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Modified kinetics of platelet-derived growth factor-induced Ca^{2+} increases in NIH-3T3 cells overexpressing phospholipase $C\gamma_1$

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The effects of platelet-derived growth factor (PDGF) on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and inositol phosphates were studied in NIH-3T3 fibroblasts transfected with cDNA for phospholipase $C\gamma_1$ ($PLC\gamma_1$) to yield a 7-fold overexpression of this enzyme, compared with cells containing normal levels of $PLC\gamma_1$. In a study published recently [Margolis, Zilberstein, Franks, Felder, Kremer, Ullrich, Rhee, Skorecki & Schlessinger (1990) Science **248**, 607–610] it was reported that this overexpression of $PLC\gamma_1$ caused a specific potentiation of the inositol phosphate response to PDGF, but this was not associated with an enhancement of the $[Ca^{2+}]_i$ response. In the present study, measurements of the time course and isomeric profile of PDGF-induced inositol phosphate formation demonstrated that the initial rate of Ins(1,4,5)P_3 formation was also enhanced in the $PLC\gamma_1$-overexpressing cells, yielding a 10-fold greater increase at 1 min compared with the parental NIH-3T3 cells. By contrast, bradykinin-induced phosphoinositide metabolism was unchanged in $PLC\gamma_1$-transfected cells. Measurements of $[Ca^{2+}]_i$ in cell populations and single cells showed a significant latent period following PDGF addition prior to the $[Ca^{2+}]_i$ increases in both cell lines, which decreased in a dose-dependent manner with increasing PDGF concentration. The duration of the latent period was decreased and the maximal rate of $[Ca^{2+}]_i$ rise was increased in the $PLC\gamma_1$-overexpressing cells at all doses of PDGF examined. In single-cell measurements these cells also responded to PDGF with a greater peak amplitude of $[Ca^{2+}]_i$. Both intracellular Ca^{2+} mobilization and Ca^{2+} influx across the plasma membrane were enhanced in the $PLC\gamma_1$-overexpressing cells. There was no difference between the two cell lines in either the latency or the magnitude of the $[Ca^{2+}]_i$ increases induced by bradykinin. These data provide further evidence that $PLC\gamma_1$ is responsible for the PDGF-induced stimulation of Ins(1,4,5)P_3 formation. Moreover, in contrast to earlier conclusions, the modified kinetics of the $[Ca^{2+}]_i$ changes in $PLC\gamma_1$-overexpressing cells suggest that Ins(1,4,5)P_3 does play a predominant second messenger role in the PDGF-induced $[Ca^{2+}]_i$ increases. The data also indicate that the latent period may be a function of the time required to reach a threshold level of Ins(1,4,5)P_3, rather than an intrinsic property of the PDGF receptor.

INTRODUCTION

At least two classes of mitogens can be distinguished which appear to act through different signal transduction pathways (for reviews see Boyer *et al.*, 1989; Rana & Hokin, 1990). Once class constitutes the family of peptide growth factors that bind to receptors with intrinsic protein tyrosine kinase activity, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Hunter & Cooper, 1985; Carpenter, 1987). A number of substrates of these tyrosine kinases have been identified which could have regulatory functions within the cell, including phospholipase $C\gamma_1$ (Kumjian *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Rhee *et al.*, 1989; Wahl *et al.*, 1989, 1990; Ullrich & Schlessinger, 1990). Another widely studied class of mitogens activates a phosphoinositide-specific phospholipase C through the mediation of a receptor-linked GTP-binding protein. Both classes of mitogen stimulate the formation of the second messengers Ins(1,4,5)P_3 and diacylglycerol, which activate intracellular Ca^{2+}-release channels and protein kinase C respectively (Nishizuka, 1984; Berridge, 1987).

A number of studies have shown that PDGF causes an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in fibroblasts (Moolenaar *et al.*, 1984; McNeil *et al.*, 1985; Nanberg & Rozengurt, 1988; Tucker *et al.*, 1989) and that this $[Ca^{2+}]_i$ increase is associated with polyphosphoinositide breakdown and the production of Ins(1,4,5)P_3 (Berridge *et al.*, 1984; Besterman *et al.*, 1986; Nanberg & Rozengurt, 1988). Several lines of evidence have led to the conclusion that $PLC\gamma_1$ is the enzyme responsible for growth factor-induced effects on inositol lipid-dependent signalling. It has been shown that $PLC\gamma_1$ is a substrate for the tyrosine kinase activity of both PDGF and EGF receptors in cell-free systems and in intact cells (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1990). In addition, $PLC\gamma_1$ associates with these growth factor receptors, as shown by the co-precipitation of these proteins using antibodies to either the receptor or $PLC\gamma_1$ (Margolis *et al.*, 1989; Kumjian *et al.*, 1989). Antibodies to PtdInsP_2 inhibit bombesin- and PDGF-induced DNA synthesis (Matuoka *et al.*, 1988) and also block mitogenesis when injected into *ras*, *src* and *erbB* oncogene-transformed NIH-3T3 cells, whereas *myc*-transformed NIH-3T3 cells are not affected (Fukami *et al.*, 1988). Site-directed mutagenesis to inactivate the tyrosine kinase domain of the EGF receptor abolishes the ability of this receptor to activate the PLC/Ca^{2+} signal transduction pathway (Moolenaar *et al.*, 1988).

Recently, Margolis *et al.* (1990) demonstrated that overexpression of $PLC\gamma_1$ in NIH-3T3 cells results in an enhancement of the ability of PDGF to elevate inositol phosphate levels (measured at 30 min) without altering the responses to bradykinin or lysophosphatidic acid, agonists which do not utilize a tyrosine kinase-dependent mechanism of PLC activation.

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PLC, phospholipase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; fura-2/AM, fura-2 acetoxymethyl ester.

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These data suggest that distinct PLCs may be involved in the actions of the different classes of mitogens. In contrast to the clear enhancement of the effects of PDGF on inositol phosphate formation, PLCγ_1 overexpression did not result in a potentiation of the $[Ca^{2+}]_i$ responses to PDGF (measured in cell populations) or of the mitogenic potency of this growth factor. The present study was undertaken to examine the apparent contradiction between the effects of PLCγ_1 overexpression on inositol phosphate formation and the lack of effect on $[Ca^{2+}]_i$ changes. The cell line derived by Margolis *et al.* (1990), with a 7-fold overexpression of PLCγ_1 (labelled 3T-P1 in that study but designated NIH-3T3-γ here), was compared with a cell line transfected with the vector alone (designated NIH-3T3 here). The kinetics of $[Ca^{2+}]_i$ changes at the single cell level were examined by digital imaging fluorescence microscopy, and in parallel experiments the early kinetics and isomeric profile of inositol phosphate formation were measured in these cells. Comparison of the NIH-3T3 and NIH-3T3-γ cells demonstrated that Ins(1,4,5)P_3 production was increased by 10-fold in NIH-3T3-γ cells at early time points. The increased rate of Ins(1,4,5)P_3 formation was correlated with a decrease in the latent period which preceded the $[Ca^{2+}]_i$ responses to PDGF, as well as in an increase in the magnitude of the $[Ca^{2+}]_i$ responses. The Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ responses to bradykinin were not different in the two cell lines. These data confirm that PLCγ_1 overexpression causes a specific enhancement in the inositol lipid-dependent second messenger system stimulated by growth factor receptors. Furthermore, the kinetic studies of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 formation indicate that there is a direct relationship between these pathways, in contrast to the previous conclusions from population studies of $[Ca^{2+}]_i$ (Margolis *et al.*, 1990).

