

A Transient Rise in Cytosolic Calcium Follows Stimulation of Quiescent Cells with Growth Factors and Is Inhibitable with Phorbol Myristate Acetate

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ABSTRACT We have used aequorin as an indicator for the intracellular free calcium ion concentration ($[Ca^{++}]_i$) of Swiss 3T3 fibroblasts. Estimated $[Ca^{++}]_i$ of serum-deprived, subconfluent fibroblasts was 89 (± 20) nM, almost twofold higher than that of subconfluent cells growing in serum, whose $[Ca^{++}]_i$ was 50 (± 19) nM. Serum, partially purified platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) stimulated DNA synthesis by the serum-deprived cells, whereas epidermal growth factor (EGF) did not. Serum immediately and transiently elevated the $[Ca^{++}]_i$ of serum-deprived cells, which reached a maximal value of 5.3 μM at 18 s poststimulation but returned to near prestimulatory levels within 3 min. Moreover, no further changes in $[Ca^{++}]_i$ were observed during 12 subsequent h of continuous recording. PDGF produced a peak rise in $[Ca^{++}]_i$ to $\sim 1.4 \mu M$ at 115 s after stimulation, and FGF to $\sim 1.2 \mu M$ at 135 s after stimulation. EGF caused no change in $[Ca^{++}]_i$. The primary source of calcium for these transients was intracellular, since the magnitude of the serum-induced rise in $[Ca^{++}]_i$ was reduced by only 30% in the absence of exogenous calcium.

Phorbol 12-myristate 13-acetate (PMA) had no effect on resting $[Ca^{++}]_i$. When, however, quiescent cells were treated for 30 min with 100 nM PMA, serum-induced rises in $[Ca^{++}]_i$ were reduced by sevenfold. PMA did not inhibit growth factor-induced DNA synthesis and was by itself partially mitogenic.

We suggest that if calcium is involved as a cytoplasmic signal for mitogenic activation of quiescent fibroblasts, its action is early, transient, and can be partially substituted for by PMA. Activated protein kinase C may regulate growth factor-induced increases in $[Ca^{++}]_i$.

Growth factors bind to specific receptors on the surface of quiescent mammalian cells in tissue culture, initiating a mitogenic response that results, some 12–24 h later, in resumption of DNA synthesis (see references 13 and 52). Other cellular responses to growth factors are more rapid, including expression of the *c-fos* gene (26, 33), changes in cell shape and cytoskeleton (10, 39, 47, 48), and enhancement of pinocytotic rate (15, 21), all of which occur within minutes after stimulation. Presumably, cytoplasmic signals generated as a consequence of receptor binding are responsible for mediating the mitogenic and related responses. Several ions have recently been under investigation as possible cytoplasmic signals for growth factors (30–32, 40, 41).

There is some evidence that the calcium ion could be

involved in mitogenesis. (a) Growth of normal (4, 9) but not transformed (35, 53) fibroblasts is inhibited in low-calcium medium. (b) A transcellular flux of the $^{45}Ca^{++}$ calcium isotope is associated with mitogenic stimulation (38, 46, 58). (c) Additions of a supranormal concentration of calcium (17), or of calcium phosphate precipitate (7, 44) to medium are sufficient for mitogenic stimulation of some density-inhibited cells. Moreover, calcium's role as a possible secondary messenger is well documented in a variety of cellular responses. Changes in cytosolic calcium are clearly involved in lymphocyte stimulation with mitogenic lectins (55) and in activation of sea urchin and mammalian eggs during fertilization (18), to name just two of many such examples (see reference 12).

It will therefore be important to accurately measure the

magnitude and time course of changes in cytosolic calcium, if any, occurring as a consequence of mitogenic stimulation of quiescent cells. We describe here measurements of intracellular free calcium ion concentration ($[Ca^{++}]_i$)¹ of serum-deprived Swiss 3T3 fibroblasts stimulated with growth factors using aequorin as the calcium indicator. Aequorin was selected for these measurements because: (a) it is nontoxic and should not strongly buffer cytosolic calcium (5); (b) it can measure calcium transients as high as 100 μ M and with excellent sensitivity; and (c) it does not require intense illumination of cells with a potentially damaging light of short wavelength and so permits long-term, nondisruptive measurements of $[Ca^{++}]_i$. A major drawback in using aequorin as a calcium indicator has, until recently (6, 51), been the need to microinject it into cells, and we have described in a separate paper our method for loading aequorin into populations of viable fibroblasts (28).

MATERIALS AND METHODS

Cell Culture: Swiss 3T3 fibroblasts from American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum (Gibco Laboratories), 0.3 mg/ml L-glutamine, 50 U/ml penicillin, and 0.05 mg/ml streptomycin. 3T3 cells were routinely passaged before confluence by trypsinization in 0.05% trypsin and 0.02% EDTA in Ca^{++} -, Mg^{++} -free saline. Passage number of these cells ranged from 126 to 131.

Introduction of Aequorin into Fibroblast Cytoplasm: We have described in detail the technique for loading aequorin into cell cytoplasm and have characterized the properties of such aequorin-loaded cells in a separate paper (28). Our method is based on the previously published technique of scrape-loading (29). Briefly stated, fibroblasts to be loaded with aequorin were grown on plastic culture dishes, washed several times in calcium-free saline, and then scraped from the substratum in the presence of 20–100 μ l of a calcium-free solution of 1 mg/ml aequorin. DME containing 10% calf serum was then added back to the loaded cells, and 10^4 cells were introduced into each sterile Sykes-Moore chamber to be used in subsequent measurements of luminescence. The cells adhered to the lower glass coverslip of this chamber (2.0-cm diam) and were allowed to recover in a CO_2 (5%) incubator at 37°C for 24 h before any measurements of $[Ca^{++}]_i$ were performed.

