Intracellular Free Ca^{2+} in the Cell Cycle in Human Fibroblasts: Transitions Between G_1 and G_0 and Progression into S Phase

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Intracellular free calcium ($[\text{Ca}^{2+}]_i$) has been proposed to play an important part in the regulation of the cell cycle. Although a number of studies have shown that stimulation of quiescent cells with growth factors causes an immediate rise in $[\text{Ca}^{2+}]_i$ (Rabinovitch *et al.*, 1986; Vincentini and Villereal, 1986; Hesketh *et al.*, 1988; Tucker *et al.*, 1989; Wahl *et al.*, 1990), a causal relationship between the $[\text{Ca}^{2+}]_i$ transient and the ability of the cells to reenter the cell cycle has not been firmly established. We have found that blocking the mitogen-induced elevation of $[\text{Ca}^{2+}]_i$ with the cytoplasmic $[\text{Ca}^{2+}]_i$ buffer dimethyl BAPTA (dmBAPTA) also blocks subsequent entry of cells into S phase. The dose response curves for inhibition of serum stimulation of $[\text{Ca}^{2+}]_i$ and DNA synthesis by dmBAPTA are virtually identical including an anomalous stimulation observed at low levels of dmBAPTA. Reversal of the $[\text{Ca}^{2+}]_i$ buffering effect of dmBAPTA by transient exposure of the cells to the Ca^{2+} ionophore ionomycin also reverses the inhibition of DNA synthesis 20–24 h later. Ionomycin by itself does not stimulate DNA synthesis. These data are consistent with the conclusion that a transient increase in $[\text{Ca}^{2+}]_i$ occurring shortly after serum stimulation of quiescent fibroblasts is necessary but not sufficient for subsequent entry of the cells into S phase. This study is the first to show a direct relationship between early serum stimulated Ca_i^{2+} increase and subsequent DNA synthesis in human cells. It also goes beyond recent studies on BALB/3T3 cells by providing dose response data and demonstrating reversibility, which are strong indications of a cause and effect relationship.

INTRODUCTION

Intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) signaling plays a critical role in the regulation of a wide variety of cellular responses to external stimuli. The role of $[\text{Ca}^{2+}]_i$ as a second messenger in mitosis has been characterized by a number of laboratories (Hepler, 1988; Wolniak *et al.*, 1983; Keith *et al.*, 1985; Ratan *et al.*, 1986; Poenie *et al.*, 1986; Ratan *et al.*, 1988; Volpi and Berlin, 1988), but the role of $[\text{Ca}^{2+}]_i$ as a regulator of events in other parts of the cell cycle has been studied to a lesser degree (Zagari *et al.*, 1989; Diliberto *et al.*, 1990). With the use of image analysis and dual photometry with the flu-

orescent Ca^{2+} indicator dyes, fura-2 and indo-1, we have measured the early changes in $[\text{Ca}^{2+}]_i$ as cells emerge from quiescence.

$[\text{Ca}^{2+}]_i$ is a second messenger in the transduction of external stimuli that stimulate cell proliferation. Changes in $[\text{Ca}^{2+}]_i$ associated with progression through the cell division cycle have been observed in several systems. In sea urchin eggs, there is a small influx of extracellular Ca_i^{2+} within seconds of sperm binding, followed by a massive $[\text{Ca}^{2+}]_i$ increase (Epel, 1980; Jaffe, 1980). The large increase at the beginning of fertilization is followed by a smaller series of $[\text{Ca}^{2+}]_i$ oscillations corresponding to specific morphological stages, leading to cell cleavage (Poenie *et al.*, 1985).

$[\text{Ca}^{2+}]_i$ transients have also been observed in a variety of cells during the course of mitosis. For critical reviews dealing with the role of $[\text{Ca}^{2+}]_i$ in the regulation of mitosis see Hepler (1988). In other parts of the cell cycle,

¹ Abbreviations used: AM, acetyoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular free calcium; dmBAPTA, dimethylBAPTA; DMSO, dimethylsulfoxide; EGTA, ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; PDGF, platelet-derived growth factor.