MATERIALS AND METHODS

Materials

myo-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals; fura-2 acetoxymethyl ester (fura-2/AM) and Pluronic F-127 were obtained from Molecular Probes and Dulbecco's modified Eagle's medium, trypsin and EDTA were from GIBCO. All other chemicals were obtained from Sigma or Fisher.

Cell culture

Two cell lines were used: NIH-3T3-γ, which is the PLCγ_1-overexpressing line 3T-P1 of Margolis *et al.* (1990), and NIH-3T3, which was used as the control cell line. NIH-3T3-γ cells were obtained by cotransfection of NIH-3T3 cells with a mammalian expression vector containing bovine PLCγ_1 cDNA together with pSVneo, and selected by their resistance to geneticin (G418) (Margolis *et al.*, 1990). The control cell line NIH-3T3 was transfected with pSVneo vector only. NIH-3T3-γ cells have a 7-fold increase in PLCγ_1 expression compared with the control cell line, as determined by immunoblotting. Tyrosine phosphorylation of PLCγ_1 and ability to co-precipitate the PDGF receptor were enhanced in parallel with the overexpression of PLCγ_1 (Margolis *et al.*, 1990). By contrast, immunoblotting using anti-(PDGF receptor) antibodies revealed no difference in the total cellular complement of PDGF receptors between the two cell lines. The cells were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2 in DMEM containing 10% fetal calf serum, 50 units of penicillin/ml, 50 μg of streptomycin/ml, 2.5 μg of fungizone/ml and 2.5 μg of amphotericin B/ml. They were subcultured twice weekly at 10^5 cells/flask (75 cm^2; Corning). The medium was changed to DMEM containing 1% fetal calf serum 24 h before carrying out the experiments, to minimize desensitization of the PDGF receptor by growth factors in the serum.

Measurement of $[Ca^{2+}]_i$ in cell suspensions

Suspensions of the cells were obtained by a brief trypsin/EDTA treatment (0.05% trypsin, 0.53 mM-EDTA) of the monolayers. The cells were washed twice with DMEM containing 1% fetal calf serum and then incubated for 1 h at 37 °C with shaking, to allow recovery from the trypsin treatment. The cells were washed twice with Hepes buffer (10 mM-Hepes, 121 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 2.0 mM-$CaCl_2$, 5.0 mM-$NaHCO_3$, 10 mM-glucose, 2% dialysed BSA, pH 7.4), and incubated for a further 30 min at 37 °C with shaking. Fura-2 loading was carried out by incubation with fura-2/AM (3 μM) in Hepes buffer for 20 min. After washing to remove unincorporated dye, the cells were incubated in the cuvette of a dual-excitation channel fluorimeter (Deltascan Photon Technology Inc.) maintained at 37 °C with continuous stirring. The excitation wavelengths were 340 nm and 380 nm, and the emission wavelength was 510 nm. Calibration of the ratio of fluorescence signals in terms of $[Ca^{2+}]_i$ was done using a K_d for fura-2–Ca^{2+} binding of 224 nM, as described previously (Grynkiewicz *et al.*, 1985; Rooney *et al.*, 1989*b*).

Measurement of inositol phosphates and phospholipids

At 3 days before carrying out the experiment, the cell culture medium was replaced with medium containing modified DMEM (inositol reduced to 10 μM) and supplemented with 10 μCi of *myo*-[2-^{3}H]inositol/ml. The initial plating density was selected to allow the cells to grow to near confluence by the time they were harvested for the experiment. Cells were resuspended as described above and incubated in the modified DMEM without [^{3}H]inositol for 1 h at 37 °C. The cell suspension was washed twice with Hepes buffer and incubated in the same buffer containing 10 mM-LiCl. Incubations were carried out in plastic scintillation vials containing the cells (5×10^5 cells/ml) maintained at 37 °C in a shaking water bath. After preincubation for 60 min, time courses were initiated by addition of agonist and the incubations were terminated at the required times by addition of $HClO_4$ (4% final concentration). The $HClO_4$ precipitates were sedimented by centrifugation and the supernatants were neutralized by addition of a freshly prepared 1:1 (v/v) mixture of Freon/tri-n-octylamine as described previously (Downes *et al.*, 1986; Rooney *et al.*, 1989*a*). Inositol phosphates were separated by h.p.l.c. using a Partisil SAX-10 anion-exchange column by a method based on that described by Irvine *et al.* (1985), with the modification described previously (Hoek *et al.*, 1987). Fractions were collected for the determination of [^{3}H]inositol phosphates by liquid scintillation counting (Hoek *et al.*, 1987). For measurement of [^{3}H]inositol phospholipids, the phospholipids were extracted from the $HClO_4$ precipitate (Downes & Wusteman, 1983), deacylated (Weels & Dittmer, 1965), and then the glycerophosphoinositols were separated by anion-exchange chromatography on columns of Dowex 1X-8 anion-exchange resin (200–400 mesh, formate form) (Berridge 1983; Creba *et al.*, 1983).

Measurements of $[Ca^{2+}]_i$ in individual cells using fluorescence imaging

Cells were plated on glass coverslips coated with poly(D-lysine) (5 μg/cm^3) in 35 mm Petri dishes (5×10^4 cells/dish). The cells were loaded with fura-2 by incubation with 4.6 μM-fura-2/AM in Hepes buffer containing 2% BSA and 0.03% Pluronic F-127 (Molecular Probes) for 30 min at 37 °C. The buffer was also supplemented with 200 μM-sulfinpyrazone to prevent leakage and redistribution of the fura-2 (Di Virgilio *et al.*, 1988). Images of fura-2 fluorescence were obtained as described previously