Light Collection, Signal Recording, and Calibration: The aequorin luminescence from fibroblasts was detected, recorded, and calibrated as described (28). Luminescent signals representing resting levels of $[Ca^{++}]_i$ were often measurable in the apparatus for up to 48 h after loading of fibroblasts with aequorin. The temperature of the chamber containing the cells was 37°C. Luminescence from cells was converted to estimates of $[Ca^{++}]_i$ by the calibration method of Allen and Blinks (1).

Presentation of Growth Factors to Cells: Growth factors were diluted into 3 ml of DME (without phenol red) gassed with 5% CO_2 and warmed to 37°C. This volume was then injected directly into the Sykes-Moore chamber (volume, 0.5 ml) at a rate of 0.2 ml/s through a stainless steel needle. Perfusion at this rate did not affect cell $[Ca^{++}]_i$, density, or health, but higher rates were avoided as they tended to break chamber coverslips.

Measurement of Growth Factor-stimulated DNA Synthesis: Cells scraped or trypsinized from culture dishes were plated in DME containing 10% calf serum on 35-mm petri dishes ($\sim 5 \times 10^4$ cells/dish), rinsed 2 h later with 2 ml of serum-free DME, and then given a further 2 ml of serum-free DME. 24 h later, 1.0 ml of growth factors in serum-free DME, or DME alone, was added to triplicate dishes along with 1 μ Ci [3H]thymidine (New England Nuclear, Boston, MA). Parallel dishes to be used for counting cell number received growth factors or serum-free medium but no [3H]thymidine. 24 h after addition of growth factors, the dishes were rinsed three times with 2 ml of cold phosphate-buffered saline (PBS), cells were solubilized with 0.5 ml of 0.1% SDS, and dishes were rinsed with a second 0.5 ml of SDS. 1 ml of 1 mg/ml bovine serum albumin (BSA) was added to the solubilized cells in

¹ *Abbreviations used in this paper:* $[Ca^{++}]_i$, intracellular free calcium ion concentration; DCS, dialyzed calf serum; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate.

conical 15-ml centrifuge tubes, 100% trichloroacetic acid (TCA) (wt/vol) was added to give a final concentration of 10% TCA, and the precipitate collected by centrifugation at 2,000 rpm for 10 min. Supernatants were then aspirated, and the pellets were resuspended in 0.5 ml of 0.1 N NaOH and added to 10 ml of scintillant. Counts per minute (cpm) were made on a Beckman LS7000 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Parallel cultures that received no [3H]thymidine were trypsinized and counted in a Coulter counter so that [3H]thymidine incorporation into DNA could be expressed as cpm per cell.

Biochemicals: Partially purified platelet-derived growth factor (PDGF), epidermal growth factor from mouse submaxillary gland (EGF; receptor grade), and fibroblast growth factor from bovine pituitary gland (FGF) were all purchased from Collaborative Research Associates (Lexington, MA). Calf serum (DCS) was dialyzed extensively against DME before use as a mitogen. Ca^{++} -free DCS was produced by extensive dialysis against Ca^{++} -free DME until the $[Ca^{++}]_i$, checked with a calcium electrode (3), was $< 1 \mu$ M. Aequorin was obtained from Dr. J. Blinks (Mayo Clinic, Rochester, MN). PMA (Sigma Chemical Co., St. Louis, MO) was dissolved at 1 mg/ml in dimethyl sulfoxide and stored frozen at $-70^\circ C$ until use.

RESULTS

Establishment of Quiescent Populations of Fibroblasts Loaded by Scraping

Serum deprivation is one method used to establish quiescent cultures of fibroblasts that can respond to mitogens (11). As expected, we found that plating our line of Swiss 3T3 cells after scraping or trypsinization in 0.1% or serum-free medium inhibited cell growth, whereas plating the cells in 10% serum permitted normal logarithmic growth at identical rates of both scraped and trypsinized cells (data not shown). Growth of fibroblasts was not therefore affected by scraping, as opposed to trypsinization. Further, we have shown in a previous paper (28) that fibroblasts loaded with aequorin by scraping grow at rates comparable to those scraped in the absence of aequorin, implying that aequorin is not cytotoxic.

Mitogenic Responsiveness of Fibroblasts to Growth Factors after Scrape-Loading

Cells deprived of serum for 24 h after scraping or trypsinization were given fresh medium containing either serum, no serum, PDGF, FGF, or EGF. [3H]thymidine incorporation into DNA, measured 24 h later, was highest in scraped or trypsinized cells given 10% serum, but enhanced incorporation was also evident in cells given PDGF and FGF (Fig. 1). EGF from two separate sources was not mitogenic at any concentration tested (1–1,000 ng/ml). Fibroblasts loaded by scraping were therefore responsive to growth factors and, in the case of serum, at a level not reduced relative to trypsinized controls.

Resting $[Ca^{++}]_i$ Is Elevated in the Serum-deprived Fibroblast

Fig. 2 illustrates the relationship between aequorin luminescence and Ca^{++} concentration. Between 10^{-7} and 10^{-4} M Ca^{++} , the measured fractional luminescence (L/L_{max}) increases by $> 10^3$ -fold. The maximal luminescence rate (L_{max}) can be obtained by lysis of cells immediately after an experiment with Triton-X in the presence of saturating calcium as described previously (1, 28). The resting luminescence rate (L) of 10^4 fibroblasts loaded with aequorin by scraping provides a measurable analogue signal of 5–10 nA at 24 h after loading (28). Therefore, we were able to calculate the fractional luminescence of both quiescent and growing fibroblasts

