *in vivo* and *in vitro* systems (for reviews see references 3, 19, 21, and 28). Ca and Mg are required for cellular proliferation (e.g., references 11, 22, and 28), and these requirements differ in certain normal and transformed cells (2, 4, 10, 14).

Growth control appears to be manifested in early G_1 or G_0 phase of the cell cycle because normal cells grown *in vitro* come to rest at this point under a variety of conditions unfavorable

for further proliferation. Thus, we wished to evaluate the G_0 and G_1 phase requirements for extracellular Ca and Mg in both normal and transformed cells. Centrifugal elutriation has been used to isolate G_1 phase cells from exponentially growing populations of both WI-38 and SV40-transformed WI-38 cells. Using these cells as well as serum-stimulated WI-38 cell populations, we have examined the Ca and Mg requirements for successful G_1 traversal and entry into S phase. Previous studies have suggested that a single Ca-dependent period exists in late G_1 phase (5, 6). Recent observations, however, indicate that one early effect of serum or serum-derived growth

factors may involve changes in Ca binding and metabolism (9, 14, 23, 24, 26, 27). The present study indicates that Ca is required in both early and late G₁ phase.

There also exist questions regarding the specific roles of Ca and Mg in the control of cell growth. Rubin and his colleagues have suggested that intracellular Mg activity plays a central role in the proliferative response (11, 21, 22) and that the effects of Ca deprivation on normal cell growth result from competition of Ca and Mg for common intracellular binding sites. In contrast, other workers have presented data that suggest that the effects of Ca and Mg on cell growth are distinct, both functionally and temporally (14, 28). In an attempt to clarify these possibilities, we have examined the effects of Ca and Mg deprivation at various times during G₁ phase of the cell cycle in the normal and transformed WI-38 cell. Our data suggest that Ca and Mg play distinct roles in regulating G₁ progression during portions of this phase in the WI-38 cell.

MATERIALS AND METHODS

Cell Cultures

WI-38 human embryonic lung fibroblasts (passage 15, population doubling 21) and WI-38VA 13 (SV40-transformed human lung, passage 264) have been obtained from the American Type Culture Cell Repository. WI-38 cells were used up to 30 passages and then restarted from frozen stocks to eliminate variables associated with aging. Stock cultures are maintained in basal diploid medium-Eagle (BME) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin and subcultured every 3-4 d at confluence. Cultures are maintained at 37°C in a 5% CO₂ air humidified atmosphere. Medium, serum, and antibiotics were purchased from Grand Island Biological Co. (GIBCO), Grand Island, N. Y.).

Manipulation of Culture Medium Divalent Cation Content

For the majority of experiments, serum-free BME was prepared without Ca and Mg salts. Such medium routinely contained 1-5 μ M Ca and Mg, as determined by fluorometric titration of Ca using the Ca-calcien complex (see reference 27) or atomic absorption measurements of Mg. The serum levels of Ca and Mg were reduced by treatment with a divalent cation chelator (Chelex 100, Bio-Rad Laboratories, Richmond, Calif.). Resultant Ca and Mg levels were $<10^{-6}$ M as measured by fluorometric titration or atomic absorption spectroscopy, respectively. Concentrated stock solutions (10 or 100 \times) of CaCl₂ and MgCl₂·6H₂O were prepared with glass-distilled, deionized water. These concentrates were added to the "Ca- and Mg-free" BME to give the desired final concentrations. The terms "Ca-free" or "Mg-free" medium will be used in this paper to refer to the medium described above before readdition of the ion in question. Control medium was made by supplementing Ca- and Mg-free BME

with 10% chelexed FCS, then readding Ca and Mg to normal levels (2.0 and 0.96 mM, respectively). Growth experiments comparing cell proliferation in medium prepared in this manner and reconstituted to normal Ca and Mg levels vs. medium containing non-chelex-treated serum indicate that growth of both normal and transformed cells is not significantly different.

Cell Synchronization

WI-38 and SV40WI-38 cells were synchronized via centrifugal elutriation (15, 16). $1.8-2.4 \times 10^7$ exponentially growing cells were removed from the growth surface, resuspended in 10 ml of BME (10% FCS), and drawn through a 20-gauge needle to disperse the cells. These were injected into the elutriator system (JE-6 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at a flow rate of 14 ml/min (normal cells) or 22 ml/min (transformed) and a rotor speed of 2,000 rpm. The elutriation medium was BME plus 3% FCS. Subsequently, 250 ml of medium containing small, presumably G₁ phase cells were harvested at a flow rate of 18 ml/min (WI-38) or 28.2 ml/min (SV40) and a rotor speed of 2,000 rpm. Elutriated cells were then centrifuged for 10 min at 600 g and the medium was removed by aspiration. Cells were resuspended in an appropriate volume of medium, and $1-5 \times 10^5$ cells were plated on 35-mm plastic dishes. All procedures were carried out sterily.

For cell number determinations, 35-mm dishes were washed with a balanced salt solution, lifted in 1 ml of GIBCO solution A containing 0.25% trypsin and 0.25% EDTA, and counted with a Coulter electronic cell counter.

For [³H]thymidine ([³H]TdR) incorporation, cells were pulsed with [³H]TdR (5-10 μ Ci/ml) for 20 min to 1 h, depending on the experiment in question. The pulse was terminated by washing the plates once with a balanced salt solution and adding 1 ml of cold 5% TCA with 0.1 mM thymidine. Dishes were subsequently scraped with a rubber policeman and filtered on glass fiber filters in a multiwell suction device. Filters were washed four times with 5% TCA and counted in 5 ml of Instabray (Yorktown Research Inc., Hackensack, N. J.) in a liquid scintillation counter.

Autoradiography was carried out on cells pulsed for various periods (see figure legends) with [³H]TdR (10 μ Ci/ml). At the termination of the pulse, cells were lifted with solution A, spun once through an excess of balanced salt solution, and fixed with 50 μ l of 3:1 ethanol:acetic acid. In some experiments, the cells were grown on cover slips and processed without lifting. The cells were smeared on slides or the cover slips were attached to slides, and they were subsequently dipped in Kodak NTB 2 nuclear track emulsion. Slides were developed after a minimum of 5 d and scored for percent labeled nuclei under phase optics.

