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Effects of Platelet-Derived Growth Factor and Fibroblast Growth Factor on Free Intracellular Calcium and Mitogenesis

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Although increased free intracellular calcium (Ca_i) may be one of the main regulators of cell growth and differentiation, studies in cell populations have implied that not all growth factors produce Ca_i increases. In order to examine in more detail whether Ca_i increases were related to mitogenesis, we used digital image analysis of intracellular Fura-2 fluorescence to measure Ca_i in individual BALB/c 3T3 cells stimulated with either platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF). We found that PDGF induced larger and more prolonged Ca_i increases than FGF did, but that both growth factors induced an initial rapid increase in Ca_i (<2 min) followed by a later sustained increase (>20 min). Only the prolonged Ca_i increase required extracellular calcium. Following PDGF treatment (1–8 units/ml), the percentage of cells with a large peak Ca_i increase (>twofold) correlated with the percentage of cells made competent (subsequent growth in 1% platelet-poor-plasma). In contrast, purified bovine basic FGF (200–800 pg/ml) and recombinant human acidic FGF (10–300 ng/ml) produced peak Ca_i increases that were not directly correlated with mitogenesis. In addition, concentrations of intracellular Quin 2 that inhibited Ca_i transients also inhibited PDGF stimulation but not FGF stimulation of mitogenesis. Thus, Ca_i increases are necessary for mitogenesis in BALB/c 3T3 cells stimulated by PDGF, but not that stimulated by FGF.

Key words: calcium, Fura-2, growth factors, competence, PDGF, autoradiography, digital image analysis, FGF

Increases in free intracellular calcium (Ca_i) are among the earliest events stimulated by growth factors [1–3]. However, no evidence has been presented to indicate whether these early ionic signals are produced by all growth factors or whether Ca_i increases are required for mitogenic events. Platelet-derived growth factor (PDGF) definitely increases Ca_i [1–3], but Ca_i increases induced by fibroblast growth factor (FGF) are controversial. In one study of cell populations, FGF produced small increases in Ca_i [4] measured by Quin-2 fluorescence, but in another study no Ca_i change was detected using aequorin luminescence [5]. Moreover, only

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small and inconsistent increases in ^{45}Ca efflux were measured in cell populations stimulated with FGF, in contrast to large increases of ^{45}Ca efflux induced by PDGF [Tucker RW, Meade-Cobun K, Phair R; unpublished observation]. Yet, short treatment (20 min) of BALB/c 3T3 cells with FGF produced a larger mitogenic effect (more cells competent to initiate DNA synthesis) than that produced by the same short exposure to PDGF. These studies suggested that Ca_i increases that were detectable in cell populations (e.g., large synchronous increases in most cells) correlated with mitogenesis induced by PDGF but were not necessary for mitogenesis induced by FGF. However, significant questions remain about whether small, asynchronous, or localized increases in Ca_i, which are difficult to measure in cell populations, can contribute to mitogenesis induced by FGF, and whether Ca_i increases are actually required for mitogenesis induced by PDGF.

Determining the relationship between Ca_i increases and DNA synthesis in cell populations has been difficult. Both Ca_i increases and DNA synthesis can be heterogeneous and variable from time to time and from cell to cell. Thus, measurements of Ca_i in cell populations represent spatial and temporal averages only, and will underestimate asynchronous Ca_i increases or will fail to detect localized Ca_i changes. In contrast, analysis of single cells can be used to compare Ca_i changes and DNA synthesis in the same cell or population and to define spatial changes in Ca_i. In the present study we used digital image analysis of intracellular Fura-2 to measure Ca_i changes in single quiescent BALB/c 3T3 cells stimulated with either PDGF or FGF. Both PDGF and FGF induced similar spatial and temporal increases in Ca_i, but only the Ca_i increases produced by PDGF correlated with mitogenesis; moreover, buffering of Ca_i changes by intracellular Quin 2 inhibited PDGF-stimulated but not FGF-stimulated mitogenesis. Thus, Ca_i increases appear to be necessary for mitogenesis stimulated by PDGF, but not for that stimulated by FGF.

MATERIALS AND METHODS

Cell Culture and Materials

An original stock of BALB/c 3T3 cells (Clone A-31) was obtained from G. Todaro (Frederick, MD), and cells were grown in Dulbecco modified Eagle medium (DME; Gibco) supplemented with 5% calf serum (CS; Gibco). BALB/c 3T3 cells (8×10^4/dish) were plated and allowed to become quiescent during 6 days of growth in DME + 0.5% CS at 37°C and 7% CO_2.

Purified PDGF (AB heterodimer) was obtained from outdated human platelets (Dr. W.J. Pledger, Vanderbilt University) and stored as 4×10^4 units/ml in 1 M acetic acid at −30°C. Immediately before use, PDGF stock was diluted 1:400 with 0.1% bovine serum albumin (BSA) in calcium-free Krebs/Hepes buffer (in mM; NaCl 120, KCl 4, KH_2PO_4 1, $MgSO_4$ 1, Hepes 1.2) supplemented with 5 mM glucose and amino acids (minimal essential medium, Gibco). Purified basic FGF was obtained from Dr. G.D. Shipley (Oregon Health Sciences University) and was prepared as previously described from bovine brain [8]. In addition, recombinant human acidic FGF was obtained from Chiron (Emeryville, CA). Both FGF preparations were stored in 0.1% gelatin in chelexed water and were diluted in 0.1% gelatin in Krebs/Hepes buffer for experiments. Platelet-poor plasma (PPP) was prepared from human serum as previously described [7].