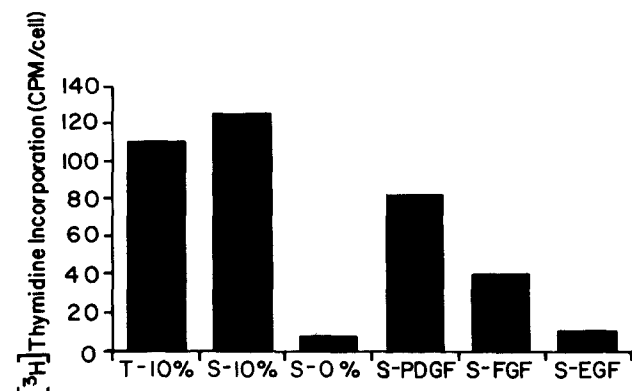


FIGURE 1 Growth factor-induced DNA synthesis by serum-deprived cells. Fibroblasts, which were scraped (S) or trypsinized (T) from 35-mm dishes and deprived of serum for 24 h, received 10% CS (10%), serum-free DME (0%), 2 U/ml PDGF (PDGF), 100 ng/ml FGF (FGF), or 100 ng/ml EGF (EGF). Stimulated DNA synthesis, measured as [³H]thymidine incorporation into TCA-insoluble material, was determined 24 h after addition of growth factor and radiolabel (see Materials and Methods for details).

and hence estimate their $[Ca^{++}]_i$ (Fig. 2). The $[Ca^{++}]_i$ of serum-deprived cells was $89 (\pm 20)$ nM, about twofold higher than that of growing cells kept in 10% serum, whose $[Ca^{++}]_i$ was $50 (\pm 19)$ nM. This and subsequent calculations of $[Ca^{++}]_i$ assume that free cytosolic Ca^{++} was uniformly distributed throughout the cytoplasmic volume containing the aequorin. Our estimates of resting $[Ca^{++}]_i$ are well within the range of those values measured independently in a variety of mammalian cells using various types of calcium indicators (6, 30, 32, 56, 57).

The $[Ca^{++}]_i$ of Quiescent Fibroblasts Increases Transiently to Micromolar Levels upon Stimulation with Growth Factors

The consequence of presenting 10% DCS to serum-deprived (24 h) fibroblasts is illustrated in Fig. 3A. Within 20 s, the aequorin-luminescence from these cells rose almost 240-fold, corresponding to an increase in $[Ca^{++}]_i$ from ~ 150 nM before stimulation to a maximum of almost $5.6 \mu M$. This rise was transient, since $[Ca^{++}]_i$ declined to within 5% of prestimulatory levels by 3 min after initial stimulation. The aequorin luminescence of these serum-stimulated cells was recorded continuously during the next 12 h, during which time no further change in cytosolic calcium was detected (Fig. 3B).

Serum was not the only demonstrable mitogen to transiently raise $[Ca^{++}]_i$. PDGF at mitogenic dosage produced a transient rise in cytosolic calcium which began ~ 27 s after stimulation and peaked at $1.3 \mu M$ $[Ca^{++}]_i$ (Fig. 4A). Perfusion of these same cells with 10% DCS, after $[Ca^{++}]_i$ had returned to near resting levels (see second arrow in Fig. 4A), raised $[Ca^{++}]_i$ only slightly. Antagonism by PDGF of the serum-induced rise in $[Ca^{++}]_i$ is not surprising, since PDGF is the major mitogen of serum (52).

FGF resulted also in a rise in $[Ca^{++}]_i$, to $\sim 0.9 \mu M$ in the record of Fig. 4B, but onset of this rise in calcium was clearly slower than that for DCS or PDGF (~ 70 s in this record). In contrast to PDGF, FGF did not inhibit subsequent transients of $[Ca^{++}]_i$, resulting from presentation of 10% DCS (data not shown).

EGF did not stimulate any change in $[Ca^{++}]_i$ when used at

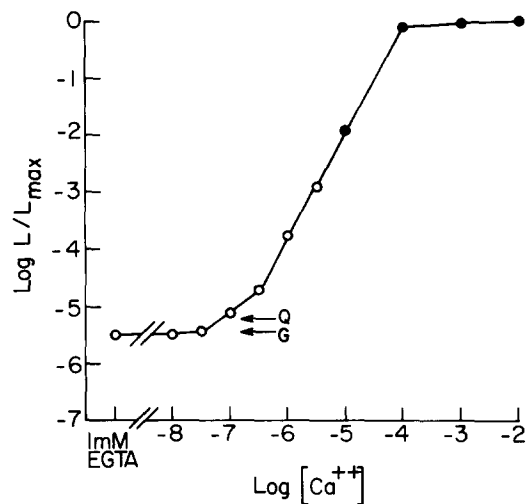


FIGURE 2 In vitro calibration of fractional aequorin luminescence (L/L_{max}) as a function of $[Ca^{++}]$. Arrows denote the mean fractional luminescence measured in vivo from serum-deprived, quiescent (Q) and growing (G) populations of fibroblasts. Calibration was performed as described previously (28) in 150 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES at pH 7.0 and $37^\circ C$. Solutions were buffered with 1.0 mM EGTA below 10^{-5} M Ca^{++} (O); solutions with $>10^{-5}$ M Ca^{++} were not buffered with EGTA (●).