Serum Stimulation

Quiescent populations of normal WI-38 cells were obtained by plating 1×10^5 cells per 35-mm dish and growing them to confluence. The cultures were then incubated in serum-free medium for 4 d. Cells were subsequently serum-stimulated with fresh medium containing 10% chelexed FCS, in which Ca and Mg levels were manipulated as described above. Specific details for each experiment are given in the figure legends.

In several experiments, cell growth was inhibited by Ca or Mg deprivation. For Ca-deprivation studies, 2×10^{-5} cells were plated in normal BME on 60-mm plastic dishes. 24 h after plating, the medium was removed and the cells were washed with a (Ca- and Mg-free) balanced salt solution and given low Ca medium. This medium contained 3×10^{-6} M Ca and 5×10^{-7} M Mg. Mg-deprivation studies were done in the same manner,

but in this case the experimental medium contained 2 mM Ca and 3×10^{-5} M Mg or "Mg-free" medium (see figure legends).

RESULTS

Cell Synchrony

The technique of centrifugal elutriation allows for the rapid harvesting of a large number of presumably G₁ phase cells from an exponentially growing population. This technique avoids some of the problems inherent in induction methods of synchrony, such as unbalanced growth. A major advantage of elutriation is that it allows both normal and transformed cells to be synchronized with the same technique (16). The elutriator is a modified continuous flow rotor designed such that cells of the same density can be separated according to size. In an exponential population of cells, theoretically the smallest cells should be in early G₁ phase and the largest in late G₂/early M phase. If early G₁ phase cells were plated, one would expect to see a lag in [³H]TdR incorporation corresponding to the length of G₁ phase followed by a synchronous entry into S phase. During this same interval, there should be no substantial change in cell number because M phase has not been reached. When the smallest fraction of elutriated cells (Fig. 1 A and B) were plated and the kinetics of [³H]TdR incorporation and cell number were monitored, the parameters varied as expected for both normal and transformed cells (Fig. 1 C and D). Cell populations treated in the same way as elutriated early G₁ phase cells, but harvested in a manner to collect the entire range of cell sizes, show no synchronous entrance to S phase (Fig. 1 D).

It should be noted that the G₁ phase observed for the normal elutriated WI-38 cells (~15 h) is longer than that observed for the transformed WI-38 cells (~9 h) and longer than that observed for cycling WI-38 cells (6–10 h; 1, 12). One possibility is that the normal elutriated cells may have fallen out of cycle and represent G₀ phase cells, which typically have extended pre-replicative phases (1). We consider this unlikely, however, since the elutriation process requires ~30 min whereas WI-38 cells exhibiting 14-h G₀–G₁ phases must remain quiescent for at least 4 d (1). Furthermore, these quiescent populations, when serum-stimulated, exhibit ~30% [³H]TdR labeled nuclei with a continuous pulse of 28 h (1). In contrast, our elutriated populations exhibit 60–80% labeled nuclei when pulsed for 30 h after plating, a value consistent

with cycling populations which have not entered G₀ phase (1).

Because WI-38 cells, like numerous other non-transformed lines, require attachment for continued proliferation, it is possible that the extended G₁ period represents the time required for complete cell attachment after elutriation, which is ~5 h. We have examined this by placing elutriated cells in suspension culture for 5 h before plating and comparing their G₁ phase to that of elutriated cells plated immediately. As is seen in Fig. 1 C, the suspension cultures are delayed in entering S phase as compared to the elutriated, nonsuspension cultures. Within the same experiment, cells were pulsed continuously from 15 to 42 h with [³H]TdR and processed for autoradiography. At the termination of the pulse, cell number was also determined. There was no significant difference in the cell number for the initially suspension vs. nonsuspension cultures (4.6×10^4 and 4.4×10^4 cells/plate, respectively) and the fraction of labeled nuclei was not significantly different (82 vs. 78%, respectively). Thus, the delay observed in Fig. 1 C is not caused by a reduction in the cycling population. These data suggest that attachment is required for G₁ progression and that the somewhat extended G₁ phase in the elutriated cells is due, in part, to this requirement. The SVWI-38 elutriated cells do not appear to exhibit this requirement, based on their 9-h G₁ phase. This observation is consistent with the absence of a strict attachment requirement for transformed cells. On the basis of these data, we shall refer to the elutriated normal cells as early G₁ phase cells throughout the text, but the data should be viewed with the apparent attachment delay in mind.

Effects of Extracellular Ca on Cell Proliferation

Initial studies were undertaken to determine the levels of extracellular Ca required for traversal of G₁ phase and entry into S, as monitored by autoradiography, in normal and transformed cells (Fig. 2). If medium Ca is 0.06 mM or lower, a substantial fraction of WI-38 cells is prevented from entering S phase. SV40-transformed WI-38 cells, on the other hand, show no decrease in the fraction of nuclei labeled with [³H]TdR even when grown in Ca-free medium (Fig. 2). The observation of this differential sensitivity to Ca deprivation between normal and transformed WI-38 cells agrees with a previous report (7).