Autoradiography

All cultures were assayed for DNA synthesis by autoradiography of cells incubated with growth factors in tritiated thymidine (1 μCi/ml; 60 Ci/mg, New England Nuclear) as previously described [8]. Briefly, after treatment with growth factor for 1 h and subsequent incubation with 1% PPP and tritiated thymidine for 48 h, cell monolayers were fixed in 95% ethanol, dipped in Kodak emulsion (NTB-2), and incubated in the dark for 48 h. Emulsion-covered slides were then developed and fixed as previously described [8], and the percentage of cells with labeled nuclei was determined. The percentage of cells made competent for DNA synthesis was measured as the percentage of labeled cells stimulated by growth factor followed by 1% PPP, minus the percentage of labeled cells stimulated by 1% PPP alone. In all experiments the percentage of cells able to initiate DNA synthesis in the presence of 1% PPP alone was less than 10%.

Fura-2 Loading

Fura-2/AM loading of BALB/c 3T3 cells was performed as a modification of previously described procedures [9]. Briefly, BALB/c 3T3 cells were cooled for 15 min on ice, then incubated for 2 h at room temperature with 25 μM Fura-2/AM, 0.03% Pluronic (Pluronic F127, BASF Wyandotte Corp.), 0.01% dimethyl sulfoxide (DMSO), and 0.75% PPP in Krebs/Hepes (in mM; NaCl 120, KCl 4, KH_2PO_4 1, $MgSO_4$ 1, $CaCl_2$ 1.3, Hepes 1.2). Fura-2 loading under these conditions did not enhance or inhibit DNA synthesis stimulated by 20% CS (data not shown).

Intracellular Fura-2 concentration (750 uM) was estimated from the fluorescence of material released from cells permeabilized with 0.001% digitonin in Krebs/Hepes buffer for 20 min. The excitation spectrum of permeabilized cell material was measured using a Perkin-Elmer fluorescence spectrophotometer (4-nm slits) with a Xenon lamp (in collaboration with Dr. Reynafarje, Johns Hopkins Medical School) and emission filters set at 500 nm.

Digital Imaging of Intracellular Fura-2

Ca_i was calculated from the fluorescence of intracellular Fura-2 as previously described [10]. Briefly, the microscope system consisted of an inverted epifluorescence microscope (Leitz Diavert), 100× or 40× Nikon UV-Fluor (N.A. 1.3) objectives, and a DC-stabilized 50-W mercury arc lamp. All optical components in the excitation pathway were either quartz or glass, with high transmittance for ultraviolet (UV) wavelengths. Fluorescent images of cells, buffer, and Fura-2 solutions were obtained at 340 and 380 nm excitation using narrow-band ($\pm$ 8 nm) interference filters (Ditric Optics, Hudson, MA), and emitted light was collected through a 500-nm broad-band filter ($\pm$ 40 nm) (Ditric Optics). All calculations and digitizations were performed using a microcomputer image-analysis system (BIAS, Loats Associates, Inc., Westminster, MD) based on an IBM/AT. An Epyx silicon video board was used to digitize a central region containing 128 horizontal × 240 vertical pixels. Thirty-two frames were averaged per wavelength, and images were acquired every 20 s, with 256 grey-level precision (8 bit). Ca_i images were photographed from the monitor using a Minolta 35-mm camera with Plus-X film.

Calculations of Ca_i

Ca_i was calculated from the relation $Ca_i = Kd\ B(R - R_{min})/(R_{max} - R)$ where K_d is the dissociation constant for Fura-2, B = Fura-2 (380 nm)/Ca Fura-2 (380 nm),

R = cell (340 nm)/cell (380 nm), R_{min} = Fura-2 (340 nm)/Fura-2 (380 nm), and R_{max} = Ca Fura-2 (340 nm)/Ca Fura-2 (380 nm) (with excitation wavelength in parentheses). Fura-2 was a solution of Fura-2 (10–100 uM) in MOPS buffer (in mM: KCl 100; KMOPS 10) containing 10 mM EGTA, and Ca Fura-2 was a Ca^{2+}-saturated solution of Fura-2 in MOPS buffer containing 1 mM $CaCl_2$. K_d = 220 nM, in agreement with previous reports [11,12]. Background images of buffer solutions in the dish were subtracted from cell images and Fura-2 solution images to correct for background noise (dark current) of the video camera and inherent fluorescence of the optical system. The spatial heterogeneity of illumination and camera sensitivity were compensated for by the inclusion of ratios of the uniform solutions of Fura-2 in the calculations of Ca_i.