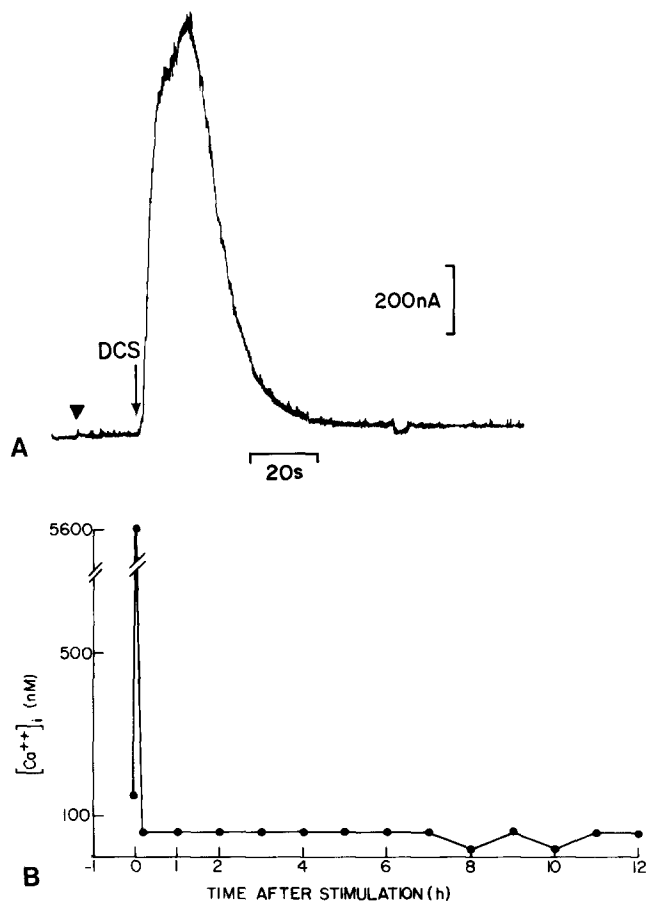


FIGURE 3 $[Ca^{++}]_i$ of serum-deprived fibroblasts stimulated with 10% serum. (A) 10% DCS, presented at the arrow, resulted in an immediate, 240-fold rise in the aequorin luminescence of these serum-deprived cells (number = 10^4). At the arrowhead, the shutter was opened, and "resting" luminescence from the cells produced an ~ 10 nA photomultiplier current. (B) Long term measurement of $[Ca^{++}]_i$ from serum-deprived cells after presentation of 10% DCS at time zero.

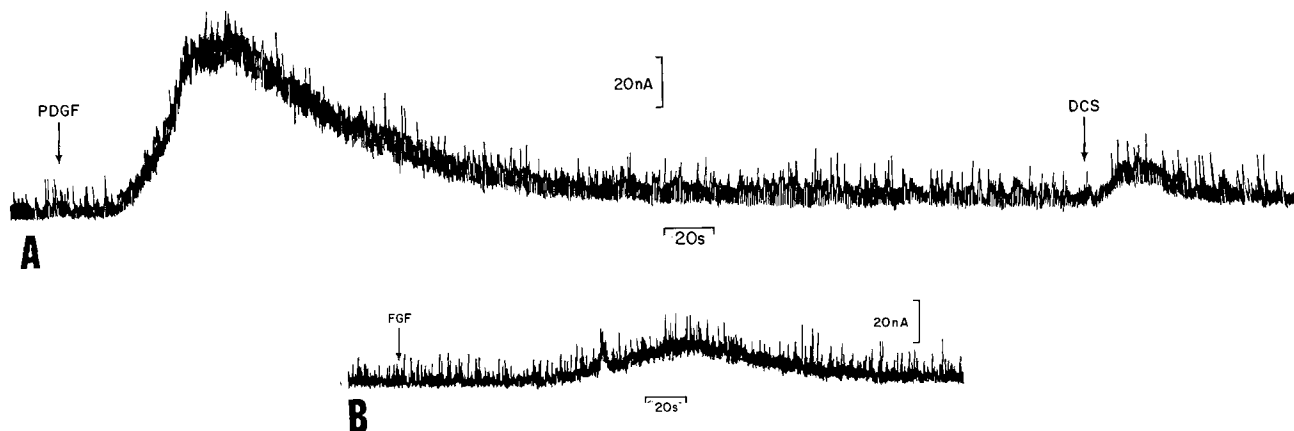


FIGURE 4 $[Ca^{++}]_i$ of serum-deprived fibroblasts stimulated with PDGF and FGF. (A) PDGF (2 U/ml), presented at the first arrow, resulted in a rise in aequorin luminescence after a delay of ~ 20 s. 10% DCS, presented at the second arrow, resulted in a rise in luminescence of only 1/50 that of cells that had not previously been presented PDGF. (B) FGF (100 ng/ml) presented at the arrow, also caused a rise in luminescence from serum-deprived cells. Since the amount of active aequorin loaded into cells varied from one experiment to another, so also did L_{max} . Thus, peak height of stimulated luminescence for two separate experiments, as here for PDGF and FGF, are not by themselves reliable indications of $[Ca^{++}]_i$.

TABLE I. Transients in $[Ca^{++}]_i$ Caused by Growth Factors

Culture	Growth factor	Maximal $[Ca^{++}]_i$ μM	Time to transient onset s	Time to maximal rise s	Total time transient* s
Growing	Serum (5) [†]	No change	—	—	—
Serum-deprived	DME only (10)	No change	—	—	—
	Serum (6)	5.3 ± 0.4 [‡]	2.5 ± 0.7	18 ± 7	180 ± 103
	PDGF (5)	1.4 ± 0.8	27 ± 12	115 ± 17	275 ± 32
	FGF (2)	1.2	70	135	240
	PMA (10)	No change	—	—	—

* Interval between initial rise and return of $[Ca^{++}]_i$ to $\leq 1\%$ of the prestimulatory level.

[†] Number of measurements.

[‡] Standard deviation of the mean.

concentrations between 1 and 1,000 ng/ml, and, as stated earlier, was also not mitogenic under the conditions we used (data not shown).

The characteristics of the calcium transients stimulated by the various growth factors are summarized in Table I.

An Internal Source of Calcium Is Mobilized by Growth Factors

A part, at least, of the calcium mobilized during the serum-induced transient appears to derive from intracellular stores. Thus, if as shown in Fig. 5, the population of serum-deprived cells was first washed with calcium-free medium containing 0.5 mM EGTA and then presented with calcium-free 10% DCS also containing 0.5 mM EGTA, the transient of $[Ca^{++}]_i$ was $4.0 \mu M$ on average, compared with $5.3 \mu M$ for controls presented with serum in the presence of 1.2 mM Ca^{++} (such as in Fig. 3A). This represents an average reduction of serum-stimulated rises in $[Ca^{++}]_i$ of only 30% in the absence of exogenous Ca^{++} .