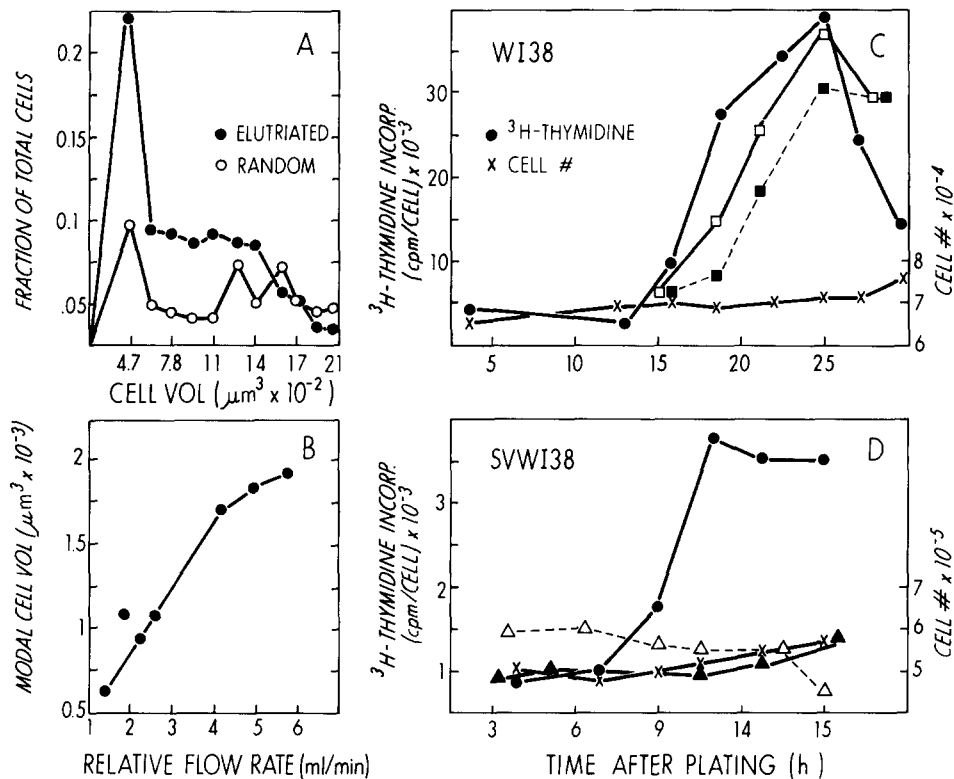


FIGURE 1 (A) Typical volume distributions for an exponential population of WI-38 cells and a G₁ phase-enriched fraction of WI-38 cells obtained by centrifugal elutriation. Cells were processed for elutriation as described in Materials and Methods, and samples were analyzed at increments of $156 \mu\text{m}^3$, with an electronic cell counter. The fraction of cells with volumes of $<1,400 \mu\text{m}^3$ represents 36% of random and 71% of elutriated populations. The modal volumes of the random and elutriated populations were $1,800$ and $950 \mu\text{m}^3$, respectively. (B) The effect of fluid flow rate on the modal volume of populations of WI-38 cells obtained by centrifugal elutriation at a rotor speed of $2,000$ rpm. The modal volume of cell populations was determined with an electronic cell counter. (C and D) Profile of [^3H]TdR incorporation and cell number after replating of early G₁ phase cells isolated from exponentially growing populations using centrifugal elutriation. Time zero represents the time of plating. At the times indicated, cells were pulsed for 20 min with $5 \mu\text{Ci/ml}$ [^3H]TdR, and acid-insoluble radioactivity (\bullet) was measured as described in Materials and Methods. Cell number (\times) was monitored with an electronic cell counter. For WI-38 (C), modal volume of the random population was $1,500 \mu\text{m}^3$; G₁ population was $875 \mu\text{m}^3$. For SV40WI-38 (D), the random population modal volume was $3,124 \mu\text{m}^3$. The G₁ population modal volume was $2,194 \mu\text{m}^3$. Data are typical of three such experiments, each composed of duplicate samples. Acid-insoluble radioactivity (Δ) and cell number (\blacktriangle) are also illustrated for a population of cells elutriated in the same way as indicated above, except that the entire cell volume distribution was collected and plated. In panel C is also shown the effect of delayed cell attachment on G₁ phase progression. WI-38 cells were elutriated as described above and plated (\square) or placed in suspension culture for 5 h and subsequently plated (\blacksquare). The cells were then pulsed as described above at the times indicated with [^3H]TdR. The data represent the mean of duplicate samples. See text for further discussion.

Using normal WI-38 cells, we carried out further studies to determine the temporal nature of the Ca requirement during G₁ phase. Initial studies showed that the synchronized WI-38 cells entered S phase 15 h after being elutriated and plated (Fig.

1C). Based on these data, two types of experiments were done within the 0 – 25 h postplating interval. In Ca readdition experiments, cells were plated in normal BME for 5 h, at which time they were washed and given Ca-free BME. At subsequent

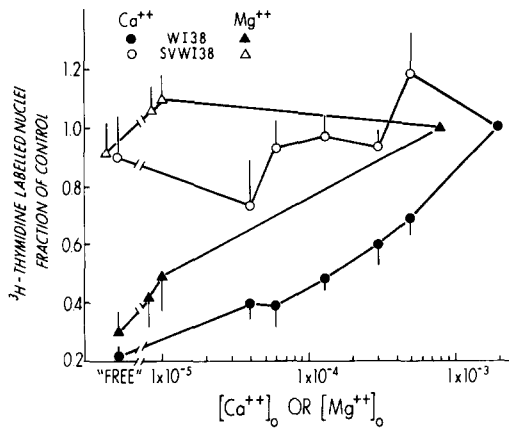


FIGURE 2 The effect of extracellular Ca or Mg concentration on the ability of elutriated G_1 phase cells to traverse G_1 and enter S phase. Cultures were elutriated and G_1 phase cells were plated in control medium (2 mM Ca, 0.96 mM Mg). 5 h after plating, cultures were washed and given fresh medium with the designated concentration of Ca or Mg. The ion which was not being manipulated was always present at control levels. Cells were pulsed continuously with $10 \mu\text{Ci/ml}$ [^3H]TdR from 5–30 h (WI-38) or 5–20 h (SV40WI-38) after plating. At 30 (20) h, all dishes were processed for autoradiography as described in Materials and Methods, and the fraction of labeled nuclei was scored under phase optics. The data from separate experiments are normalized to fraction of control, where control represents elutriated G_1 phase cells exposed to normal Ca and Mg levels throughout the experiments. All points represent the mean \pm SEM of three or four separate experiments, each containing duplicate samples for autoradiography. A minimum of 200 nuclei were scored on each sample.

times during G_1 phase, Ca levels were returned to normal by addition of a concentrated stock solution of CaCl_2 . If Ca is added back within 10 h after plating, WI-38 cells can enter S phase as well as cells that have been exposed to normal Ca since plating, as monitored by both [^3H]TdR incorporation and autoradiography (Fig. 3 A). As Ca is added beyond this 10-h period, an increasing fraction of the cells is prevented from initiating DNA synthesis. These results indicate that WI-38 cells require Ca for some indeterminate time during the last 8 h of G_1 phase. The data from these experiments give no indication of whether this requirement is brief or continuous through late G_1 (see below).