Tucker and Fay (1990) found that ~50% of 3T3 cells responded to platelet derived growth factor (PDGF) with a large, rapid increase in $[Ca^{2+}]_i$ and that a similar proportion of the cells subsequently underwent mitogenesis. However, they were not able to demonstrate that the same cells were involved in both responses. They also found that inhibition of the early $[Ca^{2+}]_i$ transient with an intracellular calcium buffer blocked mitogenesis, but they were unable to rule out the possibility of a nonspecific, toxic effect of the calcium buffer on the cells. Thus the presence of a causal relationship between the early $[Ca^{2+}]_i$ transient and the subsequent reentry of the cells into the cell cycle remains to be established.

Human fibroblasts deprived of essential growth factors will remain in G_0 until stimulated by the addition of serum to undergo the complex series of events that culminate in DNA synthesis. Among the earliest events in this process is $[Ca^{2+}]_i$ elevation. Another is the stimulation of Na^+/H^+ exchange, which has been proposed to be critical in progression of the cell cycle (Vicentini *et al.*, 1986). Recent studies on the role of pH in early reentry events have called this hypothesis into question. For example, Swzergold *et al.* (1988) have shown that cells in HCO_3^- buffered medium do not alkalinize their cytoplasm in response to mitogenic stimulation. Similarly the work of Zagari *et al.* (1989) has shown that even when alkalinization is abolished some cells still progress through S phase. These studies make the question of what role, if any, the early increase in $[Ca^{2+}]_i$ has in the transition of cells from quiescence into active growth more problematical.

In the present study, computerized image analysis was used to measure $[Ca^{2+}]_i$ with high spatial resolution during transitions from G_0 to G_1 , and from G_1 to S phase. In addition, rapid time course studies were done with indo-1, a dual emission $[Ca^{2+}]_i$ indicator, on groups of cells using dual photometry. The goal has been to determine whether there is a cause and effect relationship between early $[Ca^{2+}]_i$ changes and progression through DNA synthesis.

MATERIALS AND METHODS

Cell Culture

The human skin fibroblast line GM2987 was obtained from the Institute for Medical Research (Camden, NJ). Cells were maintained in Eagle's minimal essential medium containing 25 mM $NaHCO_3$ and supplemented with 20 mM tricine, 10% fetal bovine serum, and penicillin (100 units/ml)-streptomycin (100 μ g/ml) in a humidified incubator with 5% CO_2 -95% O_2 at 37°C. Cells between the 10th and 30th generation were harvested by trypsinization 2-4 d before experiments and seeded at $3-5 \times 10^3$ cells/cm² (subconfluent) onto 22 mm diameter circular cover glasses (Fisher Scientific, Pittsburgh, PA). The cover glasses were attached to a 0.5-inch hole in the bottom of 35 mm plastic culture dishes, using Blue RTV Silicone gasket maker (Permatex, Loctite Corp., Cleveland, OH). Cells were changed 48-72 h later to serum deprivation medium, which is the same as the growth medium but with serum reduced to 0.1%.

Cell Loading with Fluorescent Probes

Fura-2AM and Indo-1AM. Cells were loaded with 3 μ M fura-2AM or indo-1AM in culture medium for 45 min at 37°C, 5% CO_2 . Fura-2AM or indo-1AM were prepared as stock solutions of 0.67 mM dye in dry dimethylsulfoxide (DMSO) containing 8% Pluronic F127. Cells were then postincubated in Incubation Buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 , 1 mM $CaCl_2$, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], 10 mM glucose, pH 7.4) for 45 min at 37°C, ambient CO_2 .

dmBAPTA-AM. Cells that had been deprived of serum for 48 h were loaded with dmBAPTA-AM for 2 h. For experiments measuring $[Ca^{2+}]_i$, 3 μ M fura-2AM or 3 μ M indo-1AM were added 30 min after the dmBAPTA incubation had begun. Forty-five min later, the medium was replaced with Incubation Buffer containing the same concentration of dmBAPTA without fura-2 or indo-1 and the incubation was continued for an additional 45 min. For assay of DNA synthesis, cells were pulsed from 20 to 24 h after serum stimulation, during the S phase peak.

Standard Curves for Ca^{2+} Calibration

Fura-2. Solutions for in vitro standardization were prepared as described by Grynkiewicz *et al.* (1985), and the ratios of standards containing known amounts of Ca^{2+} and fura-2 (free acid) were measured under the same conditions used in experiments on the microscope. In situ calibration using fura-2 gives similar fluorescence ratios, as has been shown in previous work (Williams *et al.*, 1985; Wahl *et al.*, 1990; Wahl *et al.*, 1992).

