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Isoform-specific Induction of Nuclear Free Calcium Oscillations by Platelet-derived Growth Factor*

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Pamela A. Diliberto‡, Sumita Krishna‡, Seongwook Kwon‡, and Brian Herman‡§¶

From the ‡Laboratories for Cell Biology, Department of Cell Biology and Anatomy and the §Lineberger Cancer Research Center, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

Confocal laser scanning microscopy was used to analyze alterations in nuclear free calcium (Ca^{2+}_n) levels induced by platelet-derived growth factor (PDGF) isoforms in BALB/c3T3 fibroblasts loaded with the calcium-sensitive fluorescent indicator Fluo-3. Both AA-PDGF and BB-PDGF caused a transient increase in Ca^{2+}_n. Analysis of PDGF-induced Ca^{2+}_n alterations as a function of time revealed that BB-PDGF stimulation resulted in the generation of Ca^{2+}_n oscillations that diminished over time. The frequency of BB-PDGF-stimulated oscillations was modulated by extracellular Ca^{2+} and could not be mimicked by increasing intracellular inositol 1,4,5-trisphosphate levels in the absence of growth factor stimulation. Caffeine alone had no effect on Ca^{2+}_n levels, but exposure of cells to caffeine after BB-PDGF stimulation augmented Ca^{2+}_n oscillations, either by increasing the frequency or reinitiating preexisting oscillations. The genesis of these oscillations in Ca^{2+}_n appears to be in the region just outside of the nucleus, as perinuclear cytoplasmic free calcium (Ca^{2+}_i) increased just prior to Ca^{2+}_n. In contrast, AA-PDGF stimulation resulted in the generation of one or two irregular, transient Ca^{2+}_n spikes. Caffeine pretreatment followed by AA-PDGF stimulation resulted in Ca^{2+}_n oscillations very similar to those produced by BB-PDGF alone. Additionally, the AA-PDGF and BB-PDGF isoforms appeared to modulate distinct pools of cellular Ca^{2+}, as BB-PDGF was still capable of inducing Ca^{2+}_n oscillations subsequent to prior induction of oscillations by AA-PDGF/caffeine. These PDGF isoform-specific changes in nuclear free Ca^{2+} could serve as a mechanism by which isoform-specific cellular signaling pathways may be manifested by the growth factors.

Platelet-derived growth factor (PDGF)[1] is a potent peptide mitogen and chemotactic factor for a variety of cells of mesenchymal origin (1). The mechanism by which PDGF binding to its plasma membrane receptors is transduced into biological activity is presently unclear, a question made more complex by the existence of three PDGF isoforms that possess the capacity to interact differentially with at least two distinct but related receptors (2). PDGF molecules exist as homodimers (AA- and BB-PDGF) or heterodimers (AB-PDGF) of two disulfide-linked polypeptide chains. The A and B chain polypeptides exhibit 60% amino acid sequence identity and are the products of distinct genes with independently regulated expression. All three PDGF isoforms have been identified from natural sources. Binding studies indicate that PDGF molecules bind differentially to two distinct receptor molecules, the α and β PDGF receptors (3). The α receptor binds both PDGF A and B chains with high affinity in contrast to the β receptor, which binds only the PDGF B chain with high affinity. PDGF activity has been hypothesized to be mediated by three distinct dimeric receptor forms ($\alpha\alpha$, $\alpha\beta$, and $\beta\beta$), which differentially interact with the three PDGF isoforms (4).

Upon receptor binding, PDGF initiates a number of rapid intracellular changes that are thought to play significant roles in the mitogenic signal transduction mechanism (5). Like many ligands, one of the early cellular events initiated by PDGF is a rapid, transient elevation in cytoplasmic calcium (Ca^{2+}_i) (6, 7). PDGF-stimulated increases in Ca^{2+}_i have been strongly correlated with the mitogenic activity of the growth factor in a number of different cell types (8–13). More specifically, the *early* PDGF-stimulated increases in Ca^{2+}_i that occur immediately following PDGF exposure have been demonstrated to be necessary for PDGF-induced mitogenesis (9). PDGF and other growth factors have also been found to induce the expression of a number of protooncogenes within minutes to hours after stimulation (14). A potential interaction between PDGF-stimulated alterations in Ca^{2+}_i and gene expression is suggested by the findings that transcriptional regulation of some protooncogenes may be modulated by changes in the levels of Ca^{2+}_i under certain conditions (15). It remains to be determined, however, whether and how PDGF-induced alterations in Ca^{2+}_i are involved in PDGF-specific gene expression. One prediction of the hypothesis that PDGF-induced changes in Ca^{2+}_i regulate transcriptional activity is that PDGF would need to alter levels of Ca^{2+} within the nucleus itself.

In the present study, we have used confocal laser scanning microscopy (CLSM) in conjunction with Ca^{2+}-sensitive fluorescent probes to focus on the question of whether PDGF isoforms alter nuclear free Ca^{2+} (Ca^{2+}_n) in single intact living cells. Unlike conventional fluorescence microscopy, which gives a two-dimensional rendering of the entire three-dimensional object under study, confocal microscopy produces two-dimensional renderings of thin optical sections ($\leq$1 μm) of a specimen, rejecting light from out-of-focus planes and supplying highly detailed spatial information unobtainable by conventional microscopic methods. Thus, it is possible to examine stimulated Ca^{2+}_n alterations in relation to Ca^{2+}_i changes occurring in other subcellular compartments or areas (such as the perinuclear region or the nuclear envelope). CLSM images of Ca^{2+}-indicator fluo-

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¶ To whom correspondence should be addressed: Dept. of Cell Biology and Anatomy, CB # 7090, 232 Taylor Hall, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-962-0350; Fax: 919-966-1856.

[1] The abbreviations used are: PDGF, platelet-derived growth factor; Ca^{2+}_i, cytoplasmic free calcium; Ca^{2+}_n, nuclear free calcium; CLSM, confocal laser scanning microscopy; IP_3, inositol 1,4,5-trisphosphate. CICR, Ca^{2+}-induced Ca^{2+} release; BAPTA, dibromo-1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

rescence intensity obtained from thin optical sections passing through the level of the nuclei of single cells and analyzed as a function of time have revealed in the present study that not only do PDGF isoforms stimulate alterations in $Ca^{2+}{}_n$ but that the temporal nature and pattern of these changes (oscillatory *versus* nonoscillatory) exhibit isoform specificity.

EXPERIMENTAL PROCEDURES

Cell Culture—BALB/c murine 3T3 fibroblasts (clone A31) were grown as described (8, 16). Cells were maintained in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% heat-inactivated Colorado calf serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified 5% CO_2, 95% air at 37 °C. Cells were made quiescent prior to PDGF stimulation by placement in serum-free medium (Dulbecco's modified Eagle's medium/Ham's F-12, 1:1, supplemented with insulin/transferrin/selenium) 48 h before use. Cells used experimentally were from passage numbers 6–16.

Confocal Laser Scanning Microscopy—The confocal microscope systems used consisted of a Bio-Rad MRC-600 laser scanning confocal unit interfaced with a Nikon Diaphot inverted microscope or an Olympus LSM GB200. CLSM $Ca^{2+}{}_n$ measurements using the Bio-Rad MRC-600 were performed in live cells by scanning the specimen with the 488-nm line of the 15-milliwatt argon/krypton multiline (488, 568, and 647 nm) laser of the Bio-Rad system attenuated by means of a 0.1 or 1.0% transmittance neutral density filter and a fluorescein filter cube situated in the optical path, in order to minimize photodamage to the cells. The confocal aperture was set at ~2–3, which roughly equates to the minimum optical section thickness (0.75 μm) that can be achieved with this system using a 40× (1.3 numerical aperture) oil immersion objective. Cells cultured on glass coverslips were loaded with Ca^{2+}-sensitive fluorophore (see below), and then imaged by collecting a single 768 × 512 pixel scan from the same focal plane once every 5–15 s using the slow scan rate (~2 s/full scan) for up to 30 min.