RESULTS

PDGF and FGF Both Increase Ca_i

Average basal Ca_i was 57 ± 22 nM (S.D.) for 151 cells. For any one cell, the basal Ca_i varied by 4.6% (S.D./mean), so that a 10% increase (1.1-fold) in Ca_i was significantly different from basal Ca_i ($P < .05$). Both PDGF and FGF induced transient increases in average Ca_i in quiescent BALB/c 3T3 cells (Figs. 1, 2). The Ca_i increase appeared to be generalized, occurring in both the nucleus and cytoplasm. The Ca_i increase was also biphasic, consisting of an initial Ca_i increase lasting 2 min, followed by a second Ca_i increase persisting for at least 20 min (the period of observation) (Fig. 3). The prolonged (>20 min) elevation of Ca_i (>1.5-fold) oc-

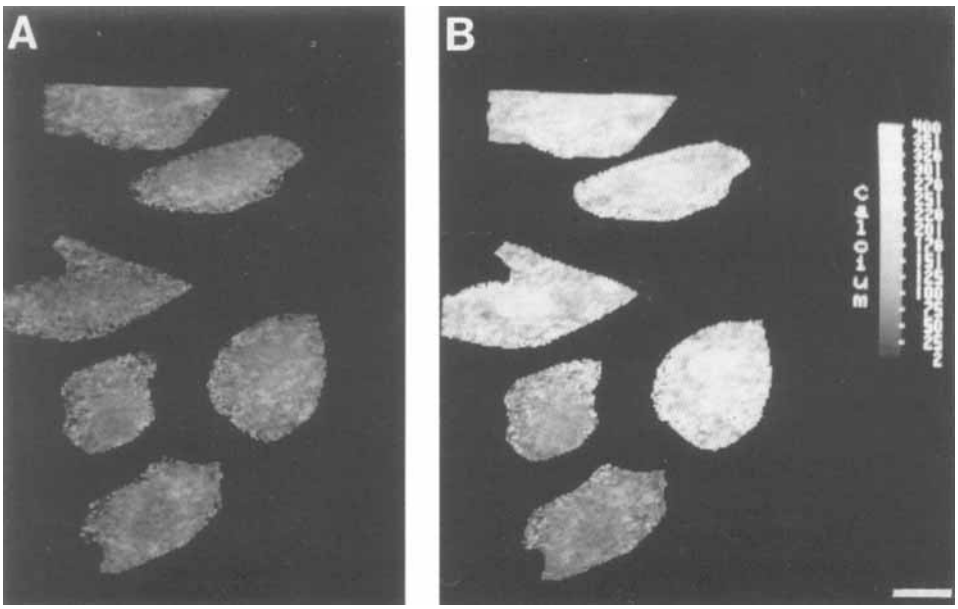

Fig. 1. PDGF increased Ca_i in both nucleus and cytoplasm. Images of Ca_i depict spatial variation of Ca_i in the cell, with Ca_i quantitated in nM and displayed as different intensities in the grey scale. **A:** Quiescent BALB/c 3T3 cells. **B:** 3 min after PDGF (8 units/ml) treatment of same cells as in A. Bar = 25 μm.

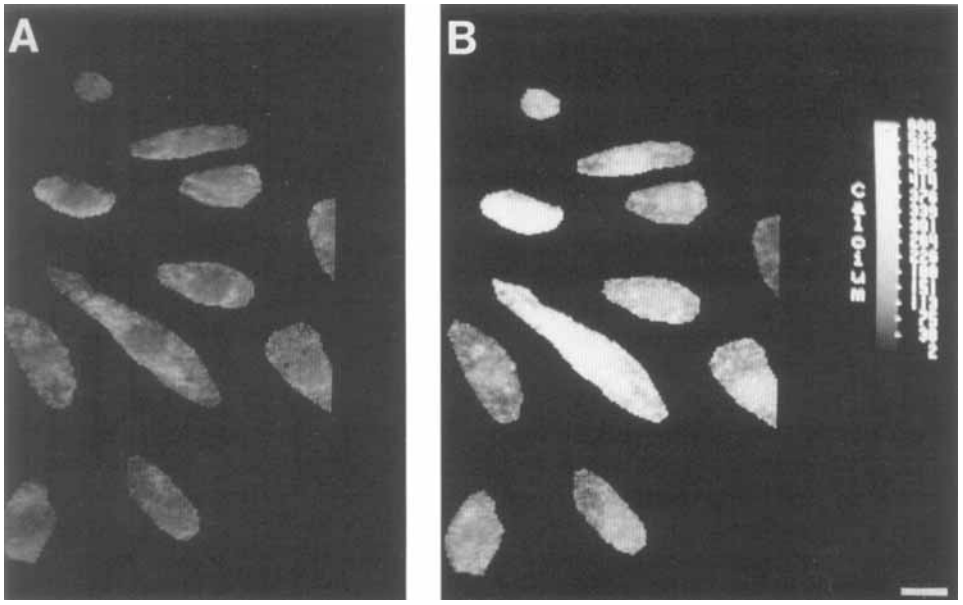

Fig. 2. FGF (human recombinant) increased Ca_i throughout the cell. **A:** Quiescent BALB/c 3T3 cells. **B:** 2.3 Min after FGF (30 ng/ml) treatment of same cells as in A. Bar = 25 μm.

curred in more than 50% of PDGF-treated cells (n = 54) and in only 10% of FGF-treated cells (n = 46). Only the initial Ca_i peak was independent of extracellular calcium (Fig. 3B,D). At maximal stimulation, PDGF produced tenfold increases in Ca_i, while FGF produced only sixfold increases (Fig. 4–6). Detailed examination of the localization of Ca_i changes did not reveal any striking spatial pattern of Ca_i increase that was consistently correlated with either growth factor stimulation. Occasionally, Ca_i increases started around the nucleus or in the periphery of the cell, but these initial changes quickly led to the more generalized Ca_i increases shown in Figures 1, 2, and at present we have not determined any pattern to these localized transient increases. Thus, detailed examination of changes in Ca_i distribution have revealed many similarities in Ca_i increases induced by PDGF and FGF. Only the increased magnitude of the initial peak Ca_i change and the presence of the prolonged Ca_i increase distinguished PDGF-treated cells from FGF-treated cells.