PMA Inhibits Serum-induced Transients of $[Ca^{++}]_i$

No change in $[Ca^{++}]_i$ was evident as a result of presentation of PMA (1–1,000 nM) by itself to serum-deprived fibroblasts (Fig. 6A). When, however, 30 min after exposure to PMA, cells were perfused with 10% DCS, there was a 40-fold inhibition of stimulated aequorin-luminescence. $[Ca^{++}]_i$ rose to only $0.8 \mu M$ in those cells pretreated with 100 nM PMA (Fig.

6B), as compared with $5.6 \mu M$ in untreated controls. This inhibitory effect of PMA pretreatment was half-maximal between 5 and 10 nM (data not shown).

PMA inhibition of the serum-induced rise in $[Ca^{++}]_i$ cannot be explained as a direct effect of this treatment on the luminescence efficiency of aequorin molecules. In the first place, there was no difference between the L_{max} values measured upon Triton lysis of equal numbers of aequorin-loaded fibroblasts that were or were not treated with 100 nM PMA for 30 min. Secondly, 100 nM PMA was without effect on the luminescence of aequorin in vitro (data not shown).

PMA Does Not Affect Mitogenic Stimulation of Quiescent Fibroblasts

Since transients of $[Ca^{++}]_i$ were associated with presentation of growth factors active in stimulating DNA synthesis in serum-deprived fibroblasts, it was of interest to determine whether PMA pretreatment, which inhibited these transients, would also inhibit stimulated DNA synthesis. We found that it did not: a 30-min pretreatment with 100 nM PMA did not significantly inhibit DNA synthesis upon stimulation with 10% DCS or PDGF, and was by itself partially mitogenic (Fig. 7).

PMA Alters the Morphology of Fibroblasts Loaded with Aequorin

There have been numerous reports that alterations in cell

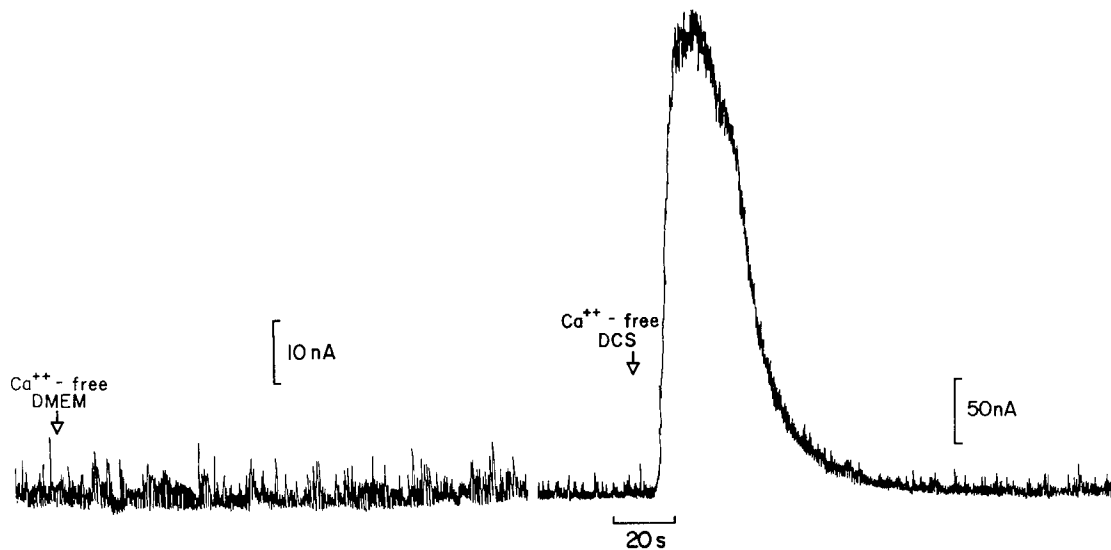


FIGURE 5 $[Ca^{++}]_i$ of serum-deprived fibroblasts stimulated with 10% serum in the absence of exogenous Ca^{++} . Cells were first rinsed with 6 ml of Ca^{++} -free DME containing 0.5 mM EGTA (first arrow), and a very slight drop in aequorin luminescence resulted. Ca^{++} -free 10% DCS (containing 0.5 mM EGTA), presented at the second arrow, resulted in a near normal rise in luminescence.

