neutralizing antibody to TGF-β. However, our initial experiments show that the effectiveness of currently available antibodies is too low to allow meaningful neutralizing experiments in vivo.

Wounding acts as a tumor promotor in many other animal models of chemical and viral carcinogenesis [referenced in (*11*)]. It has been shown that 12-O-tetradecanoyl phorbol-13-acetate (TPA) induces expression of TGF-β in mouse epidermis (*23*) and that TGF-β can mimic the promoting effects of phorbol esters in vitro (*24*) and in the mouse skin model of multistage carcinogenesis in vivo (*25*). Together with our results, TGF-β elaboration may thus offer a more general explanation for the promoting effect of wounding in conjunction with activated oncogenes or initiating chemical carcinogens. The following mechanism may be envisioned: It has been suggested that tumors subvert the host's wound-healing response by inducing a supportive stroma that resembles closely the stroma in healing wounds and that is essential for further growth (*26*). The action of growth factors, in particular TGF-β, have been implicated in the generation of such stroma (*27*). Conversely, it may be argued that an already existing stroma such as that found in a healing wound or induced by TGF-β (*10*) could provide a conducive environment for tumorigenesis.

In apparent contrast to our findings, TGF-β is a potent growth inhibitor for most epithelial cells in culture and its role in carcinogenesis has been discussed in terms of escape from this regulatory mechanism, since many tumor cell lines have been shown to have lost their responsiveness to TGF-β (*28*). The actions of TGF-β are multifunctional and dependent on the tissue context. Therefore, its role in carcinogenesis has to be evaluated cautiously, taking both epithelial and stromal elements of tumor development into consideration.


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# PDGF-Induced Activation of Phospholipase C Is Not Required for Induction of DNA Synthesis


TIMOTHY D. HILL,* NICHOLAS M. DEAN, LAWRENCE J. MORDAN, ALAN F. LAU, MARTHA Y. KANEMITSU, ALTON L. BOYNTON†



**Platelet-derived growth factor (PDGF) induction of DNA synthesis is believed to involve activation of phospholipase C (PLC) and subsequent accumulation of inositol 1,4,5-trisphosphate [I(1,4,5)$P_3$], increase in intracellular $Ca^{2+}$, activation of protein kinase C (PKC), and receptor down regulation. Generation of these events is triggered by the tyrosine protein kinase (TPK) activity of the PDGF receptor. The TPK inhibitor genistein blocked PDGF induction of these events, including DNA synthesis, with the exception of receptor down regulation. PDGF-induced phosphotyrosine phosphorylations, including receptor autophosphorylation, were inhibited by genistein. Removal of genistein and PDGF resulted in DNA synthesis without the occurrence of PLC activation. These findings indicate that these early events, with the exception of receptor down regulation, are not necessary for PDGF-induced DNA synthesis.**


THE PROCESSING OF INFORMATION across the plasma membrane is critical for successful cellular function. Mitogens, such as PDGF, bind to specific cell surface receptors that contain TPK activity. The early signals emanating from the binding of PDGF to its TPK-containing receptor are thought to be required for the initiation of DNA synthesis and cell proliferation and include increased phosphatidylinositol 4,5-bisphosphate ($PIP_2$) hydrolysis (*1–3*) resulting in the accumulation of I(1,4,5)$P_3$ and diacylglycerol (DAG), a transient I(1,4,5)$P_3$-induced increase of intracellular $Ca^{2+}$ (*2, 3*), PKC-dependent phosphorylation of an 80-kD protein (*4, 5*), and receptor down regulation (*6*). With the use of receptor mutants that lack TPK activity, PDGF-receptor TPK activity has been shown to be required for most of these early signals as well as DNA synthesis (*2, 7, 8*). An additional PDGF receptor mutant lacking the TPK insert region, but still containing TPK activity, mediated the PDGF induction of these early signals but not DNA synthesis (*9*). However, from these and other studies (*10*) it cannot be concluded that the early signals resulting from PDGF binding to its receptor are essential for a DNA synthetic response. To answer this question, we have used a pharmacological agent, genistein, which offers the advantage over re-


Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.