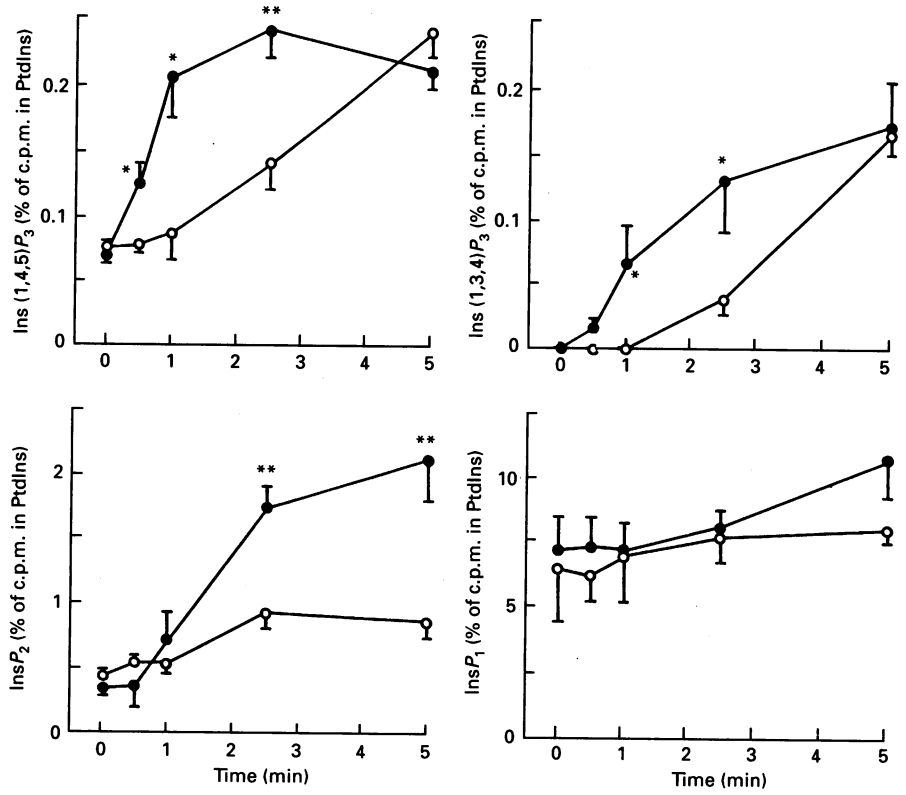

Fig. 1. Time course of production of inositol phosphates in response to PDGF

[^{3}H]Inositol-labelled NIH-3T3 and NIH-3T3-γ cells were incubated for the indicated times with 20 ng of PDGF/ml in the presence of 10 mM-LiCl. The incubations were stopped by adding $HClO_4$ and the inositol phosphates were separated by h.p.l.c. as described in the Materials and methods section. Inositol phosphate levels are expressed as a percentage of the radioactivity in PtdIns. Each point represent the mean ± S.E.M. of measurements obtained from three independent experiments using NIH-3T3 (○) and NIH-3T3-γ (●) cells. The significance of differences between the two cell lines at each time point were calculated using Student's t test; $*P < 0.05$, $**P < 0.01$.

(Rooney *et al.*, 1989*b*, 1990). Coverslips with attached fura-2-loaded NIH-3T3 cells were transferred to a chamber with 1 ml of fresh Hepes buffer (BSA concentration decreased to 0.25 %) and mounted on the stage of a Zeiss IM-35 inverted microscope. The stage, 16 × oil immersion objective and cell incubation chamber were thermostatically regulated at 30 °C. A liquid-N_2-cooled charge-coupled device camera (Photometrics Limited) was used as the imaging device. Images were digitized at 12-bit resolution and stored and analysed with a Heurikon HK68/M10 computer. Fluorescence images were obtained at excitation wavelengths 340 nm and 380 nm (10 nm bandwidth filters) using an emission band pass filter of 460–600 nm. The integration time for each image was 300–500 ms. $[Ca^{2+}]_i$ was calibrated from the ratio of fluorescence measurements at the two wavelengths, as described in detail previously (Grynkiewicz *et al.*, 1985; Rooney *et al.*, 1989*b*).

RESULTS

Effect of PLCγ_1 overexpression on inositol phosphate formation in response to PDGF and bradykinin

After labelling the cells with [^{3}H]inositol for 3 days, the basal levels of inositol phosphates, expressed as a percentage of the radioactivity in PtdIns, were identical in the NIH-3T3 and NIH-3T3-γ cell lines (see zero time points of Fig. 1). The labelling of the inositol lipids was also indistinguishable between the two cell lines (see legend to Fig. 2). Since the cells underwent three doublings during the labelling period, it can be assumed that the specific radioactivity of cellular inositol lipids was close to equilibrium with that of the medium [^{3}H]inositol. The lack of difference between the two cell lines with respect to basal levels of inositol lipids and their metabolites suggests that the transfection with PLCγ_1 cDNA did not cause any substantial change in the basal rates of polyphosphoinositide breakdown.

The effect of the overexpression of PLCγ_1 on the time course of inositol phosphate formation in response to PDGF (20 ng/ml) is shown in Fig. 1. As expected if PtdInsP_2 is the primary substrate for the PDGF-stimulated PLC, the earliest increases were observed in Ins(1,4,5)P_3, followed after a significant lag by Ins(1,3,4)P_3, InsP_2 and eventually InsP_1. The increases in InsP_1 were most apparent after 30 min of incubation in the presence of 10 mM-LiCl (results not shown). The most significant difference between the two cell lines in terms of PLC activation by PDGF was that the initial rates of increase were much faster in NIH-3T3-γ cells. At 1 min, the increase in Ins(1,4,5)P_3 formation above basal was 10-fold greater in the NIH-3T3-γ cells than in the control cells. Ins(1,3,4)P_3 levels, which were undetectable in the unstimulated cells, also increased with a much shorter lag in the NIH-3T3-γ cells. Interestingly, despite the slower rates of formation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 in the control NIH-3T3 cells, the differences between the two cell lines in the amounts of these InsP_3 isomers disappeared after about 5 min as their levels approached a steady state. By contrast, InsP_2 and InsP_1 showed substantial differences even after 30 min. At this time point InsP_2 levels were essentially similar to those at 5 min, whereas InsP_1 had increased to 15.2 and 32.1 % of the total basal radioactivity in PtdIns in NIH-3T3 and NIH-3T3-γ cells respectively (results not shown). At these later time points InsP_3 levels declined towards the basal level.

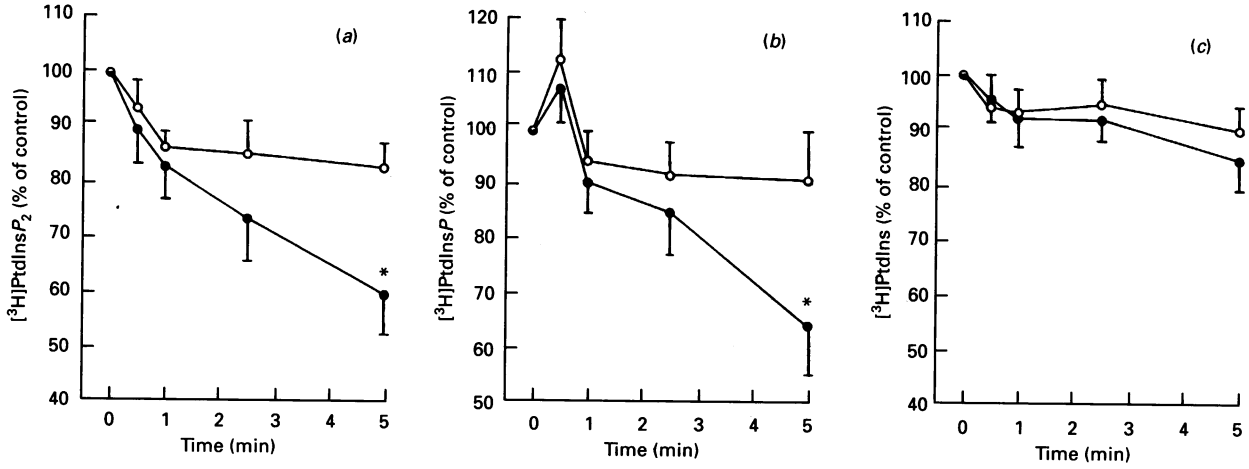

Fig. 2. Time course of inositol lipid changes in response to PDGF

Cells were incubated exactly as described for Fig. 1, and the lipids extracted from the $HClO_4$ precipitates were then deacylated and separated by anion-exchange chromatography as described in the Materials and methods section. Each point is the mean ± S.E.M. of values from three independent experiments expressed as a percentage of the radioactivity in each lipid prior to the addition of PDGF for NIH-3T3 (○) and NIH-3T3-γ (●) cells. The basal values (in c.p.m./10^6 cells) were for NIH-3T3 cells: PtdIns, 370238 ± 52338; PtdIns*P*, 12947 ± 2843; PtdInsP_2, 9931 ± 3179, and for NIH-3T3-γ cells: PtdIns, 424502 ± 47528; PtdIns*P*, 15004 ± 2980; PtdInsP_2, 10374 ± 3254. The significance of differences between the two cell lines at each time point were calculated using Student's *t* test; $*P < 0.05$.

