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# Insulin-like Growth Factor II Increases Cytoplasmic Free Calcium in Competent Balb/c 3T3 Cells Treated with Epidermal Growth Factor

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To determine the role of calcium in the action of insulin-like growth factor II (IGF-II), we have examined the effect of multiplication stimulating activity, the rat IGF-II, on cytoplasmic-free calcium concentration, $[Ca^{2+}]_c$, in aequorin-loaded Balb/c 3T3 cells. IGF-II does not cause any change in $[Ca^{2+}]_c$ in quiescent cells. By contrast, IGF-II induces changes in $[Ca^{2+}]_c$ in platelet-derived growth factor(PDGF) - pretreated competent cells: when competent cells are incubated with epidermal growth factor (EGF) for 10 min, subsequent IGF-II induces an immediate increase in $[Ca^{2+}]_c$. Without EGF treatment, IGF-II does not cause any increase in $[Ca^{2+}]_c$. The priming action of EGF is time dependent, requiring approximately 10 min for the maximum effect. The IGF-II-mediated increase in $[Ca^{2+}]_c$ is totally dependent on extracellular calcium and is blocked by lanthanum. When DNA synthesis in PDGF-treated competent cells is assessed by measuring [$^3$H]thymidine incorporation, IGF-II by itself has only a small effect. Likewise, a brief treatment with EGF results in only a small increase in [$^3$H]thymidine incorporation. By contrast, in competent cells briefly treated with EGF, IGF-II causes a marked stimulation of [$^3$H]thymidine incorporation. These results indicate that IGF-II increases $[Ca^{2+}]_c$ in competent Balb/c 3T3 cells treated with EGF by stimulating calcium influx and that IGF-II-stimulated calcium influx may be related causally to its action on cell proliferation. 

Insulin-like growth factors (IGFs) I and II are potent mitogens in various types of mammalian cells (1). In mouse Balb/c 3T3 fibroblasts, both IGFs I and II are capable of stimulating

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Abbreviations used: IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor, DMEM, Dulbecco's modified Eagle's medium containing 25 mM Hepes/NaOH (pH 7.4) and 2.5 mM $NaHCO_3$; PPP, platelet-poor plasma; CS, calf serum; PBS, phosphate-buffered saline; EDTA, ethylenediamine tetraacetic acid; TCA, trichloroacetic acid; $[Ca^{2+}]_c$, cytoplasmic free calcium concentration.

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DNA synthesis when cells are rendered "competent" by pretreatment with PDGF (2,3). Leof et al. demonstrated that EGF is required for the IGF-induced stimulation of DNA synthesis in competent cells (4). Thus, treatment with PDGF renders quiescent Balb/c 3T3 cells to become competent and competent cells progress into S phase in response to combination of EGF and IGFs. Despite that action of IGFs are matters of current interest, the mechanism of IGF action is not elucidated yet. Rubin et al. demonstrated that tyrosine-specific protein kinase activity locates in the IGF-I receptor and that IGF-I stimulates phosphorylation of its own receptor (5). Such a kinase activity, however, does not exist in IGF-II receptor, although Corvera et al. have shown recently that IGF-II receptor is phosphorylated by a tyrosine kinase in plasma membrane (6). An intracellular messenger of IGF-II action, therefore, is totally unknown at present.

It is well recognized that extracellular calcium is required for cell cycle progression: cells do not enter S phase when extracellular calcium is reduced (7). This prompted us to test a hypothesis that calcium, especially calcium influx, might be a message of IGF-II action. In the present study we examined the effect of multiplication stimulating activity, a rat homolog of human IGF-II, on cytoplasmic free calcium concentration, $[Ca^{2+}]_c$, in Balb/c 3T3 cells. Results indicate that IGF-II increases $[Ca^{2+}]_c$ in competent Balb/c 3T3 cells treated with EGF.

## EXPERIMENTAL PROCEDURES

### Materials

Aequorin was purchased from Dr. J. R. Blinks of Mayo Foundation (Rochester, MN). Partially purified PDGF was obtained from outdated human platelet rich plasma by employing CM-Sephadex chromatography and Blue-Sepharose chromatography as described by Hasegawa-Sasaki (8). Rat IGF-II (multiplication stimulating activity) and EGF were obtained from Collaborative Research (Lexington, MA), insulin from Sigma (St. Louis, MO), and [$^3$H]thymidine were from Amersham Japan (Tokyo, Japan). Synthetic IGF-I was generously provided by Fujisawa Pharmaceutical (Osaka, Japan). Nitrendipine was

donated by Yoshitomi Pharmaceutical (Tokyo, Japan). Cytodex-I microcarrier beads were purchased from Pharmacia (Uppsala, Sweden).

Methods

Cell culture Balb/c 3T3 cells (clone A31) generously provided by Dr. Shin-ichi Tominaga was grown in Dulbecco's modified Eagle's medium containing 25 mM Hepes/NaOH (pH 7.4) and 2.5 mM $NaHCO_3$ (DMEM) supplemented with 10% calf serum (CS) in a humidified atmosphere of 95% $O_2$, 5% $CO_2$ at 37°C. Quiescent cells were obtained as described by Tominaga and Lengyel (9). In short, cells were detached and seeded at a 1:5 split ratio and cultured in DMEM containing 10% CS for 5 days without renewing medium. Density arrested cells were further incubated for 24 hr in DMEM containing 5% PPP to confirm quiescency.