*Present address: Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322.
†To whom correspondence should be addressed.

ceptor mutants of being a reversible TPK inhibitor (*11*).

Platelet-derived growth factor (10 ng/ml) stimulates about 75% of confluent, proliferatively quiescent C3H10T1/2 mouse fibroblasts to enter their growth-division cycle. DNA synthesis commences 18 to 20 hours later and peaks at 24 to 28 hours (Table 1) (*12*). PDGF is required in the incubation medium for only the first 4 hours, during which time competency is induced. Thereafter, replacing the medium with one containing only 5% plasma-derived serum (PDS)—without PDGF—is sufficient to promote the progression of fibroblasts into the S phase of the cell cycle. DNA synthesis was inhibited when the TPK inhibitor genistein (100 to 200 μM) was added to confluent, proliferatively quiescent ($G_0$) C3H10T1/2 cells before the addition of PDGF in 5% PDS (Table 1). To determine whether this effect on DNA synthesis was reversible, we first treated C3H10T1/2 cells for 4 hours with genistein and PDGF in 5% PDS-containing medium, which was then replaced with genistein- and PDGF-free 5% PDS-containing medium. We observed that C3H10T1/2 cells progressed through the $G_1$ phase and replicated their chromosomes to the same extent as control cultures treated with PDGF alone in 5% PDS (Table 1). Genistein by itself was not mitogenic (Table 1).

Platelet-derived growth factor induced the typical early cellular responses in C3H10T1/2 cells, including $PIP_2$ hydrolysis (Fig. 1A), an increase in intracellular $Ca^{2+}$ (Fig. 2A), PKC-dependent phosphorylation of the 80-kD protein (Fig. 3) and receptor down regulation (Table 2). Genistein

**Table 1.** PDGF-induced DNA synthesis in C3H10T1/2 mouse fibroblasts. Confluent, proliferative quiescent cells were incubated in Eagle's basal medium (BME) containing 5% PDS (5% PDS–95% BME) with or without 100 μM genistein [ICN; genistein was dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO in medium was 0.1%] for 15 min at 37°C. Cultures were treated with genistein (B, D, and E: in DMSO) or with DMSO (A, 0.1%) only. Some of the cultures were incubated with PDGF [C, D, E: recombinant human c-*sis* BB homodimer (10 ng/ml); Amgen Biologicals] for another 4 hours, at which time the treatment medium was (E) not replaced (cells remained in the presence of genistein and PDGF), or was replaced with fresh 5% PDS–95% BME and washed free of PDGF (C) or genistein and PDGF (D). At this time [$^3$H]thymidine (1 μCi/ml) was added to each culture, and incubations were continued another 24 hours. Cells were then fixed and the percentage of total nuclei labeled with [$^3$H]thymidine was determined as previously described (*16*). Results shown are the means ± SD of values from four separate cultures.

| Treatment | Labeled nuclei (%) |
|---|---|
| A. DMSO/5% PDS (4 hours); 5% PDS (24 hours) | 3.4 ± 2.5 |
| B. Genistein/5% PDS (4 hours); 5% PDS (24 hours) | 3.9 ± 1.9 |
| C. PDGF/5% PDS (4 hours); 5% PDS (24 hours) | 75.1 ± 5.8 |
| D. Genistein/PDGF/5% PDS (4 hours); 5% PDS (24 hours) | 73.1 ± 9.4 |
| E. Genistein/PDGF/5% PDS (28 hours) | 3.2 ± 1.4 |