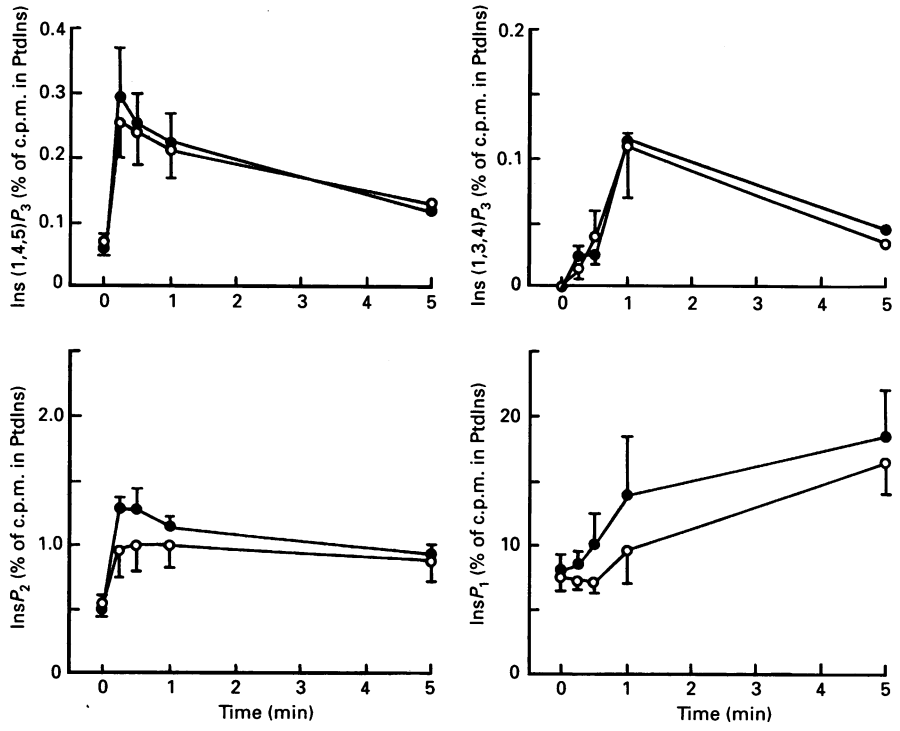

Fig. 3. Time course of production of inositol phosphates in response to bradykinin

Cells were incubated with 50 nM-bradykinin, and inositol phosphate measurements were carried out as described in the legend to Fig. 1. Each point represents the mean ± S.E.M. of measurements obtained from three independent experiments using NIH-3T3 (○) and NIH-3T3-γ (●) cells.

The changes in inositol lipid levels were determined in parallel with the inositol phosphate measurements of Fig. 1. As shown in Fig. 2, PDGF stimulated the breakdown of PtdInsP_2 and PtdIns*P*. However, in contrast to the marked difference between NIH-3T3 and NIH-3T3-γ cells in the rates of inositol phosphate formation described above, the two cell lines differed only at the later time points in the extent of polyphosphoinositide breakdown (Fig. 2). At the relatively low rates of inositol phosphate formation observed with PDGF (as compared with bradykinin, see below), it is possible that resynthesis of polyphosphoinositides from PtdIns compensated for much of the stimulated PLC activity, thus obscuring differences in breakdown rates. The small ap-

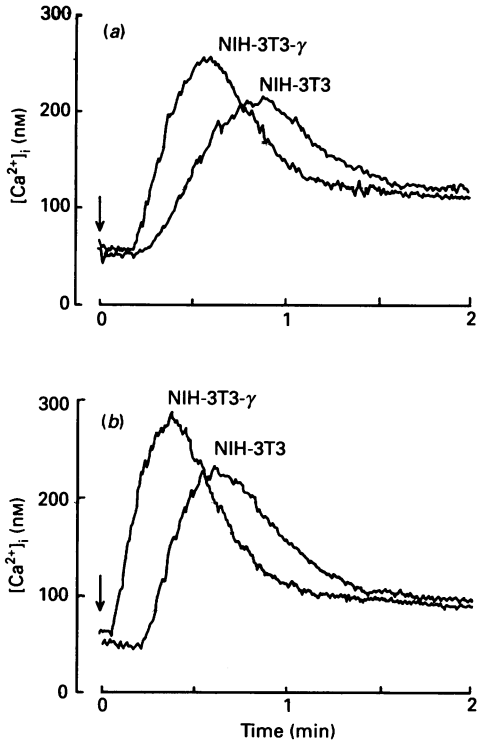

Fig. 4. $[Ca^{2+}]_i$ responses of NIH-3T3 and NIH-3T3-γ cells to PDGF in cell suspensions

Fura-2 loading and $[Ca^{2+}]_i$ measurements were carried out as described in the Materials and methods section. Traces represent typical responses to PDGF at 20 ng/ml (*a*) and 50 ng/ml (*b*) for NIH-3T3 and NIH-3T3-γ cells. PDGF was added at zero time.

parent increase in PtdIns*P* levels at 30 s after PDGF treatment was observed in all experiments, but was not significantly different from the zero time point for either cell line.

Fig. 3 shows the time course of inositol phosphate accumulation induced by 50 nM-bradykinin in the two cell lines. Bradykinin caused a much more rapid increase in Ins(1,4,5)P_3 than PDGF: at 15 s the elevation of Ins(1,4,5)P_3 was maximal, and then declined slowly during the ensuing 5 min. Ins(1,3,4)P_3, InsP_2 and InsP_1 levels followed the Ins(1,4,5)P_3 increase, but with slower kinetics. There was no significant difference in the levels of any of the inositol phosphates between the two cell lines throughout the time course. Thus the overexpression of PLCγ_1 does not affect the inositol phosphate changes induced by bradykinin, suggesting that the receptors for this agonist are coupled to a PLC isoenzyme other than PLCγ_1.