Rapid confocal line scanning was performed using the Olympus LSM GB200 with the 488-nm line of an argon ion laser attenuated using a 1% neutral density filter and a fluorescein filter cube. Cells cultured on glass coverslips were loaded with Ca^{2+}-sensitive fluorophore; from the two-dimensional image, a single line (1024 pixels), which crossed the long axis of the cell and included both the cytoplasmic and nuclear regions, was repeatedly scanned at 10-ms intervals. Two-dimensional line scan images (768 lines) were converted into tiff files and analyzed using NIH Image. Areas representing the perinuclear and nuclear regions were selected with a user-defined window, and the intensity of each line within this window was plotted as a function of time.

Determination of Nuclear Calcium by CLSM—$Ca^{2+}{}_n$ was measured using CLSM in individual or small collections of individual living cells grown on glass coverslips and loaded with the cell permeant acetomethoxyester form of the Ca^{2+}-sensitive fluorophore, Fluo-3 (Fluo-3/AM). Quiescent cells on coverslips were prechilled for 10 min at 4 °C to prevent subsequent uptake of fluorophore into intracellular organelles (17). Cells were then loaded with Fluo-3/AM (10 μM) for 35 min at 37 °C, unless otherwise indicated. This loading procedure resulted in diffuse cytoplasmic labeling of cells with an estimated intracellular concentration of Fluo-3 of 100 μM based on digitonin-releasable absorbance. Approximately 4% residual Fluo-3 fluorescence was retained in the cell pellet after digitonin (150 μM) extraction. Alternatively, cells were loaded with Fluo-3 by microinjection of a 40 mM solution of the pentapotassium salt form of the indicator dissolved in injection buffer (27 mM K_2HPO_4, 26 mM KH_2PO_4, 8 mM $NaHPO_4$, pH 7.3). Microinjection was performed using a Zeiss IM-35 inverted microscope and a General Valve Corp. Picospritzer II pressure injection system. Cells were allowed to recover for 45–60 min postinjection prior to confocal imaging.

Cells on coverslips were then washed, transferred to incubation chambers, and placed on the stage of an inverted fluorescence microscope. Incubations and washes were performed in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) supplemented with 1 mg/ml bovine serum albumin and maintained at 37 °C using an air curtain incubator. Changes in $Ca^{2+}{}_n$ were measured by changes in fluorescence intensity at emission wavelengths >520 nm. Images were collected every 15 s unless otherwise indicated. The $Ca^{2+}{}_n$ data presented represent the average pixel intensity values calculated from the mapped regions of individual nuclei obtained from an image field routinely consisting of 10–15 cells.

Materials—PDGF isoforms were purchased from Biosource International (human recombinant AA and BB homodimers) (Westlake Village, CA). PDGF isoforms were aliquotted and stored frozen, 10 μg/ml in 0.1 N acetic acid. Before addition to cells, stock solutions of PDGF were diluted to 500 ng/ml in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 0.1% bovine serum albumin and neutralized with 5 mM Na_2CO_3. Fluo-3 pentapotassium salt and Fluo-3/AM were obtained from Molecular Probes (Eugene, OR). All other reagents were purchased from Calbiochem or Sigma.

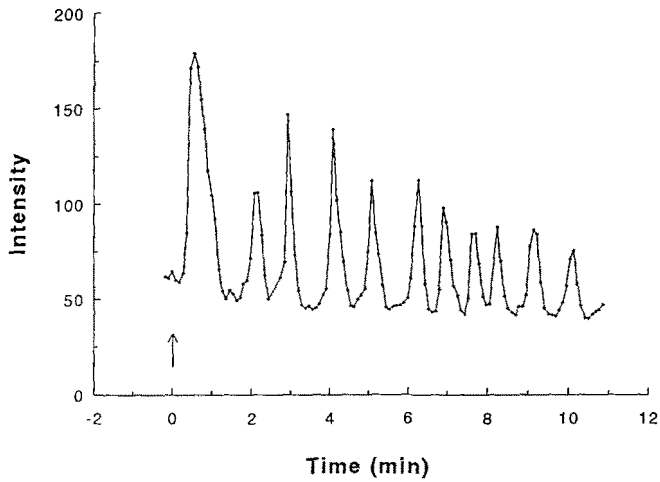

FIG. 1. **BB-PDGF-induced oscillations in nuclear free Ca^{2+} ($Ca^{2+}{}_n$).** Quiescent BALB/c3T3 cells were loaded with Fluo-3/AM, and images from an optical section through the center of the nucleus were obtained by confocal laser scanning microscopy at 5-s intervals. BB-PDGF (10 ng/ml) was added at the *arrow*. Represented on the *y* axis is the average fluorescent pixel intensity of the mapped area of a single nucleus.

RESULTS

BB-PDGF Stimulation of Ca_n^{2+} Oscillations—Cells stimulated with either AA- or BB-PDGF exhibited an increase in fluorescence intensity in the nuclear compartment in both AM-loaded and microinjected cells. No obvious heterogeneity of the $Ca^{2+}{}_n$ signal within the nucleus itself was apparent under the imaging conditions used. The increases in the nucleus were transient, returning to approximate their respective prestimulation levels within 1–2 min.

Based on the observation that AA-and BB-PDGF caused alterations in $Ca^{2+}{}_n$, further studies specifically examining the nature of PDGF-stimulated $Ca^{2+}{}_n$ alterations were undertaken. Subsequent to the initial $Ca^{2+}{}_n$ transient stimulated by BB-PDGF, oscillatory alterations in $Ca^{2+}{}_n$ were observed in cells loaded with Fluo-3/AM (Fig. 1). Generation of the oscillatory response pattern was dependent on the concentration of BB-PDGF, with optimal oscillatory activity occurring in the 10–20 ng/ml concentration range. Supraoptimal concentrations (≥20 ng/ml) resulted in nonoscillatory, sustained, monophasic $Ca^{2+}{}_n$ increases, while suboptimal (1–10 ng/ml) concentrations resulted in oscillatory responses, but in a lower percentage of the cells present. The optimal oscillatory concentration was found to vary between different cell preparations and was determined by titration for each set of experiments.

Small rising shoulders on the initial increasing phase of the individual spikes of the $Ca^{2+}{}_n$ oscillations could be discerned at the temporal resolution used to acquire images (5 s), suggesting that a threshhold level in $Ca^{2+}{}_n$ concentration was required before the large rapid increasing phase of the oscillation could be initiated. No spatial heterogeneity of $Ca^{2+}{}_n$ signals within single nuclei were observed, a finding that would be indicative of multiple nuclear Ca^{2+} pools. Oscillations within a single nucleus occurred with a fairly regular frequency of 1.19 ± 0.13/min (mean ± S.E., n = 6 experiments, totaling 65 cells) (Figs. 1 and 2). Frequency differences were observed not only between different cell preparations, but also between different cell nuclei on the same coverslip in the same image field. Thus, stimu-