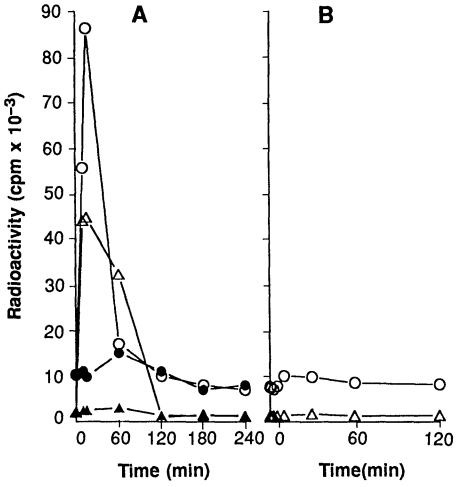


**Fig. 1.** Inhibition of the PDGF-induced increase in [$^3$H]inositol phosphates by genistein (A) and the failure of [$^3$H]inositol phosphates to accumulate after removal of genistein and PDGF (B) in C3H10T1/2 mouse fibroblasts. (**A**) Confluent, proliferatively quiescent C3H10T1/2 cells were labeled to isotopic equilibrium for 72 hours with myo-[$^3$H]inositol (914.6 Ci/mmol, 15 μCi/ml; Du Pont, Biotechnology Systems). The cells were then rinsed three times in BME containing 20 mM Hepes (pH 7.4), placed in 5% PDS–95% BME and subsequently treated with PDGF (10 ng/ml; open symbols) or 200 μM genistein plus PDGF (10 ng/ml; closed symbols) for the indicated times. For the 0-, 10- and 15-min time points, LiCl (10 mM) was added for 15 min before sampling, and for subsequent time points (that is 1, 2, 3, and 4 hours) LiCl was added 1 hour before sampling (that is at time 0 for the 60-min time point and at 180 min for the 240-min time point) in order to inhibit the dephosphorylation of inositol 1-phosphate (circles), or inositol 4-phosphate (triangles). At the appropriate time after addition of PDGF, cells were lysed by the addition of 10% trichloroacetic acid and the inositol phosphates were analyzed by high-performance liquid chromatography (HPLC) as described (*18*). The points are means of duplicate samples. The experiment was repeated twice with similar results. (**B**) Proliferatively quiescent C3H10T1/2 cells were labeled to isotopic equilibrium, rinsed, and placed in 5% PDS–95% BME as described in (A). The cells were then treated for 4 hours with 200 μM genistein plus PDGF (10 ng/ml), after which time they were rapidly rinsed three times with BME containing 20 mM Hepes, (pH 7.4) and placed in 5% PDS–95% BME (time 0 in). LiCl (10 mM) was added to some cultures—those sampled within 30 min—15 min before rinsing the cultures three times with BME containing 20 mM Hepes (pH 7.4) and LiCl (10 mM). Cultures were then placed in genistein- and PDGF-free 5% PDS–95% BME. Cultures sampled at 30, 60, 90, and 120 min after placing them in genistein- and PDGF-free 5% PDS–95% BME were incubated with LiCl (10 mM) for 30 min before sampling. Therefore, any hydrolysis of PI, PIP, or $PIP_2$ would be reflected by an increase in inositol 1-phosphate (circles) and inositol 4-phosphate (triangles). The points are means of duplicate samples. The experiment was repeated twice with similar results.