The experimental procedure used in the above experiments also provided no information concerning a requirement during the first 5 h of G_1 phase. This point is an important one in light of

the report of Boynton et al. (6) that 3T3 cells appear to require Ca only in very late G_1 phase, immediately before S. To examine the effect of Ca deprivation during this interval, we plated cells in Ca-free medium, and Ca was restored to normal 5 h after plating. As can be seen in Fig. 4 A, WI-38 cells deprived of Ca for the first 5 h of G_1 phase are delayed in entering S phase by an approximately equivalent length of time, suggesting the presence of an early G_1 Ca requirement in cycling WI-38 cells. This experiment also serves to indicate that any lag induced in G_1 progression by an attachment requirement appears to be distinct from an early Ca requirement. In this experiment, both the control and Ca-deprived cells are washed at 5 h and then control medium is readded to both sets of plates. Thus, only cells that had attached in either low Ca or normal Ca medium at 5 h were ultimately evaluated for DNA synthetic activity. Under these conditions, an effect was still clearly evident.

In Ca removal experiments, cells were maintained in control medium and, at various times during G_1 phase, plates were washed with a Ca- and Mg-free balanced salt solution and given Ca-free BME. If WI-38 cells are exposed to Ca for up to 15 h and then the Ca is removed, only ~30% of the cycling cells are able to initiate DNA synthesis (Fig. 3 A). Because WI-38 cells generally enter S phase after a 15- to 18-h G_1 phase, these results indicate a late G_1 phase Ca requirement in these cells. The increase in percent of control observed as Ca is removed at times later than 15 h is probably due, in part, to cells that have already entered S at the time of removal, because undoubtedly the length of G_1 phase is not constant for all cells.

The requirement of WI-38 cells for Ca appears to be continuous from 10 to 18 h after plating, because pulses of Ca for 0.5–3 h at various times within this interval are insufficient to allow more than 25–30% of cycling cells to enter S phase (Fig. 4 B).

We have undertaken similar experiments using serum stimulation of quiescent populations of WI-38 cells. Such experiments might reflect any differences in the Ca requirements of cycling (elutriated) cells presumably making the M- G_1 transition vs. noncycling (quiescent, serum-deprived) cells making the G_0 - G_1 transition. Serum stimulation in the presence of Ca allows for a synchronous entry into S phase after 15–18 h (Fig. 5 A). In the absence of Ca, serum-stimulated cells do

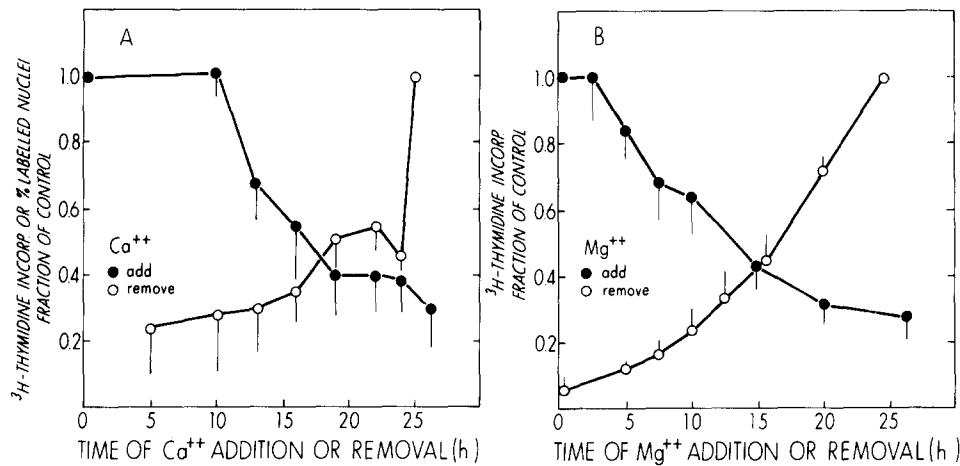


FIGURE 3 (A) Ability of WI-38 cells to enter S phase after Ca readdition to Ca-deprived cells or after removal of Ca from cells previously exposed to control medium. Exponential populations of WI-38 cells were elutriated, and early G₁ phase cells were plated in control medium. In one set of cultures, medium was replaced after 5 h with fresh Ca-free medium and, at the indicated intervals Ca was returned to normal (2 mM) by addition from a concentrated stock of CaCl₂ (●). Other cultures were left in control medium until the times indicated, when they were washed and given fresh Ca-free medium (○). The results of two readdition experiments using [³H]TdR incorporation into acid-insoluble material vs. two experiments using autoradiography were directly comparable when normalized as fraction of control, where control represents cultures exposed to Ca continuously for 26 h. Ca removal data (○) represents [³H]TdR incorporation only and are expressed as fraction of the control (same as above). All cultures were processed for [³H]TdR incorporation or autoradiography 26 h after plating as described in Materials and Methods. Cultures used to determine acid-insoluble [³H]TdR incorporation were pulsed for 1 h with 10 μCi/ml [³H]TdR. Cultures used for autoradiography were exposed to 10 μCi/ml [³H]TdR continuously from 5 to 26 h after plating. Data are the mean ± SEM of two (○) or four (●) separate experiments, each comprised of duplicate samples. (B) Ability of WI-38 cells to enter S phase after Mg readdition to Mg-deprived cells or after removal of Mg from cells previously exposed to control medium. Exponential populations of WI-38 cells were elutriated, and early G₁ phase cells were plated in control or Mg-free medium. At the times indicated, cells were either washed and given fresh Mg-free medium (○) or given Mg to 0.96 mM by addition from a concentrated stock of MgCl₂ (●). At 25 h after plating, the cultures were pulsed for 30 min with 10 μCi/ml [³H]TdR, and acid-insoluble radioactivity was measured as described in Materials and Methods. Data are the mean ± SEM of two (○) or four (●) separate experiments, each comprised of duplicate samples. Data are expressed as fraction of control, where control represents cells exposed to Mg continuously for the duration of the experiment.