Effects of PDGF on $[Ca^{2+}]_i$ in cell suspensions

When $[Ca^{2+}]_i$ measurements were carried out using fura-2-loaded cells maintained in suspension, there was no significant difference between NIH-3T3 and NIH-3T3-γ cells in basal $[Ca^{2+}]_i$ calculated on the basis of paired experiments. The basal values for $[Ca^{2+}]_i$ were 84.5 ± 23.2 nM for NIH-3T3 cells and 59.1 ± 6.8 nM for NIH-3T3-γ cells. These data, together with the imaging data discussed below, indicate that the overexpression of PLCγ_1 did not affect basal Ca^{2+} homeostasis. Our values are in the range of 60–160 nM reported previously for $[Ca^{2+}]_i$ in fibroblasts measured using fura-2 and other dyes (Moolenaar *et al.*, 1984; McNeil *et al.*, 1985; Tucker *et al.*, 1989). Fig. 4 shows typical $[Ca^{2+}]_i$ responses induced by the addition of PDGF to the two cell lines maintained in suspension. Following PDGF addition at time zero, there was a well-defined lag (> 1 min at low PDGF doses), followed by a relatively slow transient increase in $[Ca^{2+}]_i$. By comparison, the $[Ca^{2+}]_i$ increases in response to bradykinin were very rapid and showed no discernible latent period in the cell population measurements (results not shown). The rate of $[Ca^{2+}]_i$ rise following PDGF addition increased in a dose-dependent manner, and the latent period decreased with increasing PDGF dose (Table 1). The major difference between the two cell lines was that the NIH-3T3-γ cells showed a significantly shorter latent period and a faster rate of $[Ca^{2+}]_i$ rise throughout the entire dose range of PDGF (Table 1). As reported previously by Margolis *et al.* (1990), there was no significant difference in the peak levels of $[Ca^{2+}]_i$ between the two cell lines in these cell suspension measurements.

$[Ca^{2+}]_i$ responses to PDGF and bradykinin in single cells

In order to characterize further the effects of PLCγ_1 overexpression on the time course and kinetics of $[Ca^{2+}]_i$ increases in response to PDGF, digital imaging fluorescence microscopy was used to follow the $[Ca^{2+}]_i$ changes in individual cells. The average basal $[Ca^{2+}]_i$ in both NIH-3T3 and NIH-3T3-γ cells was the same (159 nM). This value is somewhat higher than those observed in cell suspensions. When $[Ca^{2+}]_i$ was measured in single cells that were allowed to settle out of suspension on to the coverslip of the imaging chamber, the basal $[Ca^{2+}]_i$ level was

Table 1. Latency and magnitude of $[Ca^{2+}]_i$ changes induced by PDGF in suspensions of NIH-3T3 and NIH-3T3-γ cells

Experiments were carried out as described in the legend to Fig. 4 and the Materials and methods section. Latency was measured as the time between the addition of PDGF and half the peak height. ΔPeak $[Ca^{2+}]_i$ is the maximal increase above basal $[Ca^{2+}]_i$, and the rate of $[Ca^{2+}]_i$ rise was measured at half height. Each value represents the mean ± S.E.M. of measurements obtained from three to seven independent experiments. The significance of differences between the two cell lines was calculated by Student's *t* test; $*P < 0.05$.

PDGF (ng/ml)	Cell line	ΔPeak $[Ca^{2+}]_i$ (nM)	Latency (s)	Rate of rise (nM/min)
5	NIH-3T3	100.5 ± 12.9	62.2 ± 9.3	88.8 ± 16.6
	NIH-3T3-γ	123.3 ± 33.6	38.3 ± 5.3*	138.6 ± 20.4*
10	NIH-3T3	96.5 ± 20.0	31.6 ± 5.7	146.2 ± 3.9
	NIH-3T3-γ	107.4 ± 42.4	22.0 ± 6.7*	191.7 ± 16.6*
20	NIH-3T3	149.9 ± 13.4	32.4 ± 7.5	248.4 ± 29.6
	NIH-3T3-γ	213.0 ± 59.5	19.5 ± 5.4*	632.4 ± 107.4*
50	NIH-3T3	178.5 ± 19.5	15.1 ± 1.6	582.0 ± 75.3
	NIH-3T3-γ	155.0 ± 43.7	10.0 ± 1.4*	804.3 ± 129.2

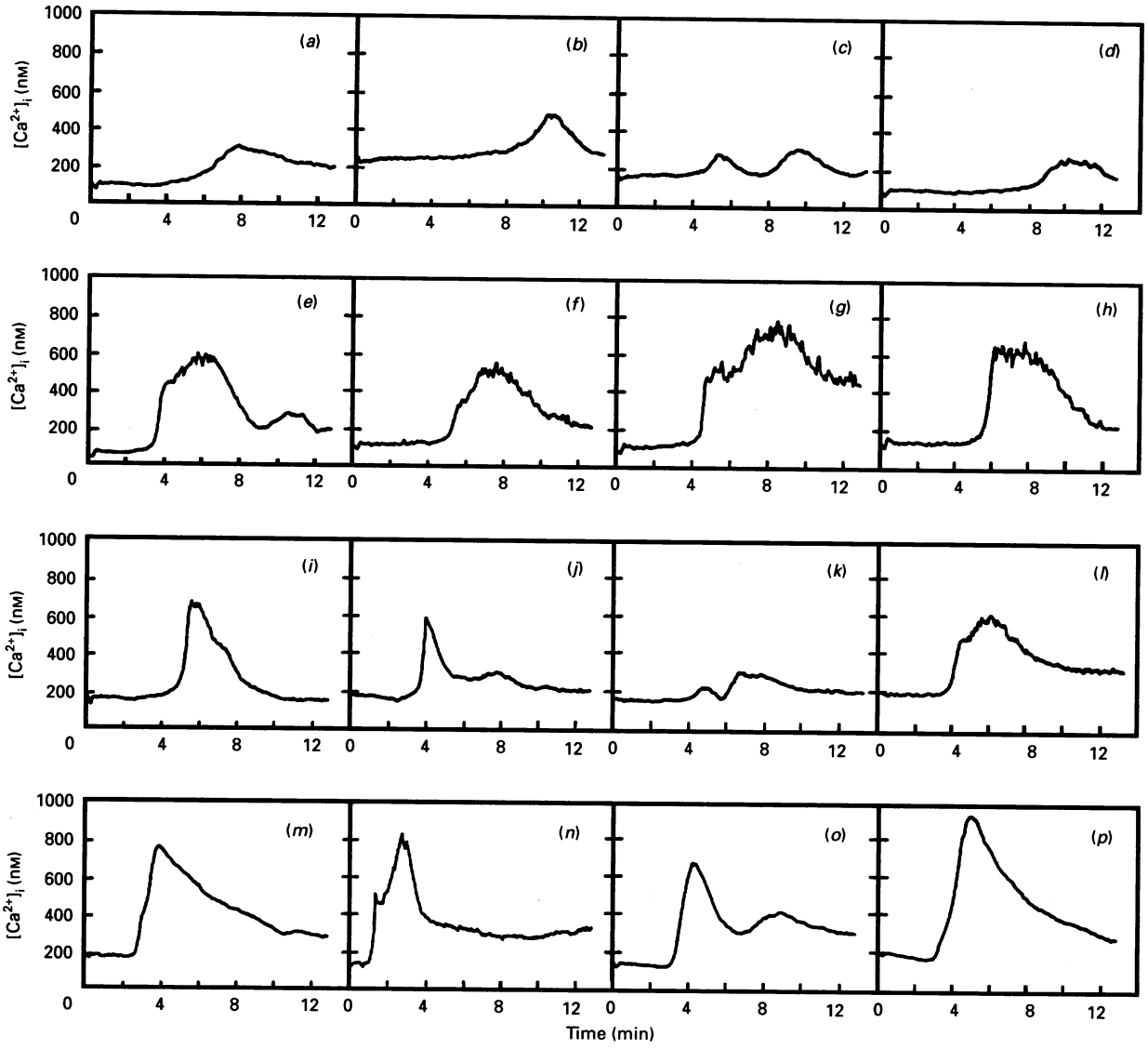

Fig. 5. $[Ca^{2+}]_i$ responses to PDGF in single cells

Monolayers of cells grown on coverslips were loaded with fura-2 as described in the Materials and methods section before being placed in the microscope incubation chamber. $[Ca^{2+}]_i$ values were calculated from the ratio of 340 nm and 380 nm fluorescence image pairs collected at 4 s intervals. PDGF was added at 20 s after the start of the time courses. The upper two panels show typical responses of NIH-3T3 (*a–d*) and NIH-3T3-γ (*e–h*) cells to PDGF at 1 ng/ml. The lower two panels show responses of NIH-3T3 (*i–l*) ad NIH-3T3-γ (*m–p*) cells to PDGF at 5 ng/ml.