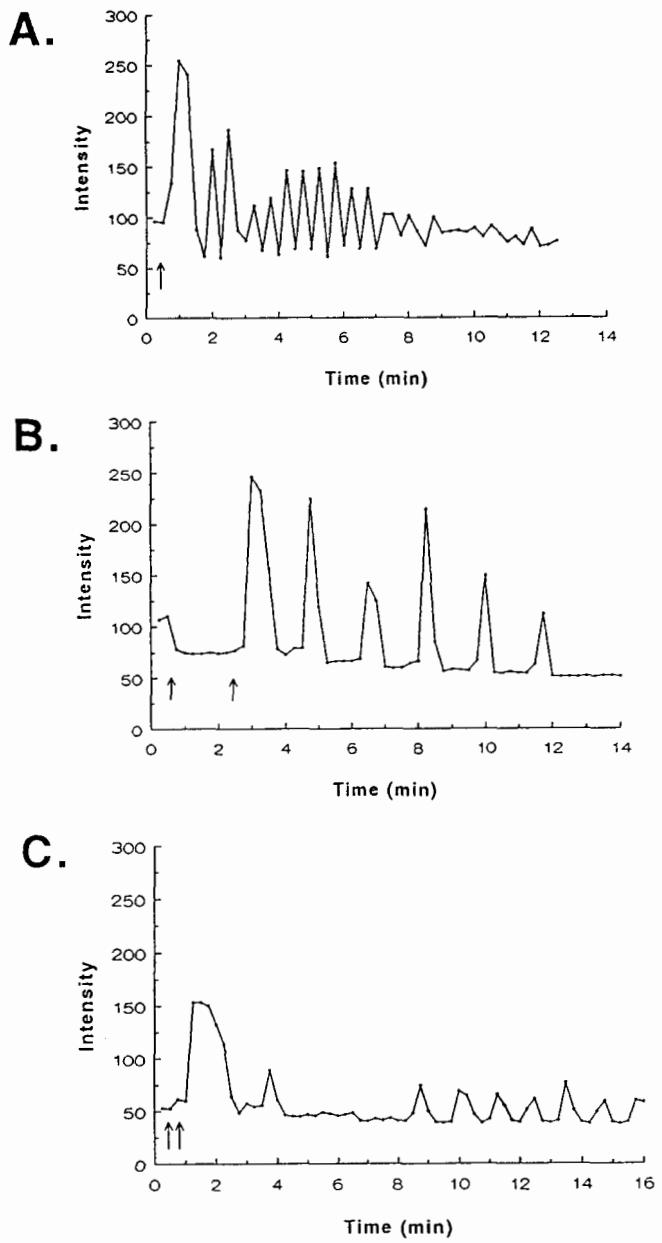

FIG. 2. **The effect of extracellular Ca^{2+} and intracellular IP_3 manipulations on $Ca^{2+}{}_n$ oscillations.** Cells were loaded with Fluo-3/AM and confocal images obtained as in Fig. 1. Individual traces represent the average fluorescent intensity (on a scale of 0–256) of the mapped area of a single nucleus. *A*, BB-PDGF (10 ng/ml) was added at the *arrow*. BB-PDGF induces $Ca^{2+}{}_n$ oscillations. *B*, EGTA (2.5 mM) was added at the *first arrow*. BB-PDGF (10 ng/ml) was added 2 min later at the *second arrow*. EGTA pretreatment decreases the frequency of BB-PDGF-induced $Ca^{2+}{}_n$ oscillations. *C*, intracellular IP_3 levels were increased by the addition of AlF_4^- at the *arrows* (10 μM $AlCl_3$, *first arrow*; 10 mM NaF, *second arrow*). Treatment of cells with AlF_4^- induces a transient increase in $Ca^{2+}{}_n$, but does not result in nuclear oscillations resembling those produced by BB-PDGF. Data represented in *Panels A–C* were obtained on the same day using cells from the same cell preparation. These data are representative of data obtained from 6–8 different cell preparations for each treatment condition, with a total of 339 cells examined.

lation with a specified dose of BB-PDGF resulted in the induction of asynchronous $Ca^{2+}{}_n$ oscillations within a given cell population but with characteristic periodicity within individual cells. On occasion, cells on the same coverslip exibited heterogeneity in the type of $Ca^{2+}{}_n$ response induced by BB-PDGF, some oscillatory, some nonoscillatory (data not shown). The duration of the oscillatory response was also somewhat variable between different cells and different cell preparations, ranging from 5 to >10 min, usually exhibiting a gradual decrease in amplitude at the later time points (Fig. 1).

Dependence of Ca_n^{2+} Oscillations on Extracellular Ca^{2+} and Release from IP_3-sensitive Intracellular Storage Sites—In BALB/c3T3 cells, PDGF-induced increases in $Ca^{2+}{}_i$ arise from the combined release of Ca^{2+} from intracellular IP_3-sensitive storage sites and influx of extracellular Ca^{2+} across the plasma membrane (13). We therefore examined the role of these two mechanisms in the generation of $Ca^{2+}{}_n$ oscillations. Removal of extracellular Ca^{2+} with the Ca^{2+} chelator, EGTA, did not inhibit the initiation of $Ca^{2+}{}_n$ oscillations by BB-PDGF but did decrease oscillation frequency when compared with cells stimulated in the presence of extracellular Ca^{2+} (compare Fig. 2, *A* and *B*). These data indicate that generation of BB-PDGF-induced $Ca^{2+}{}_n$ oscillations were not dependent on the influx of extracellular Ca^{2+} but that extracellular Ca^{2+} was required to maintain the normal frequency of oscillations. However, investigation of the role of IP_3-sensitive intracellular Ca^{2+} release in the $Ca^{2+}{}_n$ oscillatory mechanism did not indicate that this intracellular pool was solely responsible for the oscillations observed. Generation of increased levels of intracellular IP_3 by the fluoroaluminate complex, AlF_4^-, did not result in $Ca^{2+}{}_n$ oscillations similar to those produced by BB-PDGF (Fig. 2*C*). Addition of AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$), which stimulates phospholipase C activity through activation of heterotrimeric G-proteins, has been shown to increase both intracellular IP_3 (18) and $Ca^{2+}{}_i$ (16) levels in BALB/c3T3 cells to the same extent as maximal doses of BB-PDGF, although the mechanism of IP_3 generation is distinct from that of BB-PDGF, which promotes G-protein-independent tyrosine phosphorylation of phospholipase C-γ (18). IP_3 generation by AlF_4^- did result in a single transient increase in $Ca^{2+}{}_n$, somewhat longer in duration than the initial transient produced by BB-PDGF stimulation. Thus, neither the influx of extracellular Ca^{2+} nor the release of intracellular Ca^{2+} by IP_3 alone were found to be sufficient for the $Ca^{2+}{}_n$ oscillations stimulated by BB-PDGF. Extracellular Ca^{2+} appears to be involved in the regulation of the frequency of $Ca^{2+}{}_n$ oscillations, whereas IP_3-mediated release from intracellular sites may play a role in the initial $Ca^{2+}{}_n$ transient induced by BB-PDGF.

Modulation of Ca_n^{2+} Oscillations by Caffeine—In an attempt to identify the source of intracellular Ca^{2+} involved in the generation of PDGF-induced $Ca^{2+}{}_n$ oscillations, caffeine, a known intracellular Ca^{2+}-releasing agent, was employed. The effects of caffeine on $Ca^{2+}{}_i$ have most commonly been attributed to its releasing action on the calcium-induced calcium release (CICR) intracellular Ca^{2+} pool in excitable cell types. Caffeine alone has not been demonstrated to produce $Ca^{2+}{}_i$ alterations in quiescent BALB/c3T3 cells,[2] however, the effect of this agent on nuclear Ca^{2+} levels in this system has not been previously reported. Addition of caffeine alone (1–5 mM) to resting BALB/c3T3 cells for periods up to 10 min in duration did not elicit any discernible $Ca^{2+}{}_n$ alterations or oscillations ((Fig. 3*A*), data not shown for the longer time points). Additionally, no increase in cytoplasmic Ca^{2+} levels was observed. Caffeine addition *was* found to modulate the $Ca^{2+}{}_n$ response stimulated by BB-PDGF (Fig. 3, *A–C*). Preincubation of cells with caffeine (2.5 mM, 2 min) prior to BB-PDGF stimulation did not substantially alter the ability of BB-PDGF to induce $Ca^{2+}{}_n$ oscillations (Fig. 3*A*), although variable effects on the time of onset and the nature of the initial $Ca^{2+}{}_n$ increase were seen. More revealing were the effects of caffeine found on the $Ca^{2+}{}_n$ response when added at various times following BB-PDGF stimulation: 1) caffeine added to cells in which BB-PDGF-induced $Ca^{2+}{}_n$ oscillations

[2] P. A. Diliberto and B. Herman, unpublished observations.