Ca_i Increase Induced by PDGF Correlated With Mitogenesis

Increasing concentrations of PDGF stimulated a larger percentage of cells to respond with Ca_i increases, so that the percentage of cells with Ca_i increases in each of three categories (< twofold, two- to fourfold, > fourfold) rose progressively as the dose of PDGF increased (Fig. 4). Larger concentrations of PDGF also increased the magnitude of the peak Ca_i increase in each cell. In fact, cells treated with maximal doses (8 units/ml) of PDGF responded with particularly large (> fourfold) increases in peak Ca_i . Since increasing PDGF concentration (2-h exposure) also produced a progressive increase in the percentage of cells made competent for DNA synthesis (Table I) we compared Ca_i increase and DNA synthesis induction in the same cell population. For example, a high PDGF dose (8 units/ml) induced competence in the

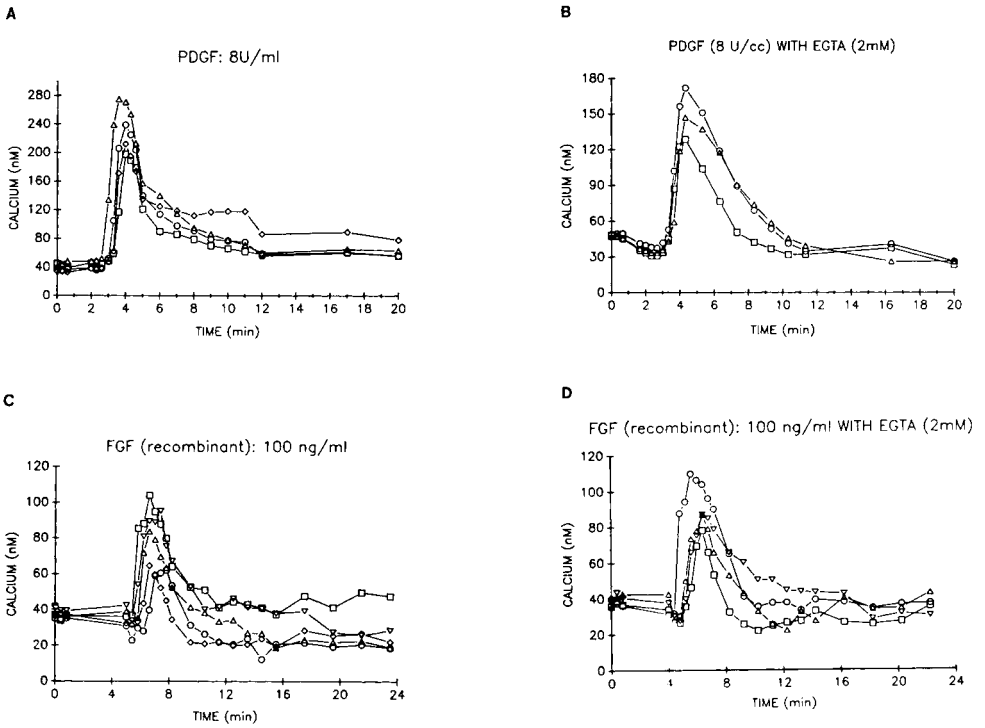

Fig. 3. PDGF and FGF produce Ca_i increases, with initial Ca_i change independent of extracellular Ca^{2+}. Time course of average cellular Ca_i in individual cells following stimulation of quiescent BALB/c 3T3 cells with treatment indicated. **A:** PDGF (8 units/ml). **B:** PDGF (8 units/ml) + EGTA (2 mM). **C:** Human recombinant FGF (100 ng/ml). **D:** Human recombinant FGF (100 ng/ml) + EGTA (2 mM). Growth factors were added 1 min after Ca_i measurement began.

majority of the quiescent cells and stimulated an increase in Ca_i to more than four times basal levels in most cells (54%) and more than two times basal levels in almost all cells (>92%). Thus, as the percentage of cells with an increase in Ca_i rose in response to increased concentrations of PDGF, so also did the percentage of cells made competent to initiate DNA synthesis.

Ca_i Increases Induced by FGF Did Not Directly Correlate With Mitogenesis

In contrast to PDGF, FGF stimulated Ca_i increases that did not correlate with the induction of competence for DNA synthesis (Table II). For human recombinant acidic FGF, there was very little difference in the Ca_i increase stimulated by low (10 ng/ml) and high (300 ng/ml) doses (Fig. 5). However, the high dose of FGF (300 ng/ml) made 75% of the cells competent, while the low dose (10 ng/ml) induced competence in only 1% of cells (Table II). Therefore, in cells stimulated by recombinant FGF there was no correlation between the percentage of cells with Ca_i increases and the percentage of cells made competent. In addition, low concentrations of purified bovine basic FGF (200 pg/ml) produced barely detectable increases (>1.2-fold) in Ca_i (Fig. 6) and yet 45% of the same cells were made competent by a 1-hr exposure to this concentration of FGF (Table II). Thus, neither purified bovine basic FGF nor recombinant human acidic FGF produced Ca_i increases in a percentage