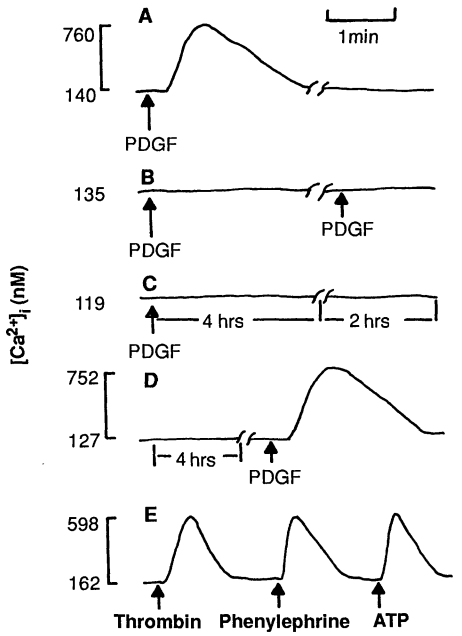


**Fig. 2.** Influence of genistein on PDGF-induced $Ca^{2+}$ release in C3H10T1/2 cells. (**A**) Typical $Ca^{2+}$ response stimulated by the addition of PDGF (10 ng/ml) to fura 2–loaded fibroblasts. After replacement of the buffer with one containing or not containing PDGF (wash, indicated by a break in the trace) no additional changes in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were detected. (B to E) Cells were incubated with 100 μM genistein for 15 min at 37°C before agonist stimulation. (**B**) PDGF (10 ng/ml) failed to induce the release of $Ca^{2+}$ in the presence of genistein. The $[Ca^{2+}]_i$ of cells washed free of genistein and PDGF did not change with a subsequent addition of PDGF. (**C**) Cells were stimulated with PDGF (10 ng/ml) in the presence of genistein for a period of 4 hours. Genistein and PDGF were then removed by perfusion with fresh buffer and the $[Ca^{2+}]_i$ was monitored for an additional 2 hours. (**D**) Unstimulated cells were washed free of genistein after 4 hours and then PDGF (10 ng/ml) was added. (**E**) Representative tracings of $[Ca^{2+}]_i$ responses stimulated by thrombin (50 U/ml), 50 μM phenylephrine, and 100 μM ATP in the presence of 100 μM genistein. Fibroblasts were grown to confluency on round glass cover slips (12 mm) and loaded with 7 μM of the acetoxymethylester of fura 2 (Molecular Probes) in culture medium. Cells were placed in cuvettes containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, and 0.55 mM glucose at 37°C and monitored spectrofluorometrically (*19*). Removal of buffer containing genistein from the cuvette during the wash step was accomplished by rapidly perfusing with fresh buffer.

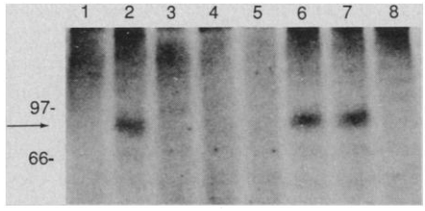


**Fig. 3.** Effect of genistein on the PDGF-induced increase in phosphorylation of an 80-kD cellular protein. Shown is an autoradiogram of $^{32}P$-labeled proteins separated on an SDS–10% polyacrylamide gel. Lane 1, unstimulated cell control; lane 2, cells incubated with PDGF for 20 min; lane 3, cells incubated with 100 μM genistein for 15 min and then PDGF (10 ng/ml) for a further 20 min in the continued presence of genistein; lane 4, cells stimulated with PDGF (10 ng/ml) in the presence of 100 μM genistein over a 20-min period, then washed to remove genistein and PDGF, and incubated for a further 20 min; lane 5, cells were stimulated with PDGF (10 ng/ml) in the presence of 100 μM genistein over a 4-hour period, washed to remove genistein and PDGF, and incubated for a further 20 min; lane 6, cells incubated for 20 min with TPA (100 ng/ml); lane 7, cells incubated with genistein (100 μM) for 20 min and then with TPA (100 ng/ml) for a further 20 min; lane 8, cells incubated with 100 ng/ml of the non–protein kinase C activator and TPA analog 4-a-phorbol 12,13-didecanoate for 20 min. Confluent, proliferatively quiescent fibroblasts grown on 100-mm petri dishes were incubated for 2 hours at 37°C with 0.5 mCi of [$^{32}P$]orthophosphate [$^{32}P_i$] to label the intracellular ATP pool. Excess [$^{32}P_i$] was removed by washing cells three times with Hepes-buffered medium (pH 7.4). Cells received fresh Hepes-buffered medium and were treated as indicated above at 37°C. At the end of each treatment period, 1.0 ml of heated SDS–sample buffer was added to each dish to solubilize cell protein. Samples were boiled for 5 min and centrifuged 20 min at 400,000*g* to remove DNA, and 50 μg of protein from each sample was applied to a SDS–10% polyacrylamide gel and separated as described previously (*20*). Molecular sizes are shown in kilodaltons and the position of the 80-kD protein is indicated (arrow).