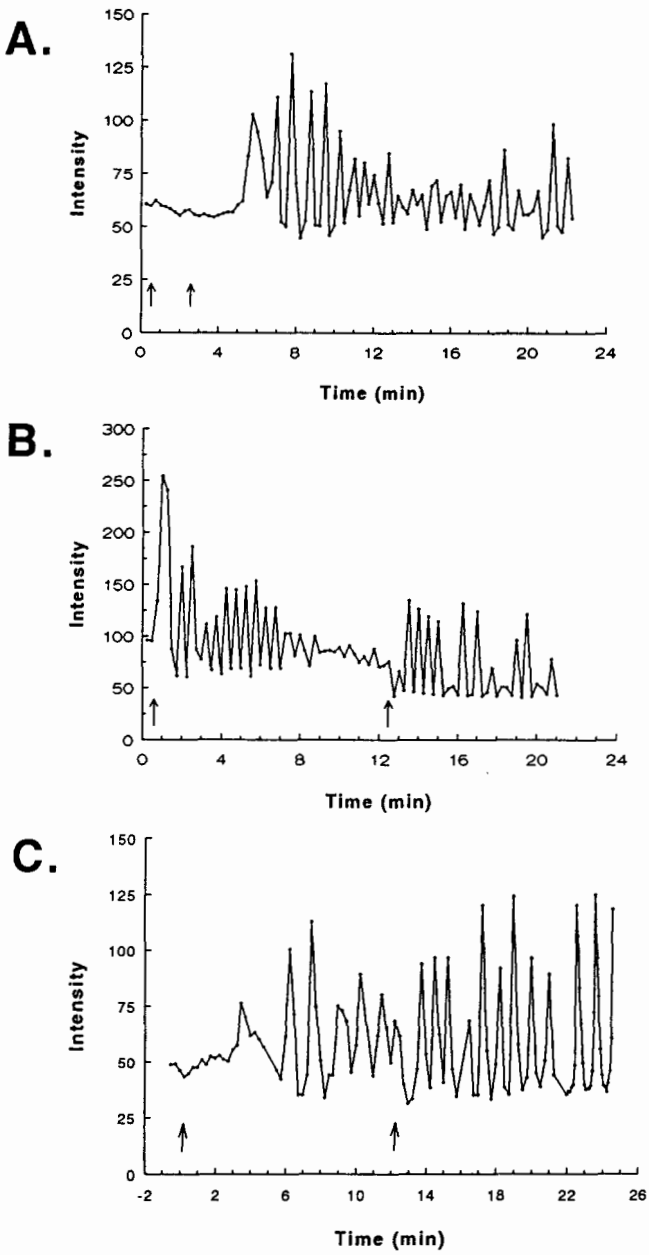

FIG. 3. **The effect of caffeine on BB-PDGF-induced Ca^{2+}_n oscillations.** Cells were loaded with Fluo-3/AM, and confocal images were obtained as in Fig. 1. Individual traces represent data obtained from a single nucleus. *A*, cells were pretreated with caffeine (2.5 mM) for 2 min by addition at the *first arrow*. BB-PDGF (10 ng/ml) was added at the *second arrow*. *B*, BB-PDGF (10 ng/ml) was added at the *first arrow*. Caffeine (2.5 mM) was added at the *second arrow*. Caffeine reinitiates BB-PDGF-induced Ca^{2+}_n oscillations. *C*, BB-PDGF (*first arrow*) and caffeine (*second arrow*) were added as in *Panel B*, but this time to cells still actively oscillating. Caffeine addition under these conditions increased the frequency of Ca^{2+}_n oscillations. These data are representative of data obtained from 7, 5, and 2 independent experiments for each treatment condition (*panels A–C*, respectively), with a total of 195 cells examined.

had been stimulated and then subsequently dissipated caused the reinitiation of oscillations with a similar frequency to those observed initially (Fig. 3*B*) and 2) caffeine added to BB-PDGF stimulated cells that were still actively oscillating resulted in a sharp and rapid decrease in Ca^{2+}_n followed by the continuation of oscillations with an increased and more regular frequency (Fig. 3*C*). In both instances, the duration of the oscillatory activity following caffeine addition was at least 10 min. When the same experiment was performed on cells preincubated in EGTA-containing (2.5 mM) medium, caffeine (2–5 mM) addition did not stimulate or reinitiate BB-PDGF stimulated Ca^{2+}_n oscillations (data not shown). These data suggest that once a Ca^{2+}_n response has been initiated by BB-PDGF, caffeine has the ability to act as a modulating agent on the intracellular Ca^{2+} pools involved in the mechanism of Ca^{2+}_n oscillation generation, and the Ca^{2+} content of these pools is partially regulated by extracellular Ca^{2+}.

AA-PDGF Induction of a Nonoscillatory Ca^{2+}_n Response and Its Conversion to an Oscillatory Response by Caffeine—Stimulation of BALB/c3T3 cells with AA-PDGF (1–80 ng/ml) did not induce Ca^{2+}_n oscillations as seen with BB-PDGF. Instead, a single, transient Ca^{2+}_n increase of brief duration was observed (Fig. 4*A*). Following the initial transient increase, Ca^{2+}_n levels returned toward but remained slightly elevated above prestimulation values. Occasionally, a second or third Ca^{2+}_n spike was seen to occur during the 10–15 min period following AA-PDGF addition. Thus, a distinct difference was observed in the nature of Ca^{2+}_n responses elicited by the two PDGF isoforms (compare Figs. 2*A* and 4*A*). However, the presence of caffeine in addition to AA-PDGF resulted in an oscillatory Ca^{2+}_n response pattern very similar to the oscillations produced by BB-PDGF stimulation alone (Fig. 4, *B* and *C*). The mechanism of this "conversion" of an AA-PDGF response into a BB-PDGF-appearing response is presently unknown, but this finding suggests that some element in the Ca^{2+}-signaling pathway activated by the BB-PDGF isoform is not activated by AA-PDGF unless it is recruited by the action of caffeine.

Sequential Initiation of Ca^{2+}_n Oscillations by PDGF Isoforms—Sequential stimulation of the same cell with AA- and BB-PDGF was performed to investigate whether the mechanisms and/or Ca^{2+} pools involved in the generation of AA- and BB-PDGF-induced Ca^{2+}_n oscillations contained overlapping or distinct elements (Fig. 5). Ca^{2+}_n oscillations were initiated by AA-PDGF addition in the presence of caffeine. The oscillations were then stopped by the addition of EGTA to the incubation medium. At this point (in the continued presence of EGTA), BB-PDGF stimulation resulted in the reinitiation of Ca^{2+}_n oscillations, which continued, roughly, for another 5 min. Reverse addition of the isoforms, BB-PDGF followed by AA-PDGF, did not result in a Ca^{2+}_n reponse upon AA-PDGF stimulation, a finding consistent with the ability of BB-PDGF to bind to both types of PDGF receptors (data not shown).