Indo-1. Unlike the situation for fura-2, the in situ fluorescence ratio as a function of Ca^{2+} curve is significantly different from the curve obtained in vitro (Popov *et al.*, 1988; Wahl *et al.*, 1990). For this reason in situ standard curves were routinely used for calculation of $[Ca^{2+}]_i$ using indo-1 with the use of a modification of the procedure of Chused *et al.* (1987) as previously described (Wahl *et al.*, 1990). Briefly, cells were treated with inhibitors of energy metabolism to block ATP synthesis and nigericin to equilibrate the intracellular and extracellular pH. Cells were then subjected to hypotonic shock in solutions containing the desired Ca^{2+} concentrations plus indo-1 (free acid) and Br-A23187 to maintain Ca^{2+} equilibration.

Fluorescence Microscopy and Video Image Analysis

Measurements of $[Ca^{2+}]_i$ levels were obtained from cells loaded with the Ca^{2+} -sensitive fluorescent dye fura-2 based on a modification of the techniques of Grynkiewicz *et al.* (1985) and Williams *et al.* (1985). Image analysis was performed as previously described (Johnson *et al.*, 1990; Wahl *et al.*, 1990). Briefly, cells were observed using a Nikon Diaphot (Melville, NY) inverted microscope equipped with epifluorescence optics and a 75 W xenon lamp. All lenses in the excitation path were made of quartz except for the objectives that were made of fluorite glass to optimize transmittance at wavelengths below 350 nm. Neutral density filters that reduce the intensity of illumination by a factor of 4 were routinely used. Cells were excited at 340 and 380 nm with filters having 10 nm half wavelength band widths. Pairs of images of cells excited at 340 and 380 nm were captured by a silicon intensified target camera (SITCAM; [Dage, Michigan City, IN]) and digitized with a Magiscan MD image analysis computer (Joyce-Loebl, Newcastle, UK). Images were analyzed with the use of the InCa program developed by the Center for Image Analysis of the University of Cincinnati College of Medicine.

Dual Wavelength Photometry

Cells were excited with the use of a xenon arc lamp and a 340 nm filter with a half wavelength of 10 nm. Emitted light was measured simultaneously at 410 and 485 nm by two separate photomultiplier tubes with the Nikon dual photometry system as previously described (Johnson *et al.*, 1990; Wahl *et al.*, 1990). Neutral density filters that reduce the intensity of light by factors of 16 or 32 were routinely

used. Data were collected with the FastInCa program developed at the University of Cincinnati College of Medicine.

DNA Synthesis Assays

Cells were plated at the same (subconfluent) density as those used for Ca²⁺ analyses, 3.5×10^3 cells/cm² on 24 well plastic culture dishes (Falcon, Lincoln Park, NJ). After 48 h, cells were serum deprived in 0.1% serum for 48 h. Cells were stimulated by the addition of fresh medium containing 10% serum or as otherwise indicated. Cells were pulsed with 1 μ Ci/ml [³H]-thymidine from 20 to 24 h after the serum had been added to each well. At the end of the 4-h pulse, the medium was aspirated and cells were rinsed with Hanks Medium/1% BSA. The [³H]-thymidine incorporated into the DNA was precipitated with the use of 5% TCA, solubilized in 0.25 N NaOH, and neutralized in 0.75 N HCl. Scintillation cocktail was added to each sample, and each was counted.

Materials

Dimethyl BAPTA-AM, fura-2AM, indo-1AM, indo-1 free acid, and Pluronic F127 were from Molecular Probes (Junction City, OR). Tissue culture media were from Gibco (Grand Island, NY); fetal bovine serum was from Hyclone Laboratories (Logan, UT); and nuclear track emulsion, developer, and fixer were from Eastman-Kodak (Rochester, NY).

RESULTS

Effects of Serum on [Ca²⁺]_i

The effect of nutritional status on basal [Ca²⁺]_i levels was examined in quiescent fibroblasts during serum deprivation. As shown in Figure 1 (■) basal levels of [Ca²⁺]_i declined substantially over the first 24 h of serum deprivation and then remained constant at ~50% of the [Ca²⁺]_i levels found in serum replete cells until 72 h. Between 72 and 96 h, there was a dramatic drop in basal levels to <5% of serum replete values. At 96 h, cells appear thin and irregular in morphology, yet maintain firm attachment and load fura-2 well. These cells will recover from this degree of deprivation if 10% serum is restored and go on to grow to confluence.