Table 2. $[Ca^{2+}]_i$ parameters in single cells stimulated with PDGF and bradykinin

Measurements of $[Ca^{2+}]_i$ in monolayers of NIH-3T3 and NIH-3T3-γ cells were carried out using digital imaging fluorescence microscopy as described in the legend to Fig. 5 and in the Materials and methods section. The ΔPeak $[Ca^{2+}]_i$ is the magnitude of the $[Ca^{2+}]_i$ change above basal, the latency is the time between the addition of agonist and the rise to 50% of maximal peak height, and the peak width represents the time that the $[Ca^{2+}]_i$ levels remained elevated to at least 50% of maximum. These parameters were measured for individual cells in the microscope field. Values represent the means ± S.E.M. of measurements obtained for the number of cells shown (*n*). The significance of differences between the two cell lines was calculated using Student's *t* test; $*P < 0.05$.

Condition	Cell line	ΔPeak $[Ca^{2+}]_i$ (nM)	Latency (s)	Peak width (s)	*n*
PDGF (1 ng/ml)	NIH-3T3	200 ± 16	416 ± 19	179 ± 19	51
	NIH-3T3-γ	477 ± 54*	318 ± 18*	213 ± 18	39
PDGF (5 ng/ml)	NIH-3T3	322 ± 21	265 ± 9	199 ± 38	124
	NIH-3T3-γ	636 ± 58*	220 ± 12*	192 ± 8	83
Bradykinin (50 nM)	NIH-3T3	930 ± 74	6 ± 1	26 ± 1	20
	NIH-3T3-γ	904 ± 101	5 ± 1	26 ± 4	20

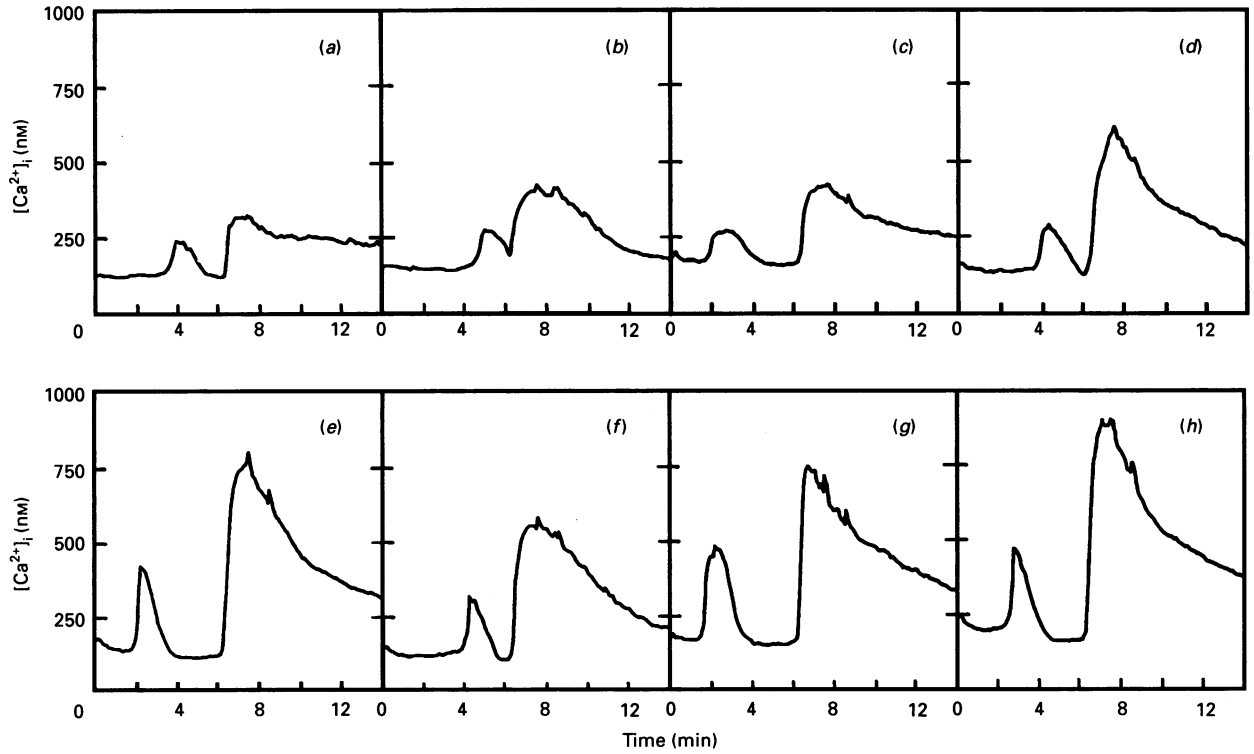

Fig. 6. Effect of extracellular Ca^{2+} depletion and re-addition on responses to PDGF in single cells

Monolayers of fura-2-loaded cells were preincubated in normal Hepes buffer in the cell incubation chamber of the imaging system. Just before the initiation of the $[Ca^{2+}]_i$ measurements, the cell incubation medium was changed to a Ca^{2+}-free Hepes buffer. PDGF (20 ng/ml) was added 1 min after Ca^{2+} removal (20 s after start of traces). After 6 min of incubation in the low-Ca^{2+} medium, the medium was changed back to the normal Hepes buffer containing 2 mM-$CaCl_2$ and 20 ng of PDGF/ml. Typical traces obtained with NIH-3T3 cells (*a–d*) and NIH-3T3-γ cells (*e–h*) are shown.

similar to that found in the cell population measurements. Thus it seems likely that the higher basal $[Ca^{2+}]_i$ calculated for cells grown on coverslips is not an artefact of the imaging measurements. Differences in basal Ca^{2+} fluxes across the plasma membrane could account for the higher $[Ca^{2+}]_i$ in the attached cells, perhaps caused by the relatively large surface area/volume ratio of these flattened cells compared with the spherical cells in suspension.