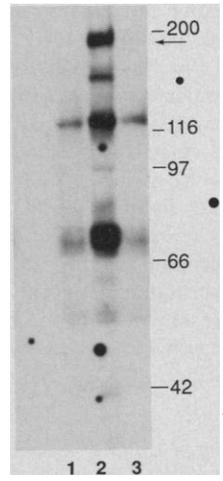


**Fig. 4.** Genistein inhibits PDGF-induced tyrosine phosphorylation of cellular proteins. Confluent, proliferatively quiescent C3H10T1/2 cells were either not treated (lane 1), stimulated with PDGF (10 ng/ml; lane 2), or treated with genistein (100 μM) for 10 min before the addition of PDGF (10 ng/ml; lane 3). Two minutes after PDGF addition, cells were lysed by the addition of hot SDS sample buffer and clarified in a Beckman TL-100 ultracentrifuge at 450,000*g* for 15 min at 4°C. Proteins were separated on SDS–7% polyacrylamide gel as described in the legend to Fig. 3. Proteins were electrotransferred to Immobilon-P membranes as previously described (*20*) and blocked overnight in 10 mM tris (pH 7.4), 0.154 M NaCl, and 5% BSA at 37°C with constant shaking. The blots were then washed three times in a solution containing 10 mM tris (pH 7.4), 0.154 M NaCl, 0.05% Tween 20, and 1 mM EDTA and immunoblotted with P-tyr antibody (2 μg/ml; ICN) for 4 hours at room temperature with constant shaking, after which they were washed six times in the above wash solution and exposed to $^{125}I$-labeled sheep antibody to mouse immunoglobulin G ($1 \times 10^6$ cpm; ICN) for 2 hours at room temperature with constant shaking. Finally, the blots were washed six times in the above wash solution and the P-tyr–containing proteins were visualized by exposure of the immunoblot to XAR-5 film overnight. The autophosphorylated PDGF receptor is visualized at 180 kD (arrow) as well as several other P-tyr–containing proteins with size of 150, 120, 85, 75, 55, and 42 kD. Molecular sizes are shown in kilodaltons.

blocked all of these early cellular responses, (Figs. 1, 2, and 3) with the exception of receptor down regulation, which occurred to the same extent either in the presence or absence of genistein (Table 2). To assess the requirement for increased $PIP_2$ turnover, intracellular $Ca^{2+}$ rise, and PKC-dependent 80-kD protein phosphorylation in the DNA synthetic response, we made use of the reversible effect of genistein on PDGF-induced DNA synthesis. Confluent, proliferatively quiescent C3H10T1/2 cells were first treated with genistein and PDGF in 5% PDS-containing medium for 4 hours, after which the medium was replaced with one containing only 5% PDS—that is, free of genistein and PDGF. This treatment resulted in a full DNA synthetic response 24 to 28 hours later (Table 1). We monitored $PIP_2$ hydrolysis by measuring the accumulation of inositol 1-monophosphate [I(1)P] and inositol 4-monophosphate [I(4)P] (the breakdown products of higher inositol polyphosphates) in the presence of LiCl, which inhibits I(1)P and I(4)P dephosphorylation to inositol. Neither of these inositol monophosphates accumulated during the initial 2 hours after genistein and PDGF removal (Fig. 1B), indicating that phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and $PIP_2$ were not hydrolyzed. It is important to emphasize that PDS did not contain agonists that could induce $PIP_2$ hydrolysis in these washout experiments.