When cells are stimulated with 10% serum after serum deprivation, the average peak [Ca²⁺]_i concentration reached was diminished with increasing time of serum deprivation. Figure 1 (□) shows the results of stimulation of cells with media containing 10% serum at various times as a function of serum deprivation. Within 2 h of the removal of serum, readdition of serum induces a substantial increase in [Ca²⁺]_i. Although the absolute magnitude of the average [Ca²⁺]_i peak decreases slowly over the next 72 h, when basal levels are taken into account the percent increase in [Ca²⁺]_i at 24–72 h is larger than the stimulation at 2 h. The large error bars associated with serum stimulation at 2 h are not due to experimental error, but rather to the heterogeneity in the timing and the magnitude of the responses in individual cells. All subsequent experiments were done on cells deprived of serum for 48 h.

The kinetics of the serum stimulation of [Ca²⁺]_i were examined for four different concentrations of serum. In all cases, maximum values of Ca²⁺ occur within 12–20

Figure 1. Long-term effects of serum deprivation or addition on Ca²⁺. Cells were plated and grown for 2–4 d in normal culture medium with 10% serum. For 1 set of cells the spent medium was replaced with low (0.1%) serum containing medium, and for a second set of cells the spent medium was replaced with high (10%) serum containing medium. Ca²⁺ was measured at the indicated times by prior loading of the cells with fura-2AM and video imaging microscopy as described in MATERIALS AND METHODS. Cross-hatched bars represent average Ca²⁺ concentration of cells in low (0.1%) serum medium. Open bars represent Ca²⁺ concentration of cells in high (10%) serum medium. Values are the means $\pm$ SD of 10–15 cells examined at each time.

s of the addition of serum. After the rapid rise to maximum [Ca²⁺]_i, there is a slower fall in [Ca²⁺]_i, which remains elevated above basal levels for >10 min. The maximum peak height of the [Ca²⁺]_i is achieved at 5% serum concentration (Figure 2A), although there is some decrease observed in standard deviation with higher serum concentrations. Examination of the [Ca²⁺]_i responses in individual cells at the time of average [Ca²⁺]_i peak (Figure 2B) shows that increased concentration of serum gives rise to an increase in the number of cells responding at this time, rather than an increase in the magnitude of the response in each cell. Because the time resolution for Ca²⁺ measured by our imaging system is limited to about one reading every 10–15 s, [Ca²⁺]_i was also measured by dual photometry. In this method, cells are loaded with indo-1, placed on the microscope stage, and the total emitted fluorescence of the entire field at 410 and 485 nm is measured simultaneously with separate photomultipliers. The results, shown in Figure 3, indicate that at the highest level of serum tested, the peak [Ca²⁺]_i achieved is actually substantially higher than that obtained at lower serum concentrations. Indeed, when peak Ca²⁺ is measured as a function of serum concentration, there is an almost linear relationship (Figure 3E). Because of the increased time resolution of this technique, it is also possible to accurately measure the average rate of rise of [Ca²⁺]_i (Figure 3F). This value also increases in a nearly linear fashion with serum concentration. We conclude from these data that increased concentration of serum recruits increased numbers of cells to increase their [Ca²⁺]_i. The observation

Figure 2. Effect of serum concentration on the kinetics of Ca_i^{2+} increases as measured by video imaging microscopy. Cells were serum deprived for 48 h, loaded with fura-2, and Ca_i^{2+} was measured by video imaging microscopy, as in Figure 1. (A) Dose-response for peak Ca_i^{2+} as a function of serum concentration. (B) Images of the cells at 340 and 380 nm excitation wavelengths were obtained every 15 s for the first 2 min after serum stimulation and at longer intervals thereafter. Calculation of the concentration of Ca_i^{2+} at each location in the cell was as described in MATERIALS AND METHODS. The images shown are for the peak of the Ca_i^{2+} responses to: (A) 1% serum, (B) 5% serum, (C) 10% serum, and (D) 20% serum.

that when $[Ca^{2+}]_i$ is measured with a high degree of temporal resolution, both the average peak height and the average rate of rise are increased, could be consistent either with an increase in the maximum $[Ca^{2+}]_i$ reached by each cell or with an increased degree of synchrony among the individual cells. At this time, we cannot distinguish between these two possibilities.