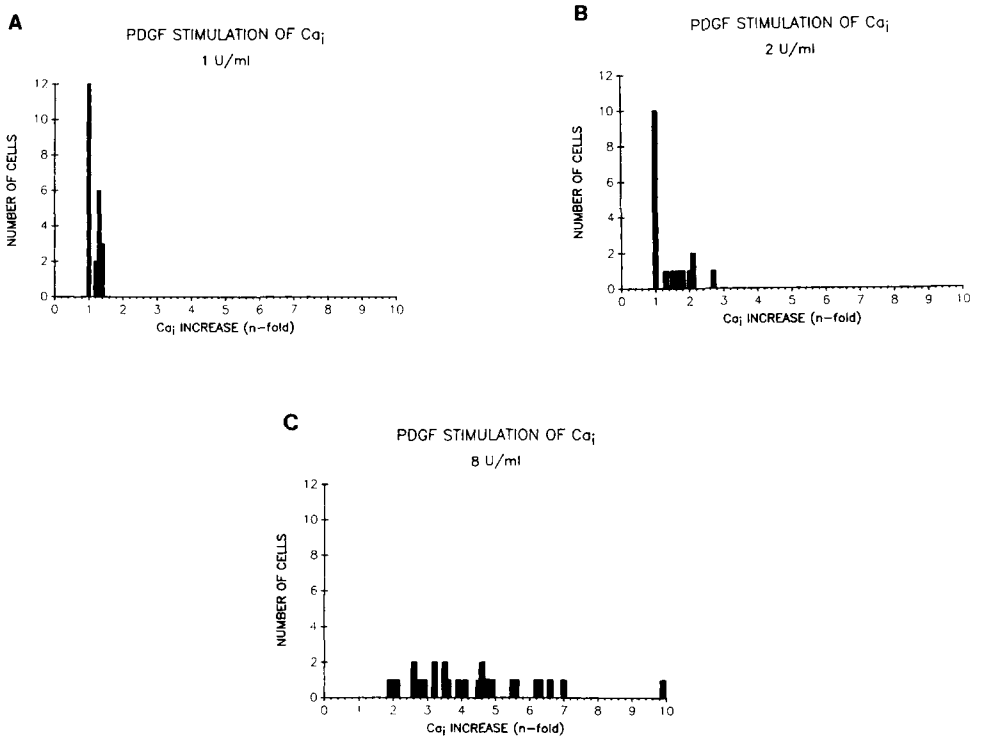

Fig. 4. PDGF produced dose-related increases in Ca_i . Frequency histograms of the magnitude of peak Ca_i after PDGF treatment. Increased Ca_i expressed as n-fold increases (stimulated average Ca_i/basal average Ca_i). Three different concentrations of PDGF were studied. **A:** 1 units/ml. **B:** 2 units/ml. **C:** 8 units/ml.

of cells that correlated with the percentage of cells made competent for DNA synthesis.

Quin 2 Inhibited Ca_i Increases Induced by PDGF and FGF

As Figure 7 illustrates, pretreatment of quiescent cells with 25 to 35 μM Quin 2/AM for 30 min resulted in obliteration of the initial transient Ca_i increase induced by both PDGF and FGF. In some cells, PDGF still increased Ca_i to a low plateau level that was not maintained. BAPTA-loaded cells showed even more profound inhibition of Ca_i increases but were not viable enough for later mitogenic studies. Permeabilization of Quin 2-loaded cells with digitonin (0.001%) showed that the intracellular concentration of Quin 2 was approximately 2 mM. Thus, this intracellular concentration of Quin 2 was sufficient to buffer Ca_i changes, thereby preventing growth factor-induced transient increases and plateau Ca_i increases.

Quin 2 Inhibited Mitogenesis Induced by PDGF but Not That Induced by FGF

Quin 2 (25 μM) loading prevented up to 90% of PDGF-stimulated competence, but less than 1% of the FGF-stimulated competence in the same cell population (Table III). In order to identify whether the inhibition of DNA synthesis was specifically