These results suggested that the $I(1,4,5)P_3$-induced $Ca^{2+}$ release would not occur after removal of genistein and PDGF. To test this, we treated the cells with fura 2 (a $Ca^{2+}$ indicator) after they had been treated with genistein and PDGF. Cells were then rapidly perfused in fresh buffer containing no genistein and PDGF, while intracellular $Ca^{2+}$ was simultaneously monitored. No change in the intracellular $Ca^{2+}$ concentration occurred during the subsequent 2-hour observation period (Fig. 2C). A second addition of PDGF to the perfused cells failed to generate a $Ca^{2+}$ response (Fig. 2B), confirming that PDGF receptors were down-regulated (Table 2). Genistein did not interfere with the ability of $I(1,4,5)P_3$ to release $Ca^{2+}$ from intracellular stores because other agonists such as thrombin, phenylephrine, and adenosine triphosphate (ATP) that also induce $Ca^{2+}$ release through $I(1,4,5)P_3$ but whose receptors are not tyrosine kinases, were not affected by genistein (Fig. 2E). These experiments could not be carried out in the presence of PDS because the high background fluorescence in the presence of PDS interfered with the fura 2 signal.

The effect of genistein in inhibiting second messenger generation was reversible. When cells were treated with genistein alone for 4 hours, after which the medium was changed to one free of genistein, subsequent exposure to PDGF caused a rapid hydrolysis of $PIP_2$ (*13*) and an increase in intracellular $Ca^{2+}$ (Fig. 2D).

Phosphorylation of an 80-kD protein induced by PDGF is dependent on PKC activation (*4*). Phosphorylation of this protein can also be induced by 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) in

**Table 2.** PDGF receptor down regulation in C3H10T1/2 fibroblasts. Cells were grown to confluency on round glass cover slips (12 mm) and placed in 5% PDS–95% BME medium immediately before treatment. Cells were treated with either 0.1% DMSO (control) or 100 μM genistein and incubated for 15 min at 37°C. PDGF (60 ng/ml) was then added to cells in the continued presence of DMSO or genistein and the incubation continued for a further 90 min, at which time the unlabeled PDGF was removed from the cells by washing twice for a total of 6 min with 0.5 M sodium acetate buffer, pH 4.5, containing 150 mM NaCl to remove PDGF bound to the cell surface receptors. The cells were then washed three times with phosphate-buffered saline, pH 7.4. Receptor binding was assayed as previously described (*17*) with $^{125}I$-labeled PDGF (0.5 Ci/μmol; 60 ng/ml). Fibroblasts not stimulated with PDGF (and previously treated with 0.1% DMSO) were used to determine maximum receptor binding. Nonspecific binding was determined by incubating cells with a tenfold excess of nonradiolabeled PDGF in the presence of $^{125}I$-PDGF, and this value was substracted from all experimental values. Genistein itself did not affect PDGF binding to its receptor. Results shown are the means ± SD of values from four separate cultures.

| Treatment | $^{125}I$-labeled PDGF bound per $10^6$ cells (fmol) |
|---|---|
| DMSO | 97.9 ± 6.3 |
| PDGF/DMSO | 49.3 ± 8.6 |
| Genistein + PDGF/DMSO | 45.4 ± 9.2 |

C3H10T1/2 cells in the absence of DNA synthesis (Fig. 3). Genistein inhibited the PDGF-induced, but not the TPA-induced phosphorylation of the 80-kD protein, confirming that its phosphorylation was not directly mediated by TPK activity (Fig. 3); rather, it resulted from indirect activation of PKC, probably through $PIP_2$ hydrolysis and DAG accumulation. PKC activity was not affected by genistein (Fig. 3, lanes 6 and 7). As with inositol phosphates and intracellular $Ca^{2+}$, replacement of the genistein- and PDGF-containing medium after 20 min or 4 hours with fresh 5% PDS-containing medium—without genistein and PDGF—did not result in PKC-dependent phosphorylation of the 80-kD protein, even after an additional 20-min incubation in the absence of genistein and PDGF (Fig. 3, lane 5). These results provide additional evidence that PLC was not activated by the down regulated PDGF receptor after genistein and PDGF removal, even though a full DNA synthetic response occurred. These results also support the conclusion of Coughlin *et al.* and others (*10*) that PKC activation alone is not sufficient for stimulation of DNA synthesis.