Progression of Cells into S Phase

The time course of $[^3H]$ -thymidine incorporation in serum deprived human fibroblasts stimulated with 10% serum was determined. Cells appear to go through S phase quite synchronously, with the peak of $[^3H]$ -thymidine incorporation occurring ~ 24 h after the addition of serum. The dose response for $[^3H]$ -thymidine incorporation as a function of serum concentration is shown in (Figure 4). Based on these data, subsequent experiments on DNA synthesis were done with $[^3H]$ -thymidine pulses between 20 and 24 h after the addition of 10% serum.

Buffering of Ca^{2+}_i

To inhibit the serum-induced stimulation of $[Ca^{2+}]_i$, cells were pretreated for 2 h with dmBAPTA, a non-fluorescent Ca^{2+} chelator with a K_d of 40 nM (Tsien, 1980). Effects of dmBAPTA on serum stimulation are shown for individual cells by image analysis in Figure 5. The normal serum response (5A) is completely inhibited in the dmBAPTA treated cells (5B). The effect of dmBAPTA on the kinetics of the $[Ca^{2+}]_i$ response (Figure 6) indicate that at doses of $0.8 \mu M$ dmBAPTA and above, the $[Ca^{2+}]_i$ response is effectively inhibited, in agreement with the imaging data in Figure 5. At lower doses, a significant response is present although the kinetics are substantially altered, especially the rate of return toward resting levels. Of particular interest is the response to $0.07 \mu M$ dmBAPTA, in which the peak height is essentially unaltered, but, because of the reduced rate of return toward basal $[Ca^{2+}]_i$, the total amount of Ca_i^{2+} integrated over time is substantially increased relative to the control. These data are summarized in Figure 6G.

To compare the effect of dmBAPTA on DNA synthesis with its effects on $[Ca^{2+}]_i$, cells were pretreated with dmBAPTA exactly as in Figure 6, and $[^3H]$ -thymidine was measured 20–24 h later (Figure 7). The dose response curve for dmBAPTA inhibition of DNA synthesis correlates well with the dose response for inhibition of $[Ca^{2+}]_i$, including the anomalous increase at $0.07 \mu M$ dmBAPTA. In these experiments, there appears to be little or no effect of dmBAPTA treatment on cell viability, as measured by fluorescein diacetate uptake and ethidium bromide exclusion.

We found that the optimum dose of dmBAPTA required for inhibition of serum-induced $[Ca^{2+}]_i$ stimulation and DNA synthesis varied slightly from ex-

Figure 3. Effect of serum concentration on the kinetics of Ca^{2+} increase as measured by dual photometry. Cells were serum deprived for 48 h, loaded with indo-1, and Ca^{2+} was determined from the ratio of the fluorescence intensities at 410 and 485 nm, measured simultaneously at 400-ms intervals by dual photometric microscopy, as described in MATERIALS AND METHODS. Arrows indicate time of serum addition. (A) 0.1% serum, (B) 2% serum, (C) 5% serum, (D) 10% serum, (E) dose-response for peak Ca^{2+} , and (F) dose-response for initial rate of change of Ca^{2+} .

periment to experiment but always fell in the range of 0.4–1.0 μ M and always affected both parameters to the same degree. We believe that the variation in maximally effective dose was due almost entirely to modest differences in cell density and to whether or not the dmBAPTA stock solution had been refrozen. Treatment of cells with dmBAPTA doses above 1 μ M

for 2 h resulted in noticeable loss of cell viability. Because the effective and toxic doses are quite close, and because of the day-to-day variability in the effective dose, it is important to use several different concentrations of dmBAPTA in the optimal range to achieve the maximum, nontoxic inhibition in each experimental trial.

Figure 4. Effect of serum stimulation on DNA synthesis. Cells were serum deprived for 48 h and then stimulated by replacement of medium with fresh medium containing 10% serum. Cells were pulsed with [³H]-thymidine between 20 and 24 h, and incorporation of radioactivity into DNA was measured as described in MATERIALS AND METHODS.