Relationship Between Alterations in Cytoplasmic and Nuclear Free Ca^{2+}—The mechanism(s) underlying PDGF isoform specific alterations in Ca^{2+}_n are not known. A major question, which still needs to be resolved, is whether alterations in Ca^{2+}_n are regulated locally in the nucleus or reflect alterations in cytoplasmic Ca^{2+}, which are transmitted to the nucleus through freely permeable nuclear pores. Fig. 6 demonstrates the relationship between BB-PDGF-induced alterations in Ca^{2+}_i and Ca^{2+}_n. The data in this figure demonstrate a similar pattern of BB-PDGF-induced oscillations in Ca^{2+}_i and Ca^{2+}_n. To better define the temporal relationship between BB-PDGF-induced alterations in Ca^{2+}_i and Ca^{2+}_n (*i.e.* to determine whether the oscillations in the nucleus were identical, similar, delayed or totally independent from changes in Ca^{2+}_i), we performed rapid line scan confocal microscopy (Fig. 7). In each case examined, Ca^{2+}_i increased in the perinuclear area 50–300 ms before an increase in Ca^{2+}_n was observed.

DISCUSSION

The existence of agonist-stimulated oscillations in *cytoplasmic* free Ca^{2+} has been documented in a wide variety of systems, and the mechanisms generating and regulating these oscillations are currently the subject of extensive investigation

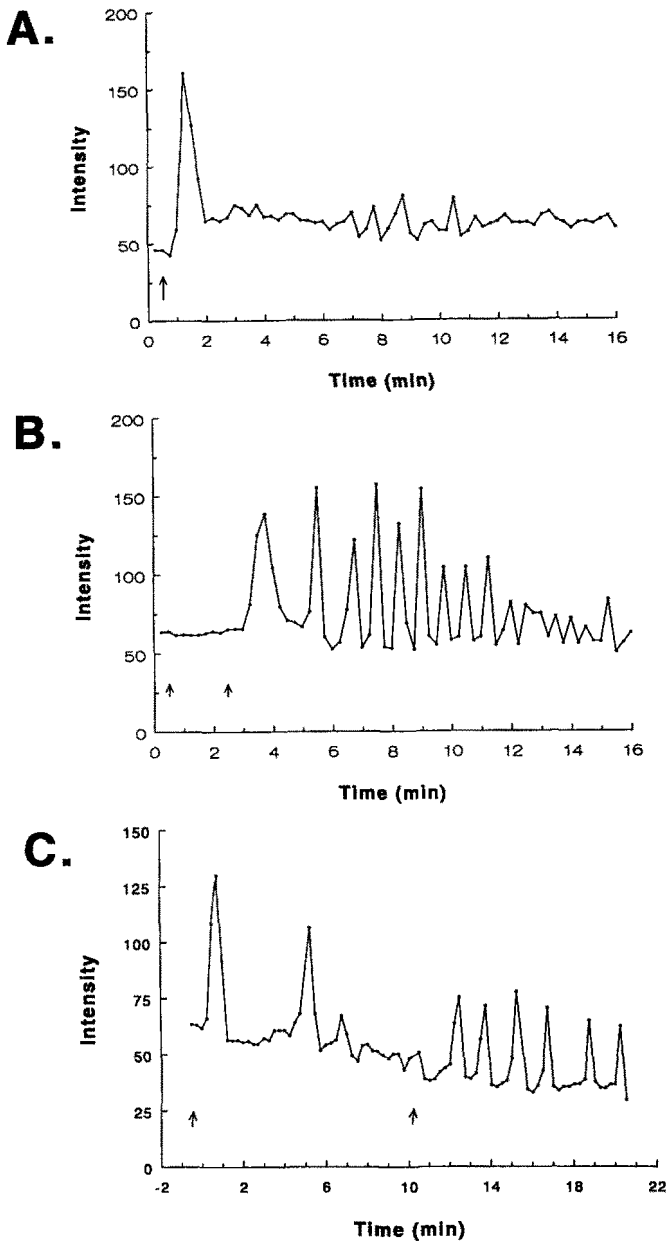

FIG. 4. **The effect of caffeine on AA-PDGF-induced Ca^{2+}_n alterations.** Cells were loaded with Fluo-3/AM, and confocal images were obtained as in Fig. 1. Individual traces represent data obtained from a single nucleus. *A*, AA-PDGF (20 ng/ml) was added at the *arrow*. AA-PDGF induced a transient increase in Ca^{2+}_n but did not result in oscillations. *B*, cells pretreated with caffeine (2.5 mM; *first arrow*) for 2 min exhibited AA-PDGF (20 ng/ml, *second arrow*) -induced Ca^{2+}_n oscillations similar to those seen upon BB-PDGF addition. *C*, AA-PDGF (20 ng/ml), was added at the first *arrow*, and caffeine (2.5 mM) was added at the second *arrow*. Treatment of cells with caffeine results in induction of Ca^{2+}_n oscillations by AA-PDGF. These data are representative of data obtained from 5 or more different cell preparations for each treatment condition, with a total of 220 cells examined.

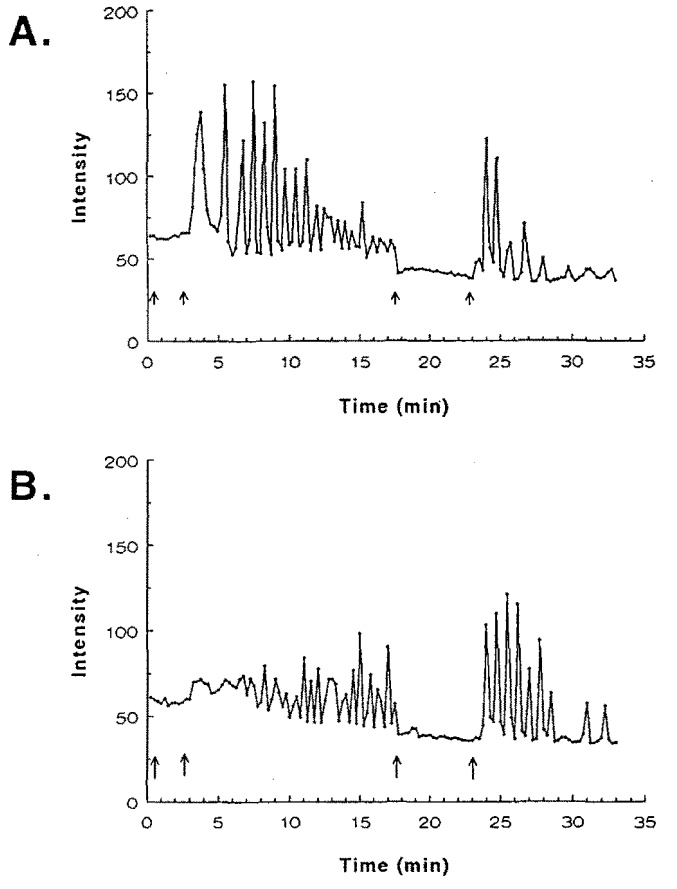

FIG. 5. **Sequential induction of Ca^{2+}_n oscillations by sequential addition of PDGF isoforms.** Cells were loaded with Fluo-3/AM, and confocal were images obtained as in Fig. 1. *Panels A* and *B* represent single nucleus traces obtained simultaneously from two different cells on the same coverslip. Caffeine (2.5 mM) was added at the *first arrow*, and AA-PDGF (20 ng/ml) was added at the *second arrow*, with resulting AA-PDGF-induced Ca^{2+}_n oscillations. Oscillations were then stopped by the addition of 2.5 mM EGTA (*third arrow*). Addition of BB-PDGF (10 ng/ml, *fourth arrow*) 5 min later induced a second round of Ca^{2+}_n oscillations. Qualitatively similar results were obtained independently with a different cell preparation, with a total of 33 cells examined in the two experiments.