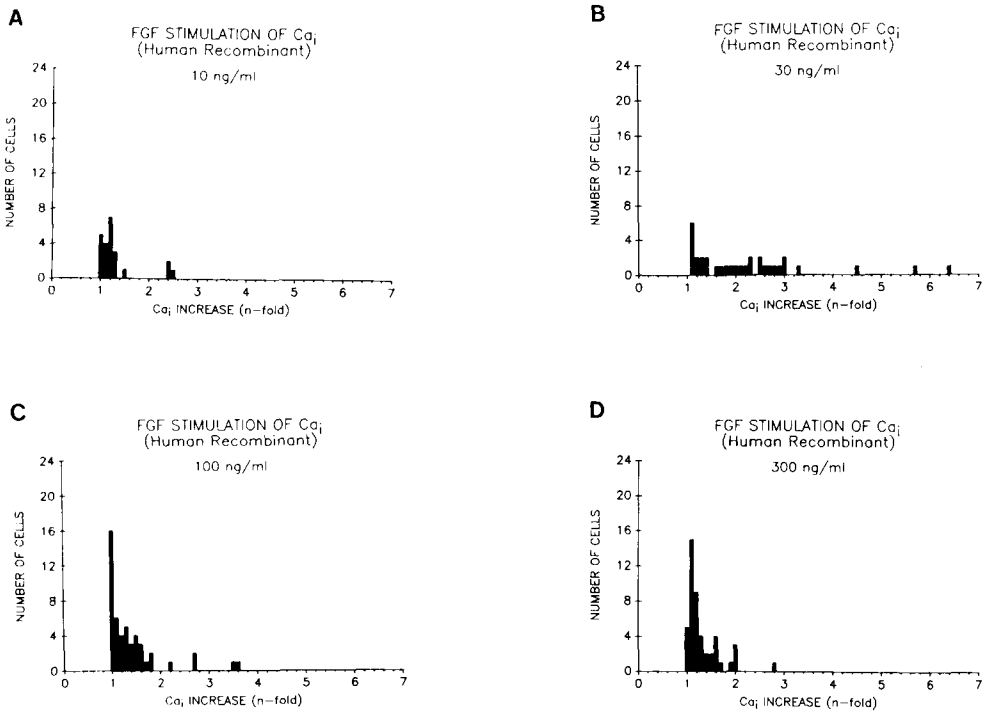

Fig. 5. Human recombinant FGF stimulated increases in Ca_i that were not dose-related. Frequency histograms of magnitude of peak Ca_i after treatment with FGF. **A**:10 ng/ml. **B**:30 ng/ml. **C**:100 ng/ml. **D**:300 ng/ml. Ca_i changes defined as n-fold increases (stimulated average Ca_i /basal average Ca_i).

related to the prevention of Ca_i increases, we performed three controls. *First*, we showed that inhibition of DNA synthesis was totally reversible, by using large concentrations (20%) of serum to stimulate DNA synthesis in Quin 2-loaded cells (data not shown). In addition, the continued stimulation of Quin 2-loaded cells by FGF also indicated the lack of nonspecific inhibition (Table III). *Second*, we used two pH indicators (calcein blue and carboxy-calcein blue) to load cells under the same conditions as those used for Quin 2 loading. These compounds contain three ester groups and following deesterification would produce amounts of intracellular formaldehyde similar to that produced by Quin 2, which contains four ester groups. Unlike Quin 2, however, calcein blue and carboxy-calcein blue do not bind calcium. We found that calcein blue and carboxy-calcein blue did not inhibit calcium transients and did not prevent competence induced by PDGF (Table III). Therefore, the production of intracellular formaldehyde by deesterification of Quin 2/AM cannot account for inhibition of DNA synthesis by Quin 2/AM. *Third*, TPEN (10–35 μM), a chelator of intracellular heavy metals, also did not inhibit Ca_i transients or DNA synthesis (Table III). Thus, Quin 2 inhibits competence for DNA synthesis induced by PDGF but not that induced by FGF. The mechanism of inhibition is independent of heavy metal chelation and intracellular formaldehyde production and most probably depends on the buffering of Ca_i increases.

DISCUSSION

By using digital image analysis of intracellular Fura-2 fluorescence in single cells, we have been able to examine the relationship between Ca_i increases and DNA

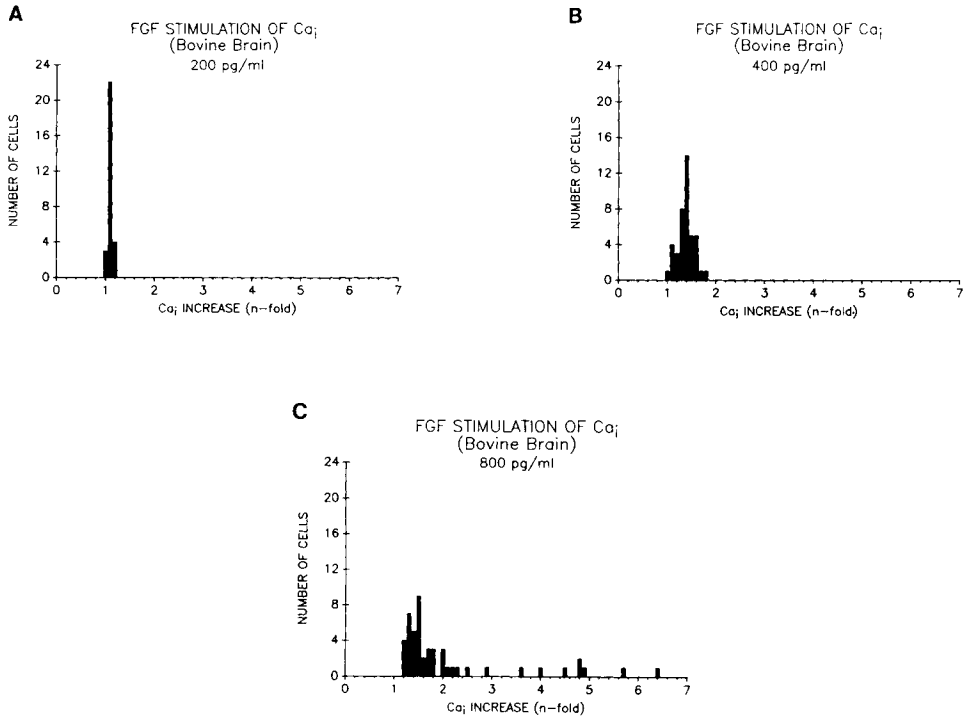

Fig. 6. Bovine brain FGF stimulated increases in Ca_i .Frequency histograms of the magnitude of peak Ca_i after treatment with different concentrations of FGF. **A:** 200 pg/ml. **B:** 400 pg/ml. **C:** 800 pg/ml. Ca_i responses expressed as n-fold increases (stimulated average Ca_i /basal average Ca_i).