not enter S over a 33-h period. If Ca is re-added at various times after serum stimulation, the cells' entrance into S phase is delayed respectively (Fig. 5 A). If WI-38 cells are exposed to Ca from 0 to 5 h after serum stimulation, and then Ca is removed, there is no increase in [³H]TdR incorporation over 42 h (Fig. 5 B). Exposure to Ca between 0 and 5 h and then again from 10 h on allows for an entrance into S phase which closely mimics that of control cultures receiving Ca for this entire period (Fig. 5 B). The apparent early G₁ requirement for Ca is further supported by the data in Fig. 6 A. In this experiment, serum-stimulated cells were exposed to Ca for 0–1 h, and then again from 7.5 h on. [³H]TdR incorporation of cells exposed

to this regimen was higher than that of cells receiving Ca only from 7.5 h on, but lower than that of controls.

If WI-38 cells are deprived of Ca for 4 d, they cease proliferating. If Ca is readded to such Ca-deprived cultures, the cells enter S phase synchronously, after a lag period of ~18 h (Fig. 6B). The kinetics of [³H]TdR incorporation in this instance are very similar to those seen with serum-stimulated cells.

Effects of Extracellular Mg on Cell Proliferation

It has been suggested that Mg may play a central role in the regulation of cell growth and that effects

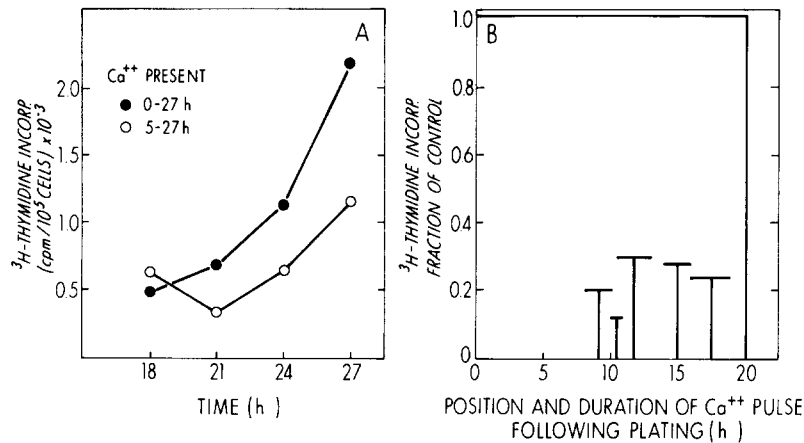


FIGURE 4 (A) The effect of Ca deprivation during the first 5 h of G_1 phase on the kinetics of WI-38 cell entry into S phase. Exponential cultures were elutriated as described in Materials and Methods, and early G_1 phase cells were plated in control (●) or Ca-free (○) medium. At 5 h after plating, all plates were washed and cultures in Ca-free medium received Ca to 2 mM by addition from a concentrated stock of CaCl_2 (○). At the times indicated, cultures were pulsed for 30 min with $10 \mu\text{Ci}/\text{ml}$ [^3H]TdR, and acid-insoluble radioactivity was measured as described in Materials and Methods. Data are typical of two such experiments, each composed of duplicate samples. In an identical experiment, cells were grown on cover slips and treated as described above. They were pulsed from 15 to 42 h with [^3H]TdR and processed for autoradiography as described in the text. The fraction of labeled nuclei for the control vs. 5-h-delayed Ca addition were $78 \pm 5\%$ and $82 \pm 2\%$ S.E., respectively, indicating that the elutriated cells were not irreversibly damaged by the delayed Ca addition. (B) The effect of limited exposures to Ca on G_1 phase progression. Exponential cultures were elutriated as described above, and early G_1 phase cells were plated in control medium. At 5 h after plating, cultures were washed and given fresh Ca-free medium. At the times indicated, cultures were given Ca to 2 mM by addition from a concentrated stock of CaCl_2 . The Ca pulse was terminated by washing the cultures and giving them fresh Ca-free medium. Horizontal lines denote the position and duration of the Ca pulse. Vertical lines indicate the fraction of [^3H]TdR incorporation as compared to control, where control represents cultures exposed to Ca for the duration of the experiment (25 h). Cultures were pulsed for 25 h after plating with $10 \mu\text{Ci}/\text{ml}$ [^3H]TdR for 30 min, and acid-insoluble radioactivity was measured as described in Materials and Methods. Data are typical of four separate experiments, each comprised of duplicate samples. Data were corrected for any differences in cell number.

of Ca deprivation on proliferation may be due to competition between Ca and Mg for common cellular binding sites (21). If the roles of these two cations are tightly linked, it is conceivable that the temporal profile of a G_1 phase Mg requirement could parallel that of Ca. It is possible, however, that Mg may be a permissive factor for G_1 progression with no specific temporal requirement during G_1 . These possibilities have been examined.

WI-38 cells were brought to quiescence by serum deprivation and were subsequently serum-stimulated in the presence or absence of Mg. If Mg is removed at the time of serum stimulation, the cells are prevented from entering S (Fig. 7). Readdition of Mg to the cultures at a time after addition of fresh serum results in a delay in the cell population's entering the S phase. However,

an equivalent fraction of the population ultimately enters DNA synthesis (Fig. 7). These data indicate that, as with Ca, Mg is required for normal exit from the quiescent state. We also observe that when cycling cells are deprived of Mg, they come to rest in the early G_1 or G_0 phase, as observed upon Ca deprivation (Fig. 6 B).