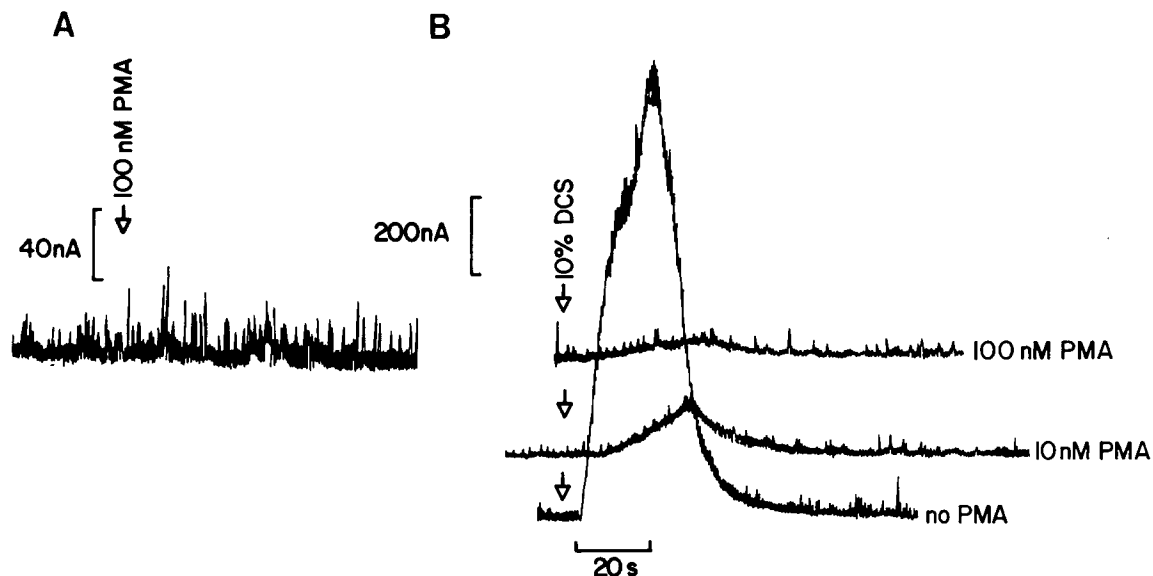


FIGURE 6 $[Ca^{++}]_i$ of serum-deprived cells stimulated first with PMA and then with 10% serum. (A) 100 nM PMA in serum-free DME, presented at the arrow, resulted in no change in aequorin luminescence. (B) Cells from A, and cells presented with 10 nM or no PMA, were stimulated 30-min later with 10% DCS. In comparison with the untreated controls, serum-induced luminescence of the 10 and 100 nM PMA-treated cells was delayed in onset and greatly reduced in magnitude.

shape and cytoskeletal architecture are a consequence of PMA treatment (39, 48). We recorded a change in the shape of serum-deprived fibroblasts after their incubation with 100 nM PMA for 30 min (Fig. 8). The PMA-induced change in cell shape of the cells in Fig. 8 was not associated with a change in $[Ca^{++}]_i$.

DISCUSSION

We have shown that an increase in the $[Ca^{++}]_i$ of serum-deprived, subconfluent Swiss 3T3 fibroblasts, measured as increase in the luminescence rate of aequorin, results from stimulation with serum, PDGF, and FGF. Each of these growth factors were demonstrably mitogenic under the conditions used. EGF, which was not mitogenic, did not raise

$[Ca^{++}]_i$. Three recent studies have similarly shown that some growth factors increase $[Ca^{++}]_i$: two employed the fluorescent calcium indicator, quin 2 (30, 32), and the third, aequorin (57), as in the present study.

There are at least two clear differences in the nature of calcium changes measured with quin 2 and those we have measured with aequorin. The first is the magnitude of the rise in $[Ca^{++}]_i$ stimulated by growth factors, which was lower as measured with quin 2. Moolenaar et al. (30), for example, measured a 2.8-fold rise in $[Ca^{++}]_i$, from 138 nM to 386 nM, as a result of serum stimulation, compared with the ~50-fold rise, from ~90 nM to ~5.3 μ M, reported here. The rise in $[Ca^{++}]_i$ measured by quin 2 as a result of PDGF stimulation was similarly less (to 290 nM) than that measured with aequorin (to 1.3 μ M). A small, 1.3-fold rise, induced by EGF,

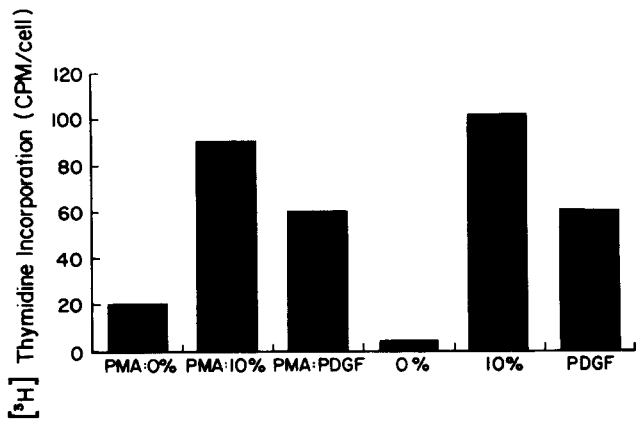


FIGURE 7 Comparison of growth factor-induced DNA synthesis by PMA-treated and untreated serum-deprived fibroblasts. Serum-deprived fibroblasts (previously scraped from the substratum as in Fig. 2) received 1 ml of 100 nM PMA in DME for 30 min (PMA), and then 2 ml of serum-free DME (0%), 10% CS (10%), or PDGF at 2 U/ml (PDGF). Control, untreated cells received 1 ml of DME without PMA for 30 min. As in Fig. 2, [³H]thymidine incorporation was measured 24 h after growth factor addition.

was also measured in their study. Morris et al. (32) measured a maximal rise in [Ca^{++}]_i above resting levels of 112–134% in response to EGF, vasopressin, and prostaglandin. The second difference is in the measured duration of the induced rises in [Ca^{++}]_i. These were longer as measured with quin 2; [Ca^{++}]_i remaining elevated over basal levels for up to 15 min after stimulation, beyond which measurements were not continued in either study (30, 32). By contrast, the rise in [Ca^{++}]_i measured with aequorin was clearly transient. [Ca^{++}]_i declined to prestimulatory levels or below within 2–4 min after growth factor stimulation, and no further rise in [Ca^{++}]_i was detected during the subsequent 12 h after initial presentation.

Because quin 2 can buffer [Ca^{++}]_i (56), it may have dampened out the growth factor-induced transients it measures. The intracellular concentration of quin 2 used by Morris et al. (32) was 2.5–3.0 mM; that used by Moolenaar et al. (30) was not stated, but similar conditions were used for loading. If, in the extreme case, the concentration of aequorin loaded into cytoplasm equaled that present during scraping, then its cytosolic concentration would have been 1 mg/ml or 0.05 mM. This is probably a gross over-estimate. We can approximate from a previous study (29) that the concentration of 70,000-mol-wt dextran loaded into cytoplasm was ~1% of that present in the medium during scraping. If loaded with an equivalent efficiency, aequorin would then have been present at ~0.5 μM in fibroblast cytoplasm. Blinks et al. (5) show, as a rough estimate, that buffering of calcium by aequorin would not be significant, in an otherwise unbuffered system, if its concentration is kept below 0.1 μM. Since also the association constant for Ca^{++} of quin 2 (52) may be considerably greater than that of aequorin (5), it is clear that artifacts of calcium buffering were far less likely to occur using aequorin under the conditions we employed.