TABLE I. Correlation Between Peak Ca_i Increase and DNA Synthesis Stimulated by PDGF

PDGF (units/ml)	N (No. cells)	Ca_i (% with n-fold increase)[a] 1.0	<2	2–4	>4	DNA synthesis (% competent)[b]
.5						0 (1)
1	23	52	48	0	0	27 ± 27 (2)
2	19	52	27	21	0	36 ± 30 (2)
8	26	0	8	38	54	77 ± 7 (4)

[a]Ca_i increase calculated as described for N (No. of cells). The n-fold increase in stimulated Ca_i was determined by stimulated peak Ca_i (cell average) divided by basal Ca_i (cell average).
[b]Percent of cells in DNA synthesis determined by autoradiography of cells stimulated with the indicated concentrations of PDGF for 2 h, followed by incubation in 1% PPP for 47 h. Results expressed as mean ± S.E.M. (No. of experiments).

synthesis in considerable detail. We found that both PDGF and FGF produced Ca_i increases in quiescent BALB/c 3T3 cells, but only PDGF treatment induced Ca_i increases that were directly dose-related and that correlated to subsequent DNA synthesis. Low concentrations of bovine brain FGF produced no cells with Ca_i increases (>1.2-fold) and a substantial percentage (>30%) of competent cells, while large concentrations of recombinant FGF produced a large percentage of competent cells but only a few cells with large (>twofold) increases in Ca_i. Moreover, buffering of Ca_i transients by intracellular Quin 2 prevented PDGF-stimulated but not FGF-

TABLE II. Correlation Between Peak Ca_i Increase and DNA Synthesis Stimulated by FGF

Stimulus (conc)	N (No. cells)	Ca_i (% with n-fold increase)[a] 1.0	<2	2–4	>4	DNA synthesis (% competent)[b]
Bovine brain FGF (pg/ml)						
50						11 (1)
100						17 ± 7 (2)
200	29	3	97	0	0	45 ± 14 (2)
400	42	2	98	0	0	34 ± 15 (3)
800	49	0	67	18	15	52 ± 15 (5)
1,600						38 ± 16 (2)
3,200						66 (1)
Human recombinant FGF (ng/ml)						
10	23	23	65	12	0	1 ± 0 (2)
30	33	0	48	42	10	19 ± 8 (3)
100	49	34	57	9	0	46 ± 1 (3)
300	49	11	81	8	0	75 ± 9 (2)

[a]Ca_i increase calculated as described for N (No. of cells). The n-fold increase in stimulated Ca_i was determined by stimulated peak Ca_i (cell average) divided by basal Ca_i (cell average).

[b]Percent of cells in DNA synthesis determined by autoradiography of cells stimulated with the indicated concentrations of FGF for 1 h, followed by incubation in 1% PPP for 48 h. Results expressed as mean ± S.E.M. (No. of experiments).

stimulated mitogenesis. Thus, both PDGF and FGF produced Ca_i increases, but only PDGF stimulation required Ca_i increases for the induction of competence for DNA synthesis.

Our documentation of the role of Ca_i in the induction of mitogenesis in PDGF-stimulated cells confirms previous work showing that PDGF stimulates increases in Ca_i [1–3] and that such changes correlate with mitogenesis in aequorin-loaded cell populations [5]. We were able to extend this analysis and use the inhibition of Ca_i increases with Quin 2 to obtain the first evidence that PDGF stimulation of mitogenesis depends on Ca_i increases. However, such clear dependence of PDGF-stimulated mitogenesis on Ca_i increases appears inconsistent with a study showing that phorbol ester (TPA) could inhibit PDGF stimulation of Ca_i increases without preventing DNA synthesis [2]. However, TPA may stimulate mitogenesis via activation of protein kinase C independently of Ca_i increases, thereby bypassing any inhibitory effect of TPA on Ca_i increase. Thus the Ca_i pathway stimulated by PDGF may not be absolutely obligatory for the stimulation of DNA synthesis when PDGF is combined with other factors. In addition, the Ca_i pathway contributing to mitogenesis can also be induced by other factors. For example, calcium ionophore A23187 induces both competence and transient Ca_i increases very similar to those stimulated by PDGF [R.W. Tucker, unpublished observations]. These results indicate that situations in which Ca_i increases are required for mitogenesis can now be documented by digital image analysis of Fura-2 fluorescence in individual cells.

This same analysis also showed that Ca_i increases are not required for FGF-stimulated mitogenesis. A low concentration of purified FGF (200 pg/ml) from bovine brain is capable of inducing competence in >45% of cells with only barely detectable (>1.2-fold) increases in Ca_i. Moreover, both low (10 ng/ml) and high (100 ng/ml)