Previous studies by us (Prentki *et al.*, 1988; Rooney *et al.*, 1989*b*) and others (Connor *et al.*, 1987; Ambler *et al.*, 1988; Jacob *et al.*, 1988; Millard *et al.*, 1988) have demonstrated a considerable cell-to-cell heterogeneity in the $[Ca^{2+}]_i$ responses of individual cells to hormonal stimulation in a variety of cell types. Similarly, PDGF treatment of NIH-3T3 cells induced a variety of complex patterns of $[Ca^{2+}]_i$ change. A representative sample of these different patterns of response for two doses of PDGF in NIH-3T3 and NIH-3T3-γ cells is shown in Fig. 5. All cells showed a substantial latent period with no change from basal $[Ca^{2+}]_i$, followed by a mono- or bi-phasic increase in $[Ca^{2+}]_i$ which subsequently declined towards basal. The two distinct phases of the initial $[Ca^{2+}]_i$ rise (e.g. Figs. 5*e*, 5*l* and 5*n*) were not always resolved, but were observed for the majority of NIH-3T3-γ cells and NIH-3T3 cells stimulated with higher PDGF doses. Some cells showed one to three subsequent transient $[Ca^{2+}]_i$ increases after the first $[Ca^{2+}]_i$ response had declined, but there were no clear-cut oscillations in $[Ca^{2+}]_i$ of the type reported previously for various G-protein-coupled receptors (Woods *et al.*, 1986; Prentki *et al.*, 1988; Rooney *et al.*, 1989*b*; Berridge, 1990; Jacob, 1990). As found for the cell suspension measurements of $[Ca^{2+}]_i$, the latent period decreased with increasing agonist dose. Furthermore the latent period for each dose of PDGF was significantly shorter in the NIH-3T3-γ cells than in the control NIH-3T3 cells (Table 2). In addition to the effects of $PLC\gamma_1$ overexpression on the latency of the PDGF-induced $[Ca^{2+}]_i$ increases, the cells plated on coverslips demonstrated significant differences in the maximal peak levels of $[Ca^{2+}]_i$ between the two cell lines (Fig. 5, Table 2). This effect was observed over the lower dose range of PDGF, where measurements in cell populations gave rather small $[Ca^{2+}]_i$ changes. The duration of the PDGF responses, measured as the peak width at half peak height, was not different between the two cell lines.

The $[Ca^{2+}]_i$ responses to bradykinin treatment measured at the single-cell level were unaffected by the overexpression of $PLC\gamma_1$. Bradykinin elicited brief transient $[Ca^{2+}]_i$ increases with peak values approaching 1 μM, which returned to the basal $[Ca^{2+}]_i$ level within 1–2 min. The kinetic parameters of the $[Ca^{2+}]_i$ increases induced by bradykinin (50 nM) are included in Table 2. The onset of the $[Ca^{2+}]_i$ rise following bradykinin treatment was very rapid, reaching a peak within 10 s after agonist addition. There was relatively little cell-to-cell heterogeneity in the kinetics of the bradykinin-induced $[Ca^{2+}]_i$ changes. The magnitude of the $[Ca^{2+}]_i$ increase, the latency and the peak width were not different between the NIH-3T3 and NIH-3T3-γ cell lines.

Effect of extracellular Ca^{2+} on PDGF responses in single cells

In order to determine the relative roles of extracellular and intracellular Ca^{2+} in the PDGF-induced $[Ca^{2+}]_i$ rise, we investigated the effects of PDGF in Ca^{2+}-depleted medium. For the experiment of Fig. 6, the cells were changed to a Ca^{2+}-free medium 1 min prior to PDGF addition and then the cells were

returned to normal Ca^{2+}-containing medium 6 min later (in the continuing presence of PDGF), after the initial responses to PDGF were completed. PDGF was still able to initiate a $[Ca^{2+}]_i$ rise in the absence of extracellular Ca^{2+}. This $[Ca^{2+}]_i$ increase must originate from intracellular stores, as reported previously (Moolenaar *et al.*, 1984; Nanberg & Rozengurt, 1988). The peak $[Ca^{2+}]_i$ levels measured in Ca^{2+}-free medium were, however, smaller and of shorter duration than those observed in medium containing normal Ca^{2+} concentration. On re-addition of Ca^{2+} to the medium, $[Ca^{2+}]_i$ was rapidly restored to a higher value which decayed with similar kinetics to the Ca^{2+} responses described above for cells incubated in medium containing normal Ca^{2+} concentrations. The difference between NIH-3T3 and NIH-3T3-γ cells with respect to latency and magnitude of $[Ca^{2+}]_i$ responses was clearly apparent in the absence of extracellular Ca^{2+}, indicating that the effects of PLCγ_1 overexpression are mediated at the level of the intracellular Ca^{2+} storage pools. However, the difference in amplitude was also apparent in the second-phase $[Ca^{2+}]_i$ increase, which presumably is dependent on PDGF-stimulated Ca^{2+} influx across the plasma membrane.

DISCUSSION

The finding that many cells contain several distinct isoenzymes of the inositol lipid-specific PLC has raised questions as to which of these plays a role in receptor-stimulated PtdInsP_2 breakdown, and whether different receptor types are coupled to distinct PLC classes (Boyer *et al.*, 1989; Rhee *et al.*, 1989). The cell line used in the present study, NIH-3T3-γ, was transfected with the gene for bovine PLCγ_1 and used to examine the role of this PLC isoenzyme in PDGF action (Margolis *et al.*, 1990). These studies demonstrated that overexpression of PLCγ_1 led to a specific enhancement in the magnitude of the PDGF-induced inositol phosphate response. However, this enhancement in second messenger generation apparently did not result in any additional increment in PDGF-induced DNA synthesis or alterations in the early $[Ca^{2+}]_i$ signals elicited by this agonist (Margolis *et al.*, 1990). While the lack of correlation of inositol phosphate second messengers with the mitogenic response is not surprising in view of the multiple tyrosine kinase substrates and consequent opportunity for parallel signalling pathways, the finding that the $[Ca^{2+}]_i$ response to PDGF was not affected along with the increase in inositol phosphate second messenger levels was unexpected. Based on these data, it was suggested that the mechanism by which PDGF elevates $[Ca^{2+}]_i$ is not dependent on the stimulation of inositol phosphate formation.

Several studies have indicated that Ca^{2+} signalling can be organized in a number of complex spatial and temporal ways at the single cell level, and much of this information is lost when measurements are carried out using cell populations (Prentki *et al.*, 1988; Rooney *et al.*, 1989*b*; Berridge, 1990). For this reason, we have examined the effects of PLCγ_1 overexpression on the $[Ca^{2+}]_i$ responses to PDGF in individual cells using digital imaging fluorescence microscopy. Moreover, in the earlier study which concluded that $[Ca^{2+}]_i$ changes in response to PDGF were not correlated with the InsP_3 increase (Margolis *et al.*, 1990), measurements were limited to a single time point. Since the effects of PDGF on $[Ca^{2+}]_i$ are relatively short-lived, and since it has been shown previously in other cell types that the rate of Ins(1,4,5)P_3 generation is the parameter most closely correlated with $[Ca^{2+}]_i$ increases (Thomas *et al.*, 1984; Williamson *et al.*, 1985), we also examined the early kinetics and isomer profile of inositol phosphate changes in NIH-3T3 and NIH-3T3-γ cells.