Quin 2 and aequorin have recently been compared directly as indicators of [Ca^{++}]_i (25). Thrombin-induced rises in platelet [Ca^{++}]_i measured with aequorin were an order of magnitude greater and also of much briefer duration than those measured with quin 2. This may in part be a consequence, as the authors suggest (25), of aequorin's larger signal to noise ratio (>10³ vs 2 for quin 2) in the range of 0.1–10 μM Ca^{++} .

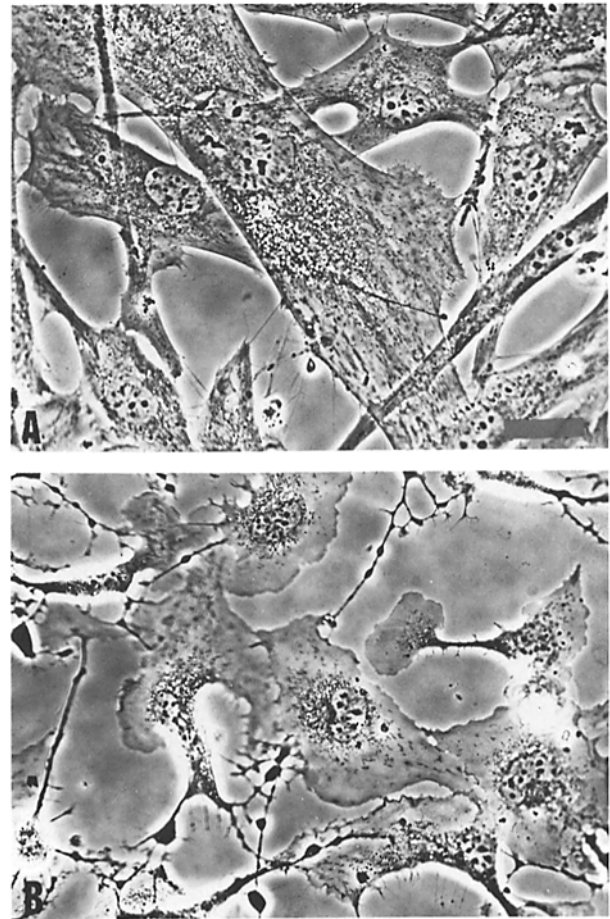


FIGURE 8 Morphological alterations induced by PMA in fibroblasts loaded with aequorin. (A) Serum-deprived cells before PMA treatment. (B) Cells from same Sykes-Moore chamber, but different microscope field, 30 min after incubation in 100 nM PMA during which continuous measurement of the aequorin luminescence of these cells indicated there was no change in [Ca^{++}]_i. Bar, 10 μm.

Hence, aequorin is far more sensitive to localized changes in [Ca^{++}]_i, and this property also could explain why it reported much higher but briefer growth factor-induced rises in [Ca^{++}]_i than did quin 2. It will be important to determine whether, in fact, growth factor-induced rises in [Ca^{++}]_i are localized, and we are presently addressing this question by light microscopic imaging of aequorin luminescence from single cells.

The different characteristics of the transients in [Ca^{++}]_i evoked by growth factors as measured with the two indicators may also be related to the different cell types used and/or to the conditions for establishing quiescence. Moolenaar et al. (30) deprived confluent cultures of human fibroblasts of serum for 24 h, whereas Morris et al. (32) grew Swiss 3T3 fibroblasts to a density-inhibited state on microcarrier beads and deprived them of serum for an unstated interval. The present study used serum deprivation of subconfluent cultures to induce quiescence. The responses of confluent cells to growth factors may differ significantly from those arrested in log-phase by serum deprivation. Another factor making comparisons difficult is that the mitogenicity of the serum, PDGF, and EGF dosages used during measurements of [Ca^{++}]_i were not measured by Moolenaar et al. (30). Morris et al. (32) found that EGF (12 ng/ml) presented with insulin (1 μg/ml) was mitogenic, but did not measure [Ca^{++}]_i after this dual stimulation. They showed that EGF by itself raised [Ca^{++}]_i,

whereas insulin alone did not. A further complication is that quin 2 itself has mitogenic activity for lymphocytes (23).

A transient rise in $[Ca^{++}]_i$ appears to be the earliest measurable effect of some growth factors on the ionic composition of cytoplasm, preceding the now well-documented Na^+/H^+ flux which results in cytoplasmic alkalinization (31, 40, 41). Increasing $[Ca^{++}]_i$ has been suggested as a trigger for cytoplasmic alkalinization induced by growth factors (38), and the role of such alkalinization as a cytoplasmic signal for mitogenesis has been emphasized (31). A recent report (31) shows that tumor-promoting phorbols or diacylglycerol can activate cytoplasmic alkalinization independently of any change in $[Ca^{++}]_i$, suggesting that Na^+/H^+ exchange is triggered by protein kinase C instead of calcium.