(19–21). The amplitude of the oscillations is usually insensitive to agonist dose, but the frequency increases in a dose-dependent manner, providing a frequency-modulated signaling system. It is now clear that oscillations in Ca^{2+}_i is a widespread phenomenon occurring in both exocrine and endocrine cells, white blood cells, fibroblasts, endothelial and epithelial cells, astrocytes, and oocytes (22–24). We have previously reported that PDGF causes oscillations in cytoplasmic Ca^{2+}_i in smooth muscle cells and that these oscillations are specific to activation of the PDGF α receptor (10). The oscillations usually took the form of discrete oscillations (*i.e.* Ca^{2+}_i returns to base line at the end of each oscillation), or sinusoidal oscillations superimposed on a sustained elevation of Ca^{2+}_i. Oscillations are thought to potentially provide extremely fine control over the signaling process (*i.e.* differential activation of Ca^{2+}-dependent processes based on the frequency or shape of the oscillations). The present study documents, for the first time, agonist-induced oscillations in *nuclear* free Ca^{2+} levels. Furthermore, we have observed differences in the propensity of two closely related agonists (the BB- and AA-PDGF isoforms) to evoke oscillations in Ca^{2+}_n. These findings strongly suggest that Ca^{2+}_n oscillations may play an important role in the different signal transduction mechanisms and biological activities of the two growth factors.

In the present study, nuclear free Ca^{2+} levels were measured by specifically mapping the nuclear region of each cell in thin optical sections through a plane in the middle of the nucleus obtained using confocal laser scanning microscopy. We have also observed alterations in nuclear Ca^{2+} levels irrespective of whether cells were loaded by microinjection of Fluo-3 salt or by Fluo-3/AM loading and have also observed qualitatively similar findings in Calcium Green-1/AM and Calcium Crimson loaded BALB/c3T3 cells (data not shown). In addition, under the conditions employed in the present study to load cells with Fluo-

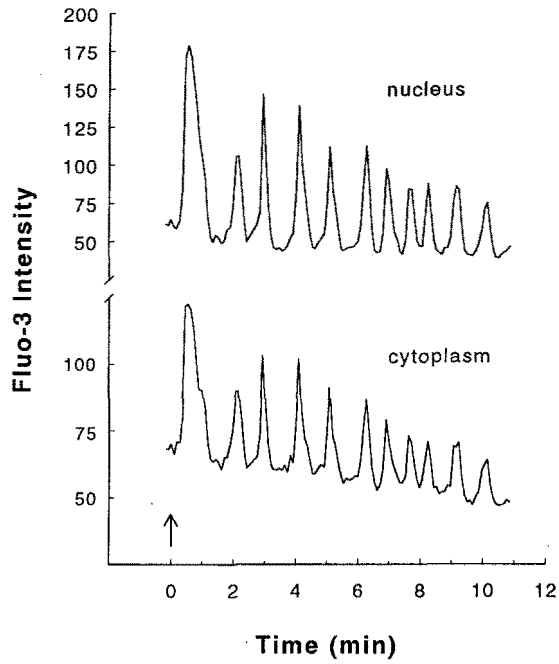

FIG. 6. **Relationship between BB-PDGF-induced oscillations in cytosolic (Ca^{2+}_i) and nuclear (Ca^{2+}_n) free Ca^{2+}.** Quiescent BALB/c3T3 cells were loaded with Fluo-3/AM, and images from an optical section through the center of the nucleus, were obtained by confocal laser scanning microscopy at 5-s intervals. BB-PDGF (10 ng/ml) was added at the *arrow*. Represented on the *y* axis is the average fluorescent pixel intensity of the mapped area of the cytoplasm and nucleus from the same cell. Qualitatively similar results were obtained independently with different cell preparations; a total of 40 cells were examined in three experiments.

3/AM, only 4% of the initial cellular Fluo-3 intensity remained following labeling with Fluo-3/AM and detergent extraction of cells under conditions in which the intracellular organelles remain intact (17). No Fluo-3 intensity remained in the nucleus after digitonin extraction. Images of thin optical sections through a plane in the middle of the nucleus demonstrated essentially homogeneous Fluo-3 intensity changes throughout the nuclear area, which would not be expected if organelle-entrapped dye was contributing significantly to the observed signal. Thus, we believe that the observed changes in Fluo-3 intensity observed represent PDGF isoform-stimulated alterations in Ca^{2+}_n.

The presence of PDGF-induced isoform-specific oscillations in Ca^{2+}_n (Figs. 1–5) argue in favor of the presence of specific cellular mechanisms for the regulation of Ca^{2+}_n levels. A major question that still needs to be resolved is whether alterations in Ca^{2+}_n are regulated locally in the nucleus or reflect alterations in cytoplasmic Ca^{2+} that are transmitted to the nucleus through freely permeable nuclear pores. Historically, the boundary between the nucleus and cytoplasm has been thought to be passive and nonselective; alterations in cytoplasmic composition could be transmitted passively to the nucleus (or *vice versa*) through the nuclear membrane and pores. However, more recent evidence has accumulated suggesting that the nuclear membrane is not a passive but rather a selective barrier that can exert control on the information flow between the nucleus and cytoplasm (25). Ca^{2+} may serve as a messenger transmitting cytoplasmic information to the nucleus (or *vice versa*).

Evidence of nuclear specific Ca^{2+} regulatory mechanisms comes from recent studies that have suggested that isolated liver nuclei may contain Ca^{2+} regulatory machinery: Ca^{2+} uptake via a Ca^{2+}-ATPase activity and IP_3-stimulated release of accumulated nuclear Ca^{2+} mediated by IP_3 receptors localized to the nuclear membrane (26–28). Furthermore, evidence supporting the existence of a nuclear inositide cycle that is unique to, and contained within, the nucleus itself has recently begun to be accumulated (for review, see Ref. 29). However, these data are difficult to interpret due to potential contamination of the isolated nuclear preparations with endoplasmic reticulum components.

Other support for the existence of nuclear specific Ca^{2+}-regulatory mechanisms has come from the use of digitized video imaging and confocal microscopy that has, in certain cases, demonstrated the existence of a concentration gradient of Ca^{2+} between the nucleus and cytoplasm. The direction of this nuclear/cytoplasmic gradient in resting cells as well as the effect of Ca^{2+}_i-stimulating agonists on this gradient, however, varies widely from study to study, possibly reflecting differences in the cell systems and agonists examined. For example, Williams and colleagues (30, 31) found higher nuclear than cytoplasmic Ca^{2+} levels in resting toad stomach smooth muscle cells loaded with fura-2 and imaged by conventional fluorescence microscopy, which responded to depolarizing stimuli with only cytoplasmic and not nuclear increases in free Ca^{2+}. Similar findings were reported for resting and stimulated human vascular smooth muscle cells loaded with fura-2 and imaged by conventional digitized video microscopy (32). In contrast, other studies examining mammalian-derived smooth muscle cell cultures using either fura-2 with conventional digitized video microscopy (33) or indo-1 with confocal laser microscopy (34, 35) have found Ca^{2+}_n to be lower than cytoplasmic levels in resting cells. In these latter studies, stimulation with a variety of Ca^{2+}-agonists resulted in increases in Ca^{2+}_n that equaled or exceeded those observed in the cytoplasm. Nuclear/cytoplasmic Ca^{2+} gradients have also been reported in fibroblast and renal epithelial cell lines, hepatocytes, pancreatoma cells, adrenal chromaffin cells, amphibian and mammalian neurons, and sea urchin eggs (36–42) with similar variation in results. Recent concerns have been raised however, regarding the accuracy of many of these measurements. Connor (43) has published data suggesting that compartmentalization of ester-loaded indicators can give rise to aberrant intracellular Ca^{2+} measurements, resulting in an apparent higher Ca^{2+}_i concentrations. A recent study published by Al-Mohanna and co-workers (44) seems to confirm these findings in that treatment of cells with anion transport inhibitors prevented the development of a cytoplasmic nuclear Ca^{2+} gradient. Thus, the existence of a cytoplasmic nuclear Ca^{2+} gradient in resting or stimulated cells and consequently evidence supporting the possibility of nuclear specific Ca^{2+} regulatory mechanisms is currently controversial.