We have examined the Mg requirement under conditions analogous to those used to determine temporal Ca requirements during G_1 phase. These data then allowed us to evaluate the possibility of competition between Ca and Mg. In Fig. 2, data similar to those seen with lowered Ca indicate that with Mg deprivation there exists a differential sensitivity between normal and SV40-transformed G_1 phase cells isolated by centrifugal elutriation. In the WI-38 cells, the effect of Mg deprivation

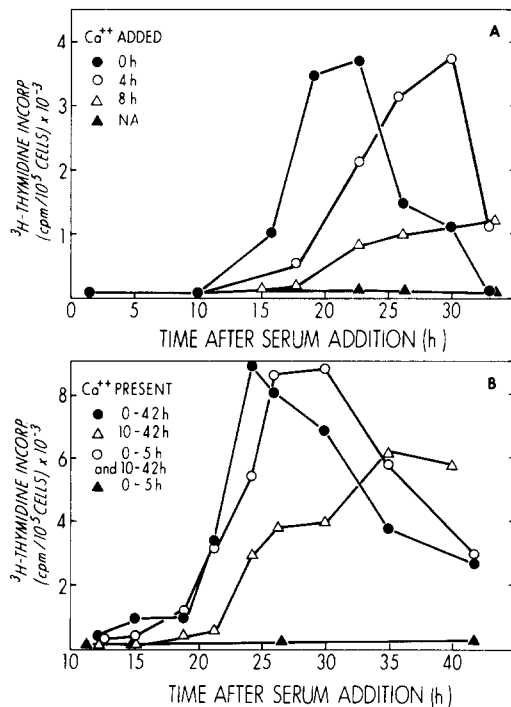


FIGURE 5 The effect of delayed Ca readdition on [³H]TdR incorporation in WI-38 cells serum-stimulated in the absence of Ca. Cells were brought to quiescence as described in Materials and Methods. At time zero, cultures were given fresh 10% serum low Ca medium (0.03 mM Ca). Ca was readded to 2 mM by addition from a concentrated stock of CaCl₂ at 0 (●), 4 (○), or 8 (△) h after serum stimulation. NA (▲) designates low Ca throughout the experiment. At the times indicated, cultures were pulsed for 1 h with 10 μCi/ml [³H]TdR, and acid-insoluble radioactivity was measured as described in Materials and Methods. (B). The effect of varying periods of exposure to Ca on [³H]TdR incorporation in serum-stimulated WI-38 cells. Cells were brought to quiescence as described in Materials and Methods. At time zero, cultures were given fresh 10% serum medium containing 0.03 mM Ca. At the time of serum addition, three sets of cultures were given Ca to 2 mM by addition from a concentrated stock of CaCl₂ (●, ○, ▲). At 5 h after serum stimulation, two sets of cultures were washed and given fresh low Ca medium (○, ▲). At 10 h, two sets of cultures received Ca to 2 mM as above (△, ○). At the times indicated, cultures were pulsed and processed for acid-insoluble radioactivity as described in A.

appears to be an actual block rather than merely a delay or elongation of G₁ phase, because Mg-deprived cells were unable to enter S phase up to 40 h after elutriation (unpublished observations). Experiments similar to those done with Ca-de-

prived cells are seen in Fig. 3 B. In both Mg-removal and Mg-readdition experiments, there does not appear to be a specific temporal requirement for Mg, contrary to our observations on the Ca requirements of WI-38 cells. We would note that the inhibition observed by Mg deprivation during G₁ phase using [³H]TdR incorporation (Fig. 3 B) is identical to that using autoradiography (Fig. 2), indicating that effects on thymidine uptake and metabolism are unlikely. The ability of WI-38 cells to enter S phase seems to be exponentially related to the length of exposure to normal levels of Mg (Fig. 8). As Mg is removed at progressively later times, an increasing fraction of cells is able to enter S. If cells are plated in Mg-free medium and if Mg is returned to normal at various times thereafter, the ability of cells to enter S decreases as the duration of Mg deprivation is prolonged. These data, in contrast to those seen under conditions of Ca deprivation, indicate that WI-38 cells require Mg through G₁ phase. They do not suggest specific times during G₁ phase in which Mg is or is not required.

DISCUSSION

The observation that extracellular Ca is able to regulate normal WI-38 cell growth much more strictly than transformed cell growth has been made previously (7) and is confirmed in the present study for WI-38 and SVWI-38 cells. However, the point (or points) in the cell cycle at which Ca exerts its effect on proliferation is unknown. This question has been previously addressed by Boynton and Whitfield (5) and Boynton et al. (6) in studies on the 3T3 cell. They have concluded that the extracellular Ca requirement for proliferative activity in the 3T3 cell resides only in late G₁ phase. This conclusion was based on the following observation (5): Quiescent 3T3 cells, when serum-stimulated, were observed to exhibit a maximum fraction of [³H]TdR-labeled nuclei ~20 h after serum addition. If Ca was not present at the time of serum addition but added back by 10 h, the peak of labeling was not significantly altered. This was interpreted as the absence of a Ca requirement during the first 10 h of G₁ phase. Examination of Fig. 1 from two such studies (5, 6), however, indicates that entrance into S phase is delayed by Ca deprivation during early G₁ phase. The fraction of labeled nuclei at 12 or 16 h is consistently below that of control values when Ca is withheld for periods after serum stimulation. Thus, in the 3T3 cell, Ca removal during early G₁ phase does ap-