Reversal of dmBAPTA Inhibition

To investigate the question of whether the inhibitory effects of dmBAPTA on DNA synthesis were related exclusively to their early effects on stimulation of $[Ca^{2+}]_i$, dmBAPTA pretreated cells were exposed to ionomycin at the time of serum stimulation (Figure 8). The dose and duration of the ionomycin treatment was chosen to roughly approximate the shape of the serum-induced $[Ca^{2+}]_i$ stimulation during the first 10 min of serum exposure. After addition of ionomycin, there was a rapid rise in $[Ca^{2+}]_i$ (~300 nM), which was sustained until the ionomycin was removed at which time $[Ca^{2+}]_i$ fell toward basal levels.

Pretreatment of cells with dmBAPTA sufficient to inhibit DNA synthesis by 70% of serum stimulated values could be reversed by ionomycin to give 82% of the serum stimulated control (Figure 9). These data indicate that the predominant inhibitory effect of dmBAPTA on DNA synthesis is due to its ability to buffer the early $[Ca^{2+}]_i$ response.

DISCUSSION

In fibroblasts that have been made quiescent by 48 h of serum deprivation, the addition of 10% serum causes an immediate increase in $[Ca^{2+}]_i$. Twenty-four h later the cells go through a synchronous round of DNA synthesis. When the serum induced increase in $[Ca^{2+}]_i$ is inhibited with the introduction of the $[Ca^{2+}]_i$ buffer dmBAPTA into the cytoplasm, there is a corresponding inhibition of subsequent synthesis of DNA. Creation of an artificial increase in $[Ca^{2+}]_i$ in dmBAPTA treated cells using the Ca^{2+} ionophore ionomycin circumvents the

dmBAPTA blockade of DNA synthesis 24 h later. We conclude from these results that early serum induced stimulation of $[Ca^{2+}]_i$ is necessary for progression of the cells into S phase.

With regard to the question of whether elevation of Ca_i^{2+} is by itself sufficient to induce progression to S phase, we have found that stimulation of Ca_i^{2+} by exposure to ionomycin for 2 min in the absence of serum does not induce DNA synthesis. However, in the absence of serum, ionomycin is toxic to the cells even at doses as low as 0.1 μ M.

Figure 5. Effect of Ca^{2+} buffering on serum stimulation of Ca^{2+} . Ten percent serum was added to 48-h serum deprived cells and Ca^{2+} was measured with fura-2 as a function of time as in Figure 3. (A) Untreated control; lower left-hand corner shows average time (s) that image pair was captured. (B) Cells pretreated for 2 h with 0.8 μ M dmBAPTA; upper left-hand corner shows average time (s) that the image pair was captured.

Figure 7. Effect of dmBAPTA on serum stimulated [³H]-thymidine incorporation into DNA. Serum deprived cells were exposed for 2 h to the indicated concentrations of dmBAPTA. The medium was then replaced with medium containing 10% serum and no dmBAPTA. Cells were pulsed with [³H]-thymidine for 4 h beginning 20 h after addition of serum, and radioactivity incorporated into DNA was measured by liquid scintillation counting.

The data on inhibition of DNA synthesis by dmBAPTA do not imply that serum does not contain any Ca^{2+} -independent growth factors or that such factors are not sufficient by themselves to induce progression to S phase in human fibroblasts. Rather, our data indicate that any growth factors that operate via Ca^{2+} -independent pathways are either present in insufficient concentrations or are overridden in the presence of Ca^{2+} -dependent growth factors such as PDGF. This suggestion is different from the conclusion reached by Tucker *et al.* (1989), who found that fibroblast growth factor (FGF) stimulation of DNA synthesis was not blocked by buffering of $[\text{Ca}^{2+}]_i$ by quin 2 in BALB/c-3T3 cells. The most likely explanation for the discrepancy between these data is to be found in the cell lines used: regulation of cell-cycle progression in 3T3 cells is known to differ in a variety of ways from that in human fibroblasts. However one cannot rule out the possibility that our results showing inhibition of serum stimulated DNA synthesis by dmBAPTA might be due to a generalized toxic effect of the Ca^{2+} chelator rather than to specific inhibition of a Ca^{2+} -dependent pathway. Arguing against such a generalized toxic effect is the reversal of dmBAPTA inhibition by ionomycin. There is no reason to believe that elevation of Ca^{2+} by ionomycin would reverse any general toxic effects of dmBAPTA.