To address the issue of whether the PDGF isoform-induced alterations in Ca^{2+}_n were distinct from alterations in Ca^{2+}_i in the same cell, we employed confocal line scanning microscopy (Fig. 7). BB-PDGF-induced oscillations in Ca^{2+}_i and Ca^{2+}_n occurred in a synchronous manner and displayed the same frequency in individual cells (Fig. 6). When a single line, which crosses both the cytoplasmic and nuclear regions of a cell, was repeatedly scanned at high temporal resolution (10 ms), we found that PDGF-BB caused an increase in Ca^{2+}_i first in the perinuclear region of cells 50–300 ms before an increase in Ca^{2+}_n was noted, suggesting that the increase in Ca^{2+}_n is due to local release of Ca^{2+} from perinuclear stores resulting in an increase in Ca^{2+}_i and diffusion of Ca^{2+} into the nucleus. These findings are consistent with recently published data indicating that in neuronal cells, the nuclear membrane is freely permeable to Ca^{2+} and that alterations in nuclear free Ca^{2+} observed in these cells in response to depolarization result from alterations in cytoplasmic Ca^{2+} (44). Allbritton and co-workers (45), using a heparin-dextran conjugate that when microinjected

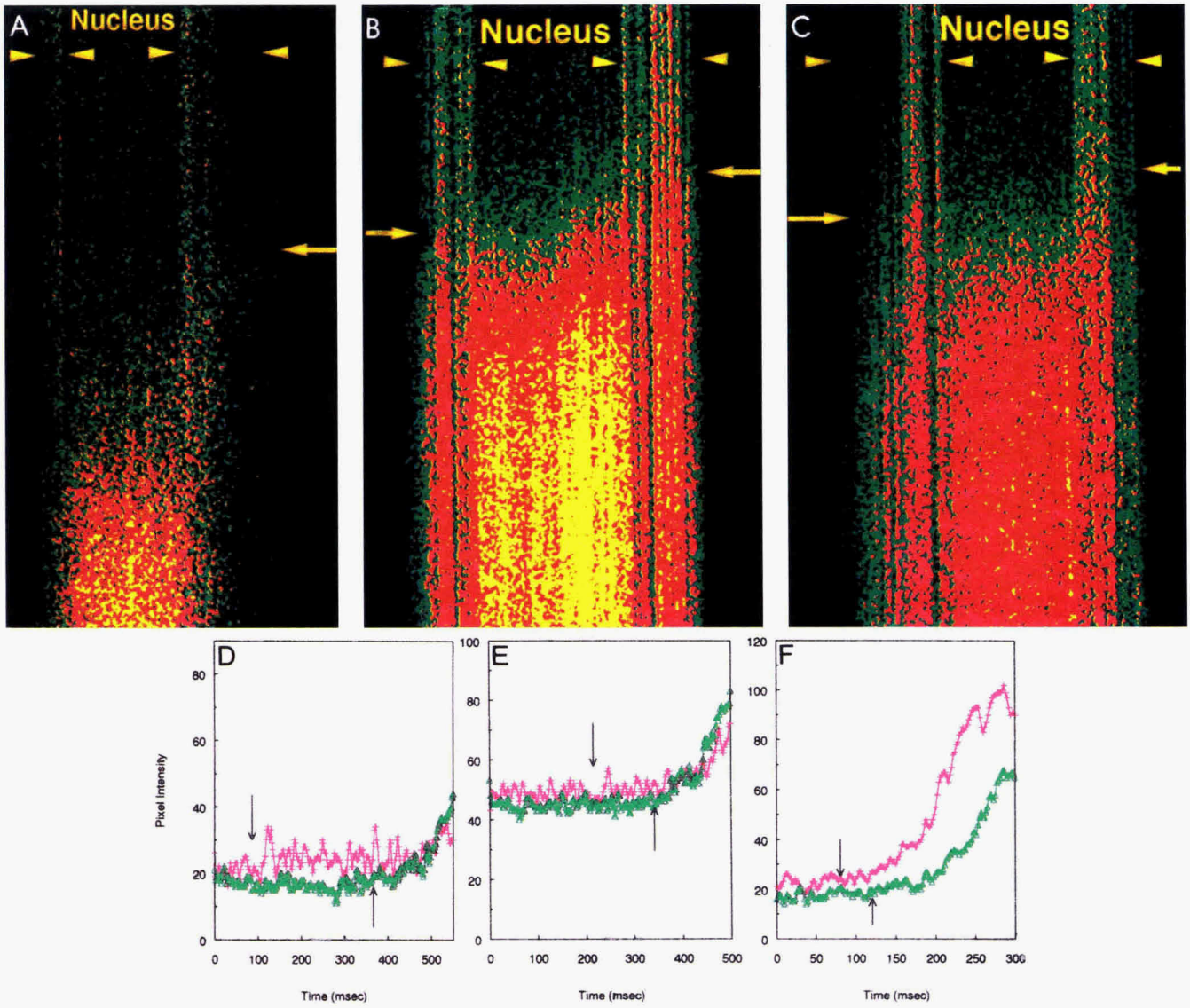

FIG. 7. **Relationship between BB-PDGF-induced increases in cytosolic (Ca^{2+}_i) and nuclear (Ca^{2+}_n) free Ca^{2+} assessed by rapid confocal line scanning microscopy.** Quiescent BALB/c3T3 cells were loaded with Fluo-3/AM and rapid confocal line scanning microscopy performed as described under "Experimental Procedures." *A–C* are representative examples of two-dimensional line scan images from BALB/c3T3 cells following addition of BB-PDGF (10 ng/ml). The area of the nucleus is labeled as such, and the area of the cytoplasm on each side of the nucleus in each cell is defined as the region in between the *arrowheads*. The *arrows* designate the point where cytoplasmic Ca^{2+}_i increased following BB-PDGF addition. *D–F* are representative plots of Fluo-3 pixel intensity *versus* time in the cytoplasmic (*red*) and nuclear (*green*) regions of the cells. The *arrows pointing down* represent the time when cytoplasmic Ca^{2+}_i increased, and the *arrows pointing up* represent the time when nuclear Ca^{2+}_n increased, both following addition of BB-PDGF. In all cases examined, Ca^{2+}_i increased in the perinuclear area 50–300 ms before an increase in Ca^{2+}_n was observed.

into cells was too large to enter the nucleus, found that both agonist-stimulated cytoplasmic and nuclear Ca^{2+} transients were suppressed. These studies and the data presented in this paper in aggregate suggest that the nuclear membrane is freely permeable to Ca^{2+} ions and that changes in Ca^{2+}_n reflect changes in Ca^{2+}_i. What is at present still unclear is the functional significance of agonist-stimulated increases in Ca^{2+}_n.