Tumor-promoting phorbol acetates stimulate the proliferation of various cell types, including fibroblasts (42). The mitogenic activity of phorbols for density-inhibited fibroblasts is dependent on serum factors or on polypeptides like EGF or insulin, which with PMA can stimulate growth in serum-free medium (16, 42). We found that PMA itself did not affect resting $[Ca^{++}]_i$, which confirms the results of both quin 2 studies (30, 32). We show, however, that PMA pretreatments of serum-deprived fibroblasts cause dramatic inhibition of growth factor-induced transients of $[Ca^{++}]_i$. One well-known effect of PMA is to activate protein kinase C. Diacylglycerol, a product of phosphoinositol breakdown at the plasma membrane, activates protein kinase C in ligand-stimulated cells (see references 27 and 36). Such breakdown of phosphoinositol has been shown to be an early consequence of stimulation with growth factors (20, 46), and diacylglycerol is mitogenic for fibroblasts (43). Activated protein kinase C phosphorylates seryl and threonyl residues of many endogenous proteins (36) including the receptors for some growth factors (24). Therefore, protein kinase C activated by PMA may inhibit growth factor-induced calcium transients by phosphorylating proteins responsible for regulating calcium efflux from internal stores, or by phosphorylating the membrane receptors for growth factors. Whitely et al. (59) have shown that the Na^+/H^+ flux normally stimulated by growth factors is inhibited when A431 cells are pretreated with PMA. Our finding supports their inference that activated protein kinase C may modulate numerous early ionic events of mitogenesis. We speculate that activation of protein kinase C may provide a mechanism for regulating the duration of growth factor-induced calcium flux, which we have shown is clearly transient. Further research will be necessary in evaluating such a role for protein kinase C. Several recent reports show that PMA inhibits other ligand-induced rises in $[Ca^{++}]_i$, and such inhibition may therefore be a general cellular phenomenon (22, 34, 45).

PMA pretreatments were here shown to inhibit serum-induced transients of $[Ca^{++}]_i$, but they did not affect the mitogenicity of serum or PDGF. Thus, for cells stimulated with PMA and subsequently with growth factors, the greater than micromolar rise in $[Ca^{++}]_i$ we have measured is not necessary for mitogenesis. In addition, the magnitudes of the rises in $[Ca^{++}]_i$ induced by serum, PDGF, and FGF (Table I) did not strictly correlate with their relative mitogenicity in the $[^3H]$ thymidine assay (Fig. 2). Thus, rises in $[Ca^{++}]_i$ cannot by themselves explain the mitogenicity of a given growth factor, whether or not the cells have been previously treated with PMA. It has however been suggested that activated protein kinase C and elevated $[Ca^{++}]_i$ work synergistically to activate

numerous cellular responses (36). Since PMA did not completely abolish the calcium transients induced by growth factors, such synergism remains a possible but unproven mechanism for cytoplasmic signaling during mitogenesis.

Using aequorin, we estimate that serum and those growth factors demonstrably mitogenic in serum-free medium and without added cofactors all raised $[Ca^{++}]_i$ to micromolar levels or above (a possible exception may be PMA, which appears to be partially mitogenic, but did not raise $[Ca^{++}]_i$). But our long term measurements (12 h) suggest that the role of Ca^{++} as a cytoplasmic signal for growth factors, if any, is transient, being limited to the initial few minutes after stimulation. Moreover, $[Ca^{++}]_i$ of growing fibroblasts was lower than that of serum-deprived cells, suggesting that elevated $[Ca^{++}]_i$ is not required for the uninterrupted, log-phase growth of subconfluent cells in serum or growth factors. The source of Ca^{++} for growth factor-induced transients of $[Ca^{++}]_i$ appears to be an internal compartment, which has not yet been identified. Inositol triphosphate, a water-soluble product of phosphoinositol turnover, has been suggested to be a signal for releasing Ca^{++} from internal stores of several cell types (see references 2 and 54). We summarize our data and speculations in Fig. 9.

DNA synthesis is not the only response of fibroblastic cells to growth factors. Chemotaxis (19, 49, 50), cell shape and cytoskeletal modifications (10, 14, 39, 47, 48), undirected motility (37), and enhanced pinocytosis (15, 21) are all rapidly activated by mitogens. It is therefore possible that any rapid change in the cytoplasmic environment stimulated by growth factors, such as increased $[Ca^{++}]_i$, may be involved in activation of the above responses by a pathway equivalent to, or divergent from, that leading to mitogenesis. For example, we found, as previously observed many times, that PMA caused a rapid (<30 min) change in cell shape (Fig. 8), and it was recently shown that such shape changes are coincident with a major reorganization of the actin-vinculin based cytoskeleton (10, 48). Our aequorin measurements show that such alterations in cell shape and cytoskeleton were not triggered by calcium, since PMA itself was without effect on $[Ca^{++}]_i$.

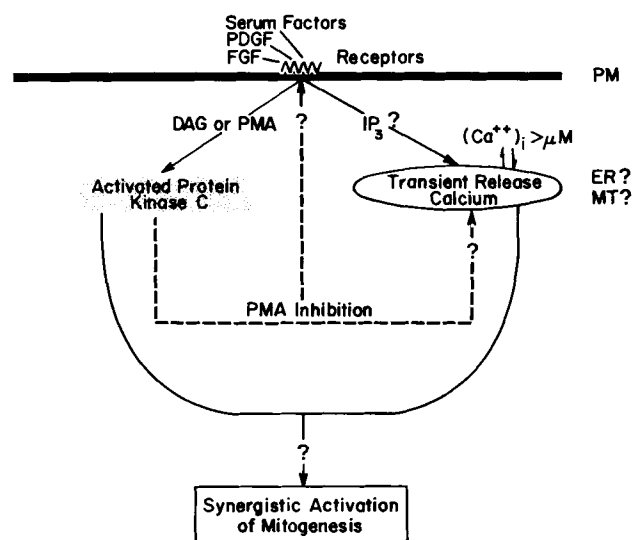


FIGURE 9 Schematic summary of data and speculations of the present paper. See text for explanation. PM, plasma membrane; ER, endoplasmic reticulum; MT, mitochondria; DAG, diacylglycerol; IP_3 , inositol triphosphate.

Phosphorylation of cytoskeletal proteins by PMA-activated protein kinase C is the suggested but unproven mechanism for PMA-induced changes in cell shape (48). Unravelling the cascade(s) of events, ionic and otherwise, initiated by growth factors may therefore bring us closer to understanding several important responses of mammalian cells.

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