The existence of a cause and effect relationship between the early elevation of Ca_i^{2+} and the subsequent synthesis of DNA is further strengthened by the close similarity between the dmBAPTA dose response curves for the inhibition of serum stimulated DNA synthesis and the inhibition of Ca_i^{2+} transients. Of particular significance is the presence of an anomalous amplification of serum stimulated $[\text{Ca}^{2+}]_i$ at low concentrations of

Figure 8. Ionomycin reversal of dmBAPTA-blocked serum stimulation of Ca_i^{2+} . Cells were serum deprived for 48 h, treated with 0.4 μM dmBAPTA for 2 h, then stimulated with 10% serum and 2 μM ionomycin ($\blacktriangle$) for 2 min and Ca_i^{2+} was measured as in Figure 3. Ionomycin was then removed ($\blacktriangledown$) and the medium was changed to fresh 10% serum. The data are representative of 3 separate experiments.

dmBAPTA, which correlates with an equally unexpected and similar amplification of DNA synthesis at the same low concentration of dmBAPTA. Although these enhancing effects of intracellular Ca^{2+} buffering have not to our knowledge been previously reported other studies have shown a correlation between the effects of growth factor induced Ca_i^{2+} elevation and subsequent DNA synthesis in 3T3 cells. Tucker and Fay (1990) have found that the buffering of $[\text{Ca}^{2+}]_i$ responses to PDGF with quin 2 inhibited DNA synthesis. Similarly, Zagari *et al.* (1989), as well as Diliberto *et al.* (1990) found that PDGF increased $[\text{Ca}^{2+}]_i$ and subsequent DNA synthesis, and

Figure 9. Ionomycin reversal of dmBAPTA-blocked serum stimulation of DNA synthesis. Cells were treated as in Figure 8 with 10% serum and 0.4 μM dmBAPTA. (A) Serum-deprived cells, (B) 10% serum stimulated cells, (C) cells treated with 0.4 μM dmBAPTA for 2 h, then stimulated with 10% serum, (D) cells treated with 0.4 μM dmBAPTA for 2 h, then stimulated with 10% serum. Ionomycin was added 1 min after the addition of serum and was removed 2 min later. DMSO solvent controls gave no stimulation. Error bars represent the standard deviation of quadruplicate wells in a representative experiment from a total of 5 such experiments.

they also showed inhibition of the PDGF induced [Ca²⁺]_i increase by TMB-8 blocked subsequent DNA synthesis. Our experience with TMB-8 in human fibroblasts, however, indicates that for any dose that inhibits Ca_i²⁺ transients, cell viability at the time of the assay for DNA synthesis 20–24 h later is extremely poor.

The anomalous augmentation of serum induced [Ca²⁺]_i stimulation by low levels of dmBAPTA can be explained on the basis of incomplete buffering by dmBAPTA. Thus, for example, consider the case in which stimulation of cells with 10% serum normally results in an increase in [Ca²⁺]_i ≤ 450 nM at which time regulatory mechanisms result in a rapid fall in [Ca²⁺]_i. In the presence of low concentrations of dmBAPTA, some of the Ca_i²⁺ released during the early stimulation phase will be bound by this buffer, thus decreasing the rate of rise of [Ca²⁺]_i at that point in time. As a consequence, the time required to reach levels of [Ca²⁺]_i sufficient to initiate the cells' regulatory response for lowering [Ca²⁺]_i will be prolonged, and the total amount of [Ca²⁺]_i released to reach that level will be greater than in the absence of the dmBAPTA buffer. In a sense, the dmBAPTA can be viewed as a pseudocompartment for the storage of [Ca²⁺]_i. When the regulatory response does become activated, and [Ca²⁺]_i starts to fall, it will do so at a slower rate because Ca²⁺ will be released from this pseudocompartment at that time. The overall result would thus be a slower rate of rise ultimately reaching the same level as the control then falling back toward normal levels, also at a slower than usual rate. Under these conditions, the total amount of [Ca²⁺]_i in the cytoplasm integrated over time would actually be increased. This is precisely what is observed in Figure 6, A and B. Only when the buffering capacity of the cytoplasmic dmBAPTA actually exceeds the amount of Ca²⁺ that can be released from intracellular stores will a true reduction in the stimulated levels of [Ca²⁺]_i be observed. We would argue that the observation that increased DNA synthesis under conditions where low levels of dmBAPTA have increased the [Ca²⁺]_i response to serum strongly suggests that the amount of Ca²⁺ release during the early phase of serum stimulation is a limiting factor in the recruitment of quiescent cells for active cycling.

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