Both AA- and BB-PDGF isoforms appear to have similar early signal transduction mechanisms in cells such as BALB/c3T3 fibroblasts, promoting receptor tyrosine autophosphorylation, phospholipase Cγ association, IP_3 generation, and increases in intracellular Ca^{2+} (5, 8, 13, 16, 18, 46). However, in the present studies, temporally distinct patterns of PDGF isoform-dependent increases in Ca^{2+}_n were observed in BALB/c3T3 cells (Figs. 2*A* and 4*A*), with BB-PDGF causing oscillations in Ca^{2+}_n. In addition, the frequency of BB-PDGF induced oscillations in Ca^{2+}_n was dependent on the presence of extracellular Ca^{2+} (Fig. 2*B*). A number of mechanisms have been proposed to explain the regulation of Ca^{2+}_i oscillations (19–22). One model suggests that oscillations in the level of IP_3 caused by cyclical activation of protein kinase C inhibit activation of phospholipase C. In another model, Ca^{2+}_i released by IP_3 causes activation of phospholipase C resulting in further release of Ca^{2+} (cooperative release). A two-pool model suggests that IP_3 initially causes release of Ca^{2+} from an IP_3-sensitive pool, which then triggers release of Ca^{2+} from a distinct CICR pool. Variations on this model include the hypotheses that the IP_3 and CICR receptor are one and the same and that Ca^{2+} can act synergistically with IP_3 either on the luminal or cytoplasmic side of the channel. In the studies reported here, increasing IP_3 levels in the absence of PDGF receptor activation was not sufficient to induce oscillations in Ca^{2+}_n in BALB/c3T3 cells, but it did generate a single transient increase in Ca^{2+}_n similar to the initial oscillations stimulated by PDGF-BB (Fig. 2*C*). Our data

also indicate that generation of Ca^{2+}_n oscillations by BB-PDGF most likely employs a mechanism involving cyclical release of Ca^{2+} from intracellular stores near the nuclear membrane whose frequency is modulated by influx of extracellular Ca^{2+} (potentially by increasing the rate of refilling of the BB-PDGF-sensitive Ca^{2+} pools). Additional support for a role for Ca^{2+} pool refilling in the mechanism of Ca^{2+}_n oscillations comes from findings that intracellularly loaded Ca^{2+}-chelating agents such as Fura-Red, BAPTA, and nonphotolyzed NITR-5 inhibit BB-PDGF-induced Ca^{2+}_n oscillations but not the initial spike in Ca^{2+}_n.[2] A similar dependence of agonist-induced cytoplasmic Ca^{2+} oscillations on extracellular Ca^{2+} has been observed in other systems (47, 48).

Caffeine was used to test for the involvement of CICR pools in BB-PDGF-induced Ca^{2+}_n oscillations (Fig. 3, *A–C*). Care must be taken in interpretation of results obtained with caffeine, as caffeine has recently been found to act as a low affinity IP_3 receptor antagonist, to inhibit agonist-induced IP_3 formation, and to inhibit G-protein-dependent phospholipase C activity (49). In addition, caffeine at high concentrations can increase cAMP levels. Alterations in cAMP levels are not thought to play a part in PDGF-induced Ca^{2+}_n alterations in the present study, as PDGF stimulation does not increase cAMP levels in BALB/c3T3 cells (50). In the present study, caffeine alone did not elicit oscillations in Ca^{2+}_n but BB-PDGF-induced Ca^{2+}_n oscillations could be either reinitiated or increased in frequency by caffeine addition (Fig. 3, *B* and *C*). These data are consistent with a mechanism for the generation of Ca^{2+}_n oscillations in which BB-PDGF-stimulated increases in intracellular IP_3 initiate the Ca^{2+}_n response and activation of caffeine-sensitive Ca^{2+} pool sustains the oscillations in Ca^{2+}_n. Whether this caffeine-sensitive pool is the same as the IP_3-sensitive pool or represents an IP_3-insensitive CICR will have to await further studies using spatially targeted perinuclear and nuclear release of caged IP_3, BAPTA, and Ca^{2+}.

In contrast to BB-PDGF, AA-PDGF did not cause oscillations in Ca^{2+}_n unless cells were exposed to caffeine. The nonoscillatory Ca^{2+}_n response to AA-PDGF was converted into an oscillatory response by caffeine treatment either before or after growth factor addition (Fig. 4, *B* and *C*). BB-PDGF was also capable of releasing Ca^{2+} from pools involved in the generation of oscillations in Ca^{2+}_n that were not released by AA-PDGF as evidenced by the finding that BB-PDGF could elicit oscillations in Ca^{2+}_n in cells in which AA-PDGF/caffeine-induced oscillations had been previously evoked (Fig. 5). In addition, inhibition of AA-PDGF/caffeine-induced oscillations in Ca^{2+}_n by EGTA did not prevent BB-PDGF from causing oscillations in Ca^{2+}_n, although the oscillations ceased within 5 min after BB-PDGF addition. These findings imply that: 1) AA- and BB-PDGF activate distinct Ca^{2+} pools; 2) AA- and BB-PDGF use the same Ca^{2+} pools but BB-PDGF acts as a more efficient stimulus of the same Ca^{2+} pool activated by AA-PDGF; or 3) AA and BB-PDGF use the same Ca^{2+} pools but BB-PDGF recruits additional Ca^{2+} pools necessary for oscillations in Ca^{2+}_n. Differentiation between these possibilities at present is not possible. The data can best be interpreted as indicating that BB-PDGF generates a "caffeine-like signal" that activates Ca^{2+} release from either additional or identical Ca^{2+} pools leading to oscillations in Ca^{2+}_n; this caffeine-like signal is not generated following AA-PDGF stimulation. While the identity of the signal by which BB-PDGF activates the release of Ca^{2+} from a pool(s) not activated by AA-PDGF is not known, recent findings examining PDGF α and β receptor substrates have shown that following activation, both PDGF receptors bind to a number of substrates: phospholipase C-γ1, phosphatidylinositol 3′-kinase, $pp60^{c\text{-}src}$, $p62^{c\text{-}yes}$, $p59^{fyn}$, *nck*, and Grb-2, a 120-kDa protein thought to be associated with phosphatidylinositol 3′-kinase and Syp (51). However one difference, in terms of substrate binding, which has been reported for the PDGF α and β receptors, is that the PDGF β receptor binds far more Ras GTPase activating protein than the PDGF α receptor following activation (52). This implies the potential involvement of Ras in the signaling pathway responsible for oscillations in Ca^{2+}_n.

A major finding of the present study is that a difference in the intracellular signal produced as a result of the two ligand-receptor interactions has been identified that is manifested at the level of nuclear Ca^{2+} alterations. Signaling differences involving nuclear second messenger levels and fluctuations within the nucleus are prime candidates for the source of differential gene activation by the two PDGF isoforms, therefore providing the basis for differences in biological activity. The prospect of nuclear Ca^{2+} levels playing a role in signal transduction processes associated with gene activation and cell growth is supported by the identification of a number of calcium-mediated enzymatic and transcriptional activities within the nucleus, including Ca^{2+}/calmodulin-dependent gene expression, Ca^{2+}-dependent regulation of transcriptional response elements similar to the cAMP response element, Ca^{2+}-dependent endonucleases, and nuclear localization of a Ca^{2+}-dependent protein kinase C isozyme. Additional evidence for this linkage is provided by recent findings of nuclear Ca^{2+} transients produced by diverse stimuli associated with cell proliferation and growth. Other mitogens, including epidermal growth factor and erythropoietin, induce Ca^{2+}_n increases (41, 53); fertilization of sea urchin eggs is accompanied by large and rapid Ca^{2+}_n transients (42); and, Ca^{2+}_n transients stimulated by depolarization are found to be quite pronounced in dorsal root ganglion neurons undergoing the early phases of neurite outgrowth and extension in contrast to greatly attenuated signals in those neurons in which neurite regeneration has already been completed (36). Insufficient data currently exists to enable one to distinguish which biological activities may be unique to the activation of specific PDGF receptor types (α *versus* β; or $\alpha\alpha$ *versus* $\alpha\beta$ *versus* $\beta\beta$). It has been reported that AA-PDGF, acting only through the α receptor, is less mitogenic than other PDGF isoforms and is unable to stimulate actin reorganization or chemotaxis in fibroblasts (54), and cells transfected with mutant PDGF β receptors lacking the ability to bind Ras GTPase activating protein have been found to be deficient in chemotaxis (55). Further information on the functional consequences of the activation of the multiple receptor types by specific PDGF isoforms will be needed to establish the significance between differences in Ca^{2+}_n signaling patterns and biological activity.

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