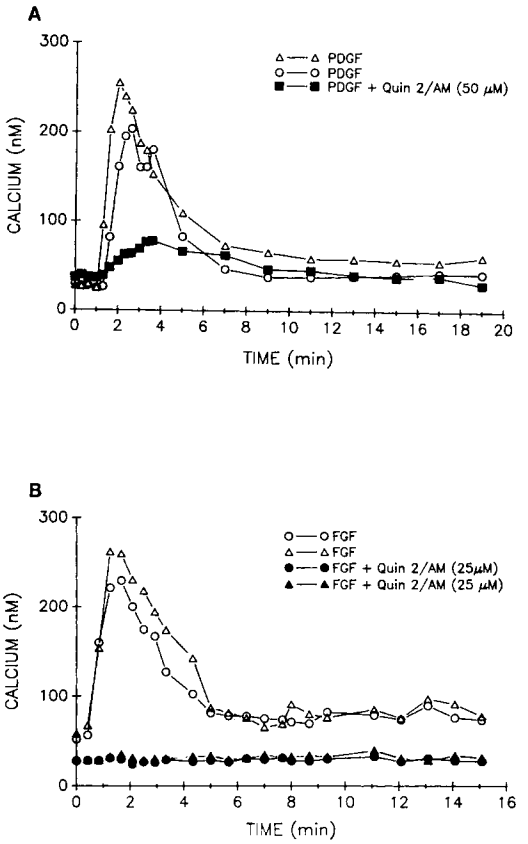

Fig. 7. Quin 2 inhibited Ca_i increases induced by PDGF and FGF. Pretreatment of quiescent BALB/c 3T3 cells with Quin 2/AM (25–50 μM) inhibited (**A**) PDGF stimulation and (**B**) FGF stimulation of Ca_i increases. Quin 2 itself was weakly fluorescent, bleached rapidly, and did not contribute significantly to the Ca^{2+}-sensitive intracellular fluorescence.

concentrations of recombinant FGF induced similar increases in Ca_i (Figs. 4, 5), but stimulated a vastly different percentage of cells to initiate DNA synthesis (1% and 46%, respectively). Finally, the concentration of recombinant FGF that induces a maximal Ca_i increase is lower than that required to stimulate maximal DNA synthesis. These results are compatible with our previous aequorin studies [5] in which large concentrations of unpurified FGF (Collaborative Research) stimulated competence without producing detectable Ca_i increases. In the present study we have extended this analysis to show that certain concentrations of purified FGF (both human recombinant and bovine brain) can cause Ca_i changes, but that the Ca_i changes are not directly related to stimulation of competence for DNA synthesis. In fact, intracellular concentrations of Quin 2 that inhibit PDGF-stimulated mitogenesis do not inhibit FGF-stimulated mitogenesis (Table III). Thus, Ca_i increases must be induced by a nonmitogenic component of purified FGF preparations. Previous workers have indeed suggested that a contaminant of purified FGF preparations produces small increases in Ca_i, as measured in hamster fibroblast populations using Quin-2 fluorescence [4]. However these Ca_i increases measured by Quin 2 were abolished by EGTA, whereas

TABLE III. Effect of Inhibitors on DNA Synthesis

Pretreatment[c] (μM)	Percent inhibition of competence stimulated by[a]	
	PDGF[b]	FGF (bovine)
Quin 2/AM	90	0
10	82	0
25	91	—
35		
Calcein blue/AM	7	—
25	1	—
35		
Carboxy-calcein blue/AM	1	—
25	0	—
35		
TPEN	0	—
10	0	—
35		

[a]Competent cells determined by percent of labeled cells as indicated in text. Percent inhibition of competence = [1 − % competence (PDGF + inhibitor)/% competence (PDGF)].
[b]Cell cultures pretreated with inhibitor for 30 min in Krebs/HCO_3- solution, at 37°C, in CO_2 incubator.
[c]PDGF (AB heterodimer) (8 units/ml) was used for Quin 2/AM experiments comparing PDGF and bovine brain FGF (800 pg/ml); PDGF (AB heterodimer) (16 units/ml) was used for Quin 2, calcein, and carboxy-calcein blue comparison, and PDGF (AA homodimer) (30 ng/ml) was used for TPEN experiments.

the FGF-stimulated Ca_i increases described in this report were not. In addition, since purified FGF from two different sources (with presumably different contaminants) both produced Ca_i increases, a contaminant of FGF preparations was probably not responsible for the Ca_i increases reported here. These discrepancies between our results and previous reports [4] may result from the study of different cell lines or from a more sensitive detection of Ca_i changes using single cell analysis of Fura-2 fluorescence in the present study. In fact, our results were recently confirmed by experiments using anti-PIP2 antibodies to prevent the generation of diacylglycerol and inositol trisphosphates (IP3) (and subsequent release of intracellular calcium stores); only PDGF-stimulated, and not FGF-stimulated, mitogenesis depended on constant PIP turnover and, by inference, on Ca_i increases [15]. Other changes in Ca^{2+} metabolism, such as depletion of Ca^{2+} stores, could still contribute to the activation of genes involved in mitogenesis. Indeed, Ca^{2+} depletion, rather than Ca_i increases, appears to be important in stimulating heat shock genes in *Drosophila* [13] and in stimulating glucose-deprivation genes in hamster fibroblasts [14]. However, appreciable Ca^{2+} depletion in BALB/c 3T3 cells can only occur if Ca^{2+} is released from an intracellular store, a finding incompatible with the negligible increase in ^{45}Ca efflux measured in FGF-stimulated 3T3 cells (R.W. Tucker, K. Meade-Cobun, R. Phair, unpublished observations). Thus, Ca^{2+} changes in both the cytosol and intracellular compartments do not appear to be important in FGF-stimulated DNA synthesis.

There are undoubtedly multiple pathways for inducing proliferation of cells. Ca_i measurements using Fura-2 in this study have shown that PDGF and FGF, two polypeptide factors that can induce competence in BALB/c 3T3 cells, use stimulatory pathways that differ in their dependence on Ca_i increases. Inhibition studies using

Quin 2 have further documented that the calcium transients are required for proliferation stimulated by PDGF, but not by FGF. Thus, Ca_i increases are not part of a common pathway used by all competence factors. In the future, digital image analysis of second messengers (e.g., Ca_i transients) in individual cells will be important in defining the relative contributions of multiple overlapping pathways to mitogenic stimulation.

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