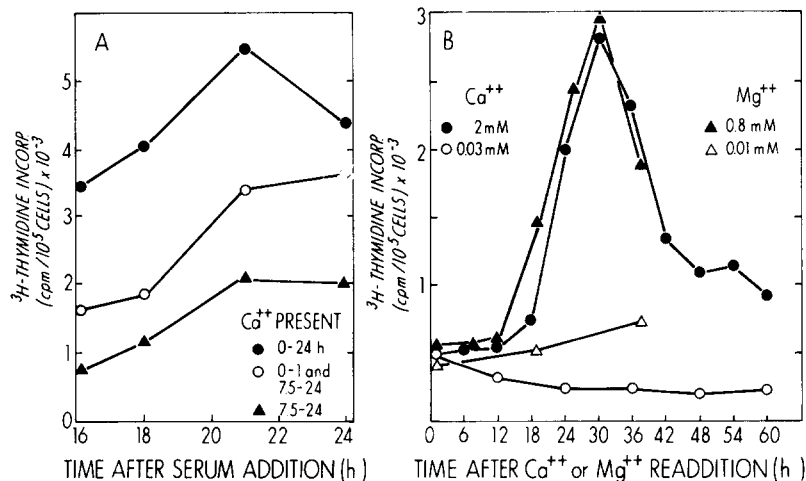


FIGURE 6 (A) The effect of varying periods of Ca deprivation on [^3H]TdR incorporation after serum stimulation of WI-38 cells. Cells were brought to quiescence as described in Materials and Methods. At time zero, cultures were given fresh 10% serum medium containing 2 mM (●, ○) or 0.03 mM (▲) Ca. At 1 h, one set of cultures was washed and given low Ca medium (○). At 7.5 h after serum stimulation, these cells (○) and another set of cultures (▲) were given Ca to 2 mM by addition from a concentrated stock of CaCl_2 . At the times indicated, cells were pulsed for 1 h with $10 \mu\text{Ci/ml}$ [^3H]TdR, and acid-insoluble radioactivity was determined as described in Materials and Methods. (B) The effect of Ca or Mg readdition on [^3H]TdR incorporation of 4-d Ca- or Mg-deprived WI-38 cells. Cells were plated at 9.5×10^3 cells/cm² in control medium, and 24 h later the cells were washed and given fresh low Ca medium or Mg medium. Cultures were maintained in these media for 4 d. At time zero, Ca or Mg was returned to normal in some cultures by addition from a concentrated stock of CaCl_2 (●) or MgCl_2 (▲). The remaining cultures received no additional Ca (○) or Mg (△). Cells were at a density of 1.7×10^4 cells/cm² at this time. At the indicated times, cells were pulsed and processed for measurement of acid-insoluble radioactivity as in A.

pear to reduce the rate at which cells enter the S phase. A recent report on the 3T3-4a clone also supports this conclusion. Paul and Ristow (17) have observed that readdition of Ca to Ca-deprived cells in the presence of excess serum results in a 14- to 16-h delay before entrance into S phase, an observation consistent with an early G₁ phase block due to Ca deprivation.

A further indication that Ca plays a role in regulating the events of early G₁ is evident from Fig. 6 B. Readdition of Ca to quiescent, Ca-deprived WI-38 cells results in the entrance to S phase ~18 h after Ca readdition. This time period is very consistent with an early G₁ or G₀ phase, Ca-dependent block because cells brought to quiescence by serum deprivation for an equivalent time period (4 d) require virtually an identical time to enter S phase after addition of fresh serum. It is generally agreed that such serum-deprived cells are blocked in the early G₁ (G₀) phase of the cell cycle. In similar studies on Ca-deprived WI-38 cells, Boynton et al. (7) have observed that readdition of Ca to 2-d Ca-deprived cells results

in two waves of DNA synthesis, one initiated within an hour after Ca readdition and a second wave ~16 h later. The ability of a fraction of the population to respond immediately to Ca readdition is consistent with a portion of these cells being blocked in late G₁ phase. Blockage of cells in early or late G₁ phase is not unique to Ca deprivation because it has been observed in a variety of cells under various experimental conditions (see reference 8 for complete references). Because in WI-38 cells the pre-replicative phase increases with the time of quiescence (1), i.e., the cells appear to move deeper into the G₀ state, the early or late G₁ phase response may be a function of the duration of Ca deprivation. Extended deprivation, like extended quiescence, may move all cells to the early G₁ or G₀ Ca-sensitive step. Preliminary experiments in our laboratory indicate this to be the case.

Recently, other data have emerged that support the concept of a very early G₁ phase requirement for Ca in normal cells. Human diploid fibroblasts show reduced growth capability in platelet-poor

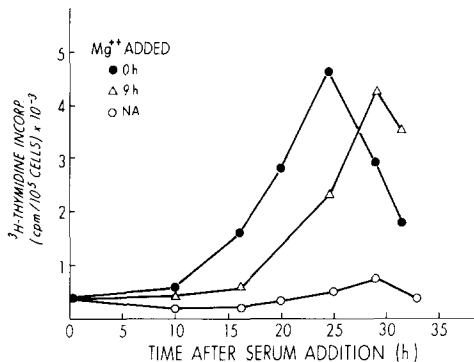


FIGURE 7 The effect of extracellular Mg on G_1 progression after serum stimulation of quiescent WI-38 cells. Cells were brought to quiescence by serum starvation as described in Materials and Methods. At zero time, fresh medium containing normal (●) or low Mg (Δ , ○) was added. In some plates, Mg was brought to normal levels at 9 h after serum addition (Δ) by addition from a concentrated stock of $MgCl_2$. NA (○) designates low Mg throughout the entire period. At the times indicated, the cultures were pulsed for 1 h with $10 \mu Ci/ml$ [3H]TdR, and acid-insoluble radioactivity was measured as described in Materials and Methods. Data are the mean of duplicate samples.

plasma as compared to serum (25). However, this occurs only at hypophysiologic levels of extracellular Ca, suggesting that Ca may substitute, in part, for platelet-derived growth factors found in serum but not platelet-poor plasma. The platelet-derived growth factors act in early G_1 or G_0 phase (e.g., reference 18). The ability of fetal bovine serum protein to support growth of diploid fibroblasts shows a dependence on the availability of extracellular Ca (14). Insulin or serum stimulation of quiescent cells in culture leads to rapid changes in Ca associated with the cell surface (24, 27). Thus, several studies indicate that serum or serum-derived growth factors, which act to effect normal cell growth in early G_1 or G_0 phase, also appear to act synergistically with extracellular Ca and to modify Ca binding and Ca transport at this phase of the cell cycle (10, 26). Also, the observation that a short Ca pulse given at the time of serum stimulation greatly enhances subsequent G_1 progression (Fig. 6 A) is consistent with the necessity of extracellular Ca for the G_0 to G_1 transition. The nature of this relatively brief Ca requirement is unknown. It may represent a role of surface Ca in growth factor binding or the necessity of surface membrane localized Ca for activation of membrane-bound, Ca-dependent regulator proteins.