The most clear-cut difference between the NIH-3T3-γ and NIH-3T3 cells was in the kinetics of InsP_3 changes during the first 1–2 min after PDGF addition. In the PLCγ_1-overexpressing cell line, Ins(1,4,5)P_3 approached a maximum level within 1 min of addition of PDGF (20 ng/ml), whereas the control cells showed a distinct lag and required about 5 min to attain a similar Ins(1,4,5)P_3 level. The increased rate of Ins(1,4,5)P_3 formation is consistent with the measured 7-fold-higher effective concentration of PLCγ_1 acting as a substrate for the PDGF receptor tyrosine kinase (Margolis *et al.*, 1990). Interestingly, even in the presence of maximal doses, the PDGF-induced stimulation of PLC activity was substantially slower than the activation by bradykinin (maximal at 15 s). This could reflect an inherently slower pathway of PLC activation via the tyrosine kinase pathway as compared with G-protein-mediated activation. The finding that PLCγ_1 overexpression had no effect on the time course of bradykinin-stimulated increases of any of the inositol phosphates measured indicates that the effects of this agonist are not mediated by PLCγ_1.

Our finding that the major effect of PLCγ_1 overexpression was to alter the initial kinetics of Ins(1,4,5)P_3 formation in response to PDGF suggested that alterations at the level of the $[Ca^{2+}]_i$ increase might include changes in the latency and rate of $[Ca^{2+}]_i$ rise. The studies with cell populations demonstrated that both of these parameters were indeed changed, with a shorter latency and faster rate of the $[Ca^{2+}]_i$ response in NIH-3T3-γ cells. As reported by Margolis *et al.* (1990), there was no significant alteration in the amplitude of the $[Ca^{2+}]_i$ changes measured in cell suspensions. Since individual cells respond asynchronously to PDGF, direct studies of this parameter are best carried out at the single-cell level. The $[Ca^{2+}]_i$ imaging experiments confirmed our data obtained using cell populations in terms of the decreased latency of the PDGF response in NIH-3T3-γ cells. They also showed that the $[Ca^{2+}]_i$ changes elicited by PDGF are quite complex, often demonstrating multiple $[Ca^{2+}]_i$ spikes and generally two phases of the initial $[Ca^{2+}]_i$ rise. It was not possible to measure the latency of bradykinin responses in suspension due to the slow mixing time and asynchronous nature of the response. However, measurements at the single-cell level revealed that the decreased latency observed in NIH-3T3-γ versus NIH-3T3 cells treated with PDGF was not apparent with bradykinin.

The $[Ca^{2+}]_i$ responses in cell populations and those measured in individual cells differed in two ways. Firstly, the latent period was significantly longer for both cell lines in the single-cell measurements, and secondly, the amplitude of the $[Ca^{2+}]_i$ increases was enhanced in the NIH-3T3-γ cells when measured at the single-cell level, while this was not apparent in population measurements. These differences are not simply the result of comparing population to single-cell $[Ca^{2+}]_i$ measurements, because they were still apparent when the time courses of all of the individual cells studied in the imaging experiments were averaged to create population $[Ca^{2+}]_i$ signals. The longer latencies for single-cell measurements probably result, at least in part, from the lower temperature used in these studies. When the cell suspension studies were carried out at 30 °C instead of 37 °C, the latent period following PDGF addition was increased by about 70% (results not shown). However, the difference between the two preparations in latency for a given PDGF dose was greater than this, suggesting that other factors also play a role (see below).

The amplitude of the $[Ca^{2+}]_i$ increase with bradykinin was unchanged under conditions where NIH-3T3 and NIH-3T3-γ cells grown on coverslips gave clearly different magnitudes of $[Ca^{2+}]_i$ increase in response to PDGF. This demonstrates that the difference between the two cell lines cannot be explained by differences in fura-2 loading or dye distribution. If both agonists rely on the same Ca^{2+} pool(s), then it can also be concluded that

the amplitude difference with PDGF is not due to alterations at this level. Compared with bradykinin, the rate of Ins(1,4,5)P_3 accumulation and the time course of the rising phase of $[Ca^{2+}]_i$ in response to PDGF are very slow, with NIH-3T3 cells giving the lowest rates. Thus one explanation for the difference between NIH-3T3 and NIH-3T3-γ cells in the amplitude of the $[Ca^{2+}]_i$ increase is that different proportions of the Ca^{2+} released from the Ins(1,4,5)P_3-sensitive store are pumped out of the cytosol before the $[Ca^{2+}]_i$ rise is complete. The smaller surface area/volume ratio of the spherical cells in suspension could decrease the effect of Ca^{2+} pumping from the cytosol, contributing to the decreased latent period and the lack of effect of PLCγ_1 overexpression on $[Ca^{2+}]_i$ amplitude in this system.

In order to determine whether the effects of PLCγ_1 overexpression on PDGF-induced $[Ca^{2+}]_i$ increases were mediated at the level of the intracellular Ca^{2+} pools, experiments were carried out in the absence of extracellular Ca^{2+}. Under this condition a single transient $[Ca^{2+}]_i$ increase was elicited by PDGF. In addition, the $[Ca^{2+}]_i$ rise was monophasic, suggesting that the second $[Ca^{2+}]_i$ increase phase observed in the presence of physiological extracellular Ca^{2+} concentrations may reflect stimulated Ca^{2+} influx across the plasma membrane. Both the amplitude and latency differences between NIH-3T3 and NIH-3T3-γ cells stimulated with PDGF were still apparent in the absence of extracellular Ca^{2+}. This indicates that the effect of PLCγ_1 overexpression on Ca^{2+} homeostasis are mediated, at least in part, at the level of the mobilization of intracellular Ca^{2+} pools. However, PLCγ_1 overexpression also appears to affect the Ca^{2+} influx component, as demonstrated by the enhanced amplitude of the secondary $[Ca^{2+}]_i$ increase observed when Ca^{2+} was added back to the cells after completion of the internal Ca^{2+} mobilization. This result could be explained either by the presence of a link between the internal Ca^{2+} pools and the Ca^{2+} entry channel, as suggested by a number of workers (Takemura *et al.*, 1989; Irvine, 1990), or by a direct role for one or more inositol phosphates in activating the Ca^{2+} influx pathway (Irvine & Moor, 1987; Kuno & Gardiner, 1987; Morris *et al.*, 1987; Irvine, 1990).

In conclusion, the data presented here provide further evidence that PLCγ_1 is the PLC isoenzyme that is responsible for the stimulation of inositol lipid metabolism by PDGF. The initial observations with NIH-3T3-γ cells suggested that PLCγ_1 overexpression did not modify the $[Ca^{2+}]_i$ changes elicited by PDGF (Margolis *et al.*, 1990), and several other studies have questioned the relationship between growth factor-stimulated inositol lipid metabolism and $[Ca^{2+}]_i$ changes (Berridge *et al.*, 1984; Nanberg & Rozengurt, 1988). However, studies of the kinetics of Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ changes demonstrate that these parameters are related, with the rate of Ins(1,4,5)P_3 formation correlating with the latency and magnitude of the $[Ca^{2+}]_i$ response. The data do not rule out the possibility that PDGF might also affect cellular Ca^{2+} homeostasis through additional mechanisms which are independent of the inositol lipid signalling system. Furthermore, it should be noted that the mitogenic effects of PDGF do not correlate well with its effects on $[Ca^{2+}]_i$ or PLCγ_1, and that additional pathways of tyrosine kinase-dependent signal transduction probably play major roles (Hill *et al.*, 1990; Margolis *et al.*, 1990).

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