Genistein did not inhibit PDGF receptor down regulation (Table 2), but it did inhibit receptor autophosphorylation as shown by immunoblots of total cellular proteins with PDGF-induced phosphotyrosine (P-tyr) antibodies (Fig. 4). Several additional proteins were phosphorylated on tyrosine by the PDGF receptor; among them are proteins with sizes of 150 kD, 120 kD, 85 kD, and 75 kD. The identity of these P-tyr–containing proteins are currently unknown, but they have a similar molecular size to phospholipase C, $p21^{ras}$ GTPase-activating protein, phosphatidylinositol 3 (PI-3)–kinase, and $p74^{raf}$, respectively (*14, 15*).

Several candidates have been discussed as mediators of PDGF receptor action involving tyrosine phosphorylation. Several novel inositol phospholipids have been identified [phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate] that are phosphorylated on the 3 position of the inositol ring by a novel PI-3–kinase (*14*). The 85-kD PI-3–kinase has been shown to be associated with and phosphorylated by the PDGF receptor (*14*). In addition, $p21^{ras}$ GTPase-activating protein and $p74^{raf}$ are associated with the activated and autophosphorylated PDGF receptor (*15*). The functional significance of these PDGF receptor complexes remains to be determined, but on the basis of our findings, these associated proteins may be involved in the mechanisms by which PDGF induces DNA synthesis. Thus, with the exception of PDGF receptor down regulation, the early biochemical events of $PIP_2$ hydrolysis—accumulation of inositol phosphates, increase in intracellular $Ca^{2+}$, and PKC-dependent 80-kD protein phosphorylation—are not, as previously believed, necessary for PDGF-induced DNA synthesis. Our results indicate the potential importance of PDGF receptor down regulation in inducing competence and of the actions of the internalized PDGF receptor TPK activity in collaboration with associated proteins in promoting mitogenesis. We cannot eliminate the possibility that genistein is functioning apart from its inhibitory effects on TPK activity or is shunting the effects of PDGF into an alternate pathway that induces DNA synthesis. However, these reservations do not preclude the conclusion that these early biochemical events are not required for PDGF-induced DNA synthesis in fibroblasts.

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# Endothelin Stimulation of Cytosolic Calcium and Gonadotropin Secretion in Anterior Pituitary Cells


STANKO S. STOJILKOVIĆ,* FRANCESCO MERELLI, TOSHIHIKO IIDA, LAZAR Z. KRSMANOVIĆ, KEVIN J. CATT



**The presence of endothelin, a vasoconstrictor peptide, in the hypothalamus and posterior pituitary suggests that it also regulates neural and other nonvascular target cells. In pituitary gonadotrophs, low doses of endothelin evoked oscillations in the intracellular calcium concentration, and high doses induced a biphasic calcium response. Mobilization of intracellular calcium predominated during the spike phase of the calcium response to endothelin, whereas calcium entry through dihydropyridine-sensitive channels contributed to both the spike and plateau phases of the calcium response. Endothelin was as potent as hypothalamic gonadotropin-releasing hormone (GnRH) in stimulation of gonadotropin release in perifused pituitary cells. Endothelin bound specifically to pituitary cells with a dissociation constant of 70 picomolar, and induced rapid formation of inositol trisphosphate and diacylglycerol. Although intracellular calcium concentration and gonadotropin secretory responses to endothelin were independent of the GnRH receptor, endothelin and GnRH appeared to have a common signal transduction mechanism. These observations suggest that endothelin can act as a neuropeptide to regulate anterior pituitary function.**


THE POTENT VASOCONSTRICTOR ACTION of endothelin (*1*) has been attributed to its promotion of calcium ($Ca^{2+}$) influx by acting directly on plasma membrane $Ca^{2+}$ channels (*2*). However, like


Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892.

*To whom correspondence should be addressed.