Numerous other possibilities exist.

It also appears that there exists a second requirement for extracellular Ca in late G_1 phase, because removal of Ca as late as 15 h into G_1 phase inhibits S phase entrance (Fig. 3 A), and short exposures to Ca in early or mid G_1 phase (Figs. 4 B and 5 B) are insufficient for complete G_1 progression. Whitfield et al. (28) have proposed that an increase in intracellular Ca activity in late G_1 phase may act to trigger the DNA synthetic process. The late G_1 requirement may be related to this. We have previously demonstrated that increases in both Ca uptake and content precede entrance into S phase in 3T3 cells progressing through G_1 phase (26). Whether the absence or presence of Ca in the extracellular medium is directly related to changes in the intracellular activity of Ca or, alternatively, to changes in surface Ca levels only, remains to be evaluated.

We believe that our observations on Mg and Ca requirements for cell proliferation are of particular interest as regards models suggesting distinct vs. related roles for these ions in growth control. Rubin and his colleagues (e.g. 21-23) suggest that because Mg ion regulates numerous intracellular metabolic processes, the availability of Mg for such events is central to the control of cell proliferation. They have also postulated that the effects of extracellular Ca ion on cell proliferation result from indirect effects on intracellular Mg activity (e.g., reference 23). In their studies on 3T3 cells (23) and in our own studies on WI-38 cells (Hazleton and Tupper, manuscript submitted for publication), Ca deprivation inhibits cell proliferation but does not significantly reduce intracellular Mg

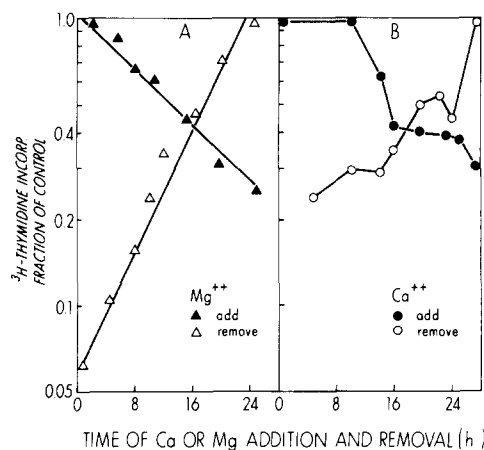


FIGURE 8 Data from Fig. 3 presented as a semi-logarithmic function. See Discussion for details.

content. Observations such as these have led them to propose that Ca and Mg compete for common intracellular binding sites (21). Thus, it is postulated that Ca deprivation inhibits cell proliferation through loss or redistribution of cell Ca such that Mg now occupies binding sites formerly occupied by Ca. Under these conditions, the availability of Mg for other processes is reduced and proliferation is inhibited. We would note, however, that it is equally plausible to argue that Ca deprivation inhibits normal cell proliferation as a result of the unavailability of Ca for critical intracellular metabolic events. This seems quite merited in view of the fact that numerous Ca-dependent regulator proteins have been identified, and some are sensitive to Ca at micromolar levels (e.g., reference 13). We would also note that the ability of supra-normal Mg levels to reverse the effects of Ca deprivation (23) could result from increased intracellular Mg activity due to an enhanced inward passive Mg flux or, alternatively, from increased Ca activity resulting from Mg displacement of bound Ca. Thus, in the absence of reliable measurements of intracellular Mg or Ca activity in cultured cells, such arguments seem equivocal.

If Mg and Ca effects on proliferation were related strictly through competition for common intracellular binding sites, one might predict that addition or removal of either of the ions during G₁ phase should produce qualitatively similar effects on cell proliferation. Our present data and one previous study (14) suggest that the roles of Ca and Mg in regulating cell proliferation are not strictly competitive and are at least temporally distinct. The ability of cells to enter S phase as a function of the availability of extracellular Mg appears to follow an exponential process (Fig. 8 A). In view of the fact that Mg is required for various aspects of macromolecular synthesis and transphosphorylation reactions, it seems quite reasonable that a continuous requirement for this ion would exist through G₁ phase because the processes themselves are continuous during this time. The Ca requirement, on the other hand, does not follow the same kinetics observed for Mg (Fig. 8 B). At this time, the functional basis of the pattern exhibited by the cells after Ca addition or removal is not clear. These data do not exclude the possibility that portions of the G₁ phase in the WI-38 cell may be influenced by competitive effects of these two ions. In fact, recent experiments in our laboratory indicate that, as observed with the 3T3 cell (23), hyperphysiological levels of Mg can re-

verse the effects of Ca deprivation in the WI-38 cell.

The present data also suggest that the ability of Ca and Mg to influence growth of the cells depends, in part, on the position in the cell cycle during which manipulation of the ion occurs. In growing populations of normal WI-38 cells, both Ca and Mg deprivation give rise to cells blocked in early G₁ or G₀ phase. Thus, under these conditions the cells appear to be capable of completing at least one cell cycle before becoming arrested. It is likely that these cells are represented in the cycling populations synchronized by elutriation as that small fraction which progresses G₁ phase under Ca or Mg deprivation (Fig. 2). In contrast, when quiescent cells presumably in G₀ are stimulated to reenter the cycle, they cannot when deprived of Ca or Mg. These data are in agreement with recent observations in 3T3 cells (20) which suggest that a fraction of serum-deprived or isoleucine-deprived cells do not become arrested upon their first G₁ encounter but are capable of continuing through at least one more cell cycle. In contrast, G₀ cells do not reenter the cycle under growth-restrictive conditions (20). Thus, extracellular Ca and Mg both appear to be required when transition from quiescence or the G₀ state is initiated, at least by serum stimulation. However, once this transition occurs, different temporal requirements, and presumably functional requirements, can be demonstrated as the cells traverse G₁ phase.

Supported by grants to J. T. Tupper from the American Cancer Society (BC-18) and the National Institutes of Health (NCI 17203-04).

Portions of this work are submitted as partial fulfillment of the requirements for the Ph.D. degree in the Department of Biology, Syracuse University.

Received for publication 2 February 1979, and in revised form 23 May 1979.

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