Measurement of cytoplasmic free calcium concentration with aequorin Quiescent cells were detached by incubating in calcium-free PBS containing 0.05% trypsin, 0.02% EDTA, and 0.2% glucose. After neutralization of trypsin, cells were centrifuged at 100g for 3 min at 4°C. The pellet was resuspended in ice-cold modified Hanks' solution containing (mM) NaCl, 137; KCl, 3.5; $KH_2PO_4$, 0.44; $NaHCO_3$, 4.2; $Na_2HPO_4$, 0.33; $CaCl_2$, 0.5: Hepes/NaOH (pH 7.4), 20. Aequorin was loaded by the method of Morgan and Morgan (10) as described previously (11). Aequorin-loaded cells were then seeded onto Cytodex-1 microcarrier beads. The number of beads was 1% of the cell number. Cells attached on beads became confluent on beads within 6 hr and were further incubated for 24 hr in DMEM containing 0.5% PPP, 3 μg/ml hypoxanthine, 10 μg/ml thymidine, Dulbecco's nonessential amino acids and 100 U/ml penicillin-G. These cells were quiescent since DNA synthesis, as assessed by [$^3$H]thymidine incorporation, was stimulated by a combination of PDGF and 5% PPP but not by 5% PPP alone (data not shown). For PDGF treatment, cells attached on beads were incubated for 3 hr in DMEM and then were incubated in DMEM containing 1.0 mM calcium. These PDGF-pretreated cells were competent since DNA synthesis was stimulated by addition of 5% PPP (data not shown). Cells attached on beads were transferred into a cuvette and incubated at 37°C under constant stirring. Aequorin bioluminescence was measured by Chrono-log PICA (Havertown, PA) as described previously (11). Aequorin bioluminescence was not calibrated in terms of free calcium concentration since spatial distribution of calcium in the cell is unknown at present and since it is possible that aequorin detects a localized increase in free calcium concentration in the cell (12). Magnitude of aequorin bioluminescence was expressed as electric current (11). Traces presented are representative of at least five experiments with similar results. Concentration of calcium in calcium-free DMEM was less than 5 μM by measurement with atomic absorption. When lanthanum was included in medium, phosphate was removed to prevent precipitation.

Measurement of DNA synthesis. DNA synthesis was assessed by measuring [$^3$H]thymidine incorporation into TCA-precipitable materials. Quiescent Balb/c 3T3 cells were obtained in a 24-well plate as described above. After an addition of the stimulant, cells were incubated for 24 hr in DMEM containing 1 μCi/ml [$^3$H]thymidine. [$^3$H]Thymidine incorporation was measured by the method of NcNiel et al. (13).

## RESULTS AND DISCUSSION

To evaluate a role of calcium as an intracellular messenger of IGF-II action on cell proliferation, we have determined whether

IGF-II induces any change in $[Ca^{2+}]_c$ using aequorin-loaded Balb/c 3T3 cells attached on microcarrier beads. IGF-II, at doses up to 100 nM, does not induce any change in $[Ca^{2+}]_c$ in quiescent Balb/c 3T3 cells (data not shown). In addition, when IGF-II is added 10 min after the addition of 5 to 50 nM EGF, neither agent affects $[Ca^{2+}]_c$ (data not shown). Since IGF-II promotes DNA synthesis in PDGF-pretreated competent Balb/c 3T3 cells but not in quiescent cells (3,4), we have studied the effect of IGF-II on $[Ca^{2+}]_c$ in PDGF-pretreated competent cells. In competent Balb/c 3T3 cells, 1 nM IGF-II does not induce any change in $[Ca^{2+}]_c$ by itself (Fig. 1A). However, when PDGF-treated cells are first treated with 10 nM EGF for 10 min, which does not affect $[Ca^{2+}]_c$, and are subsequently stimulated by 1 nM IGF-II 10 min later, addition of IGF-II results in a large increase in $[Ca^{2+}]_c$ (Fig. 1B). The effect of IGF-II is observed without any detectable lag time and $[Ca^{2+}]_c$ returns to a value which is indistinguishable from the basal level within 20 sec. Conversely, when PDGF-pretreated cells are first incubated with 1 nM IGF-II and 10 nM EGF is added subsequently, EGF does not induce any change in $[Ca^{2+}]_c$ (Fig. 1C). Thus, EGF treatment induces an action of IGF-II on $[Ca^{2+}]_c$ in competent cells. It should be noted that a few minutes of treatment is required for EGF to induce subsequent IGF-II action. As shown in Fig. 1D, when PDGF-treated cells are first stimulated by EGF and IGF-II is added after a 5-min interval, IGF-II causes only a small increase in $[Ca^{2+}]_c$. In addition, when cells contact with EGF for 10 min, subsequent IGF-II action is observed even if EGF is removed (Fig. 1E). There are at least two types of receptors on which IGF-II acts: receptors for IGF-I and receptors for IGF-II. To determine a possible type of receptor on which IGF-II acts, we have done a series of experiments. If IGF-II exerts its effect by acting on IGF-II receptor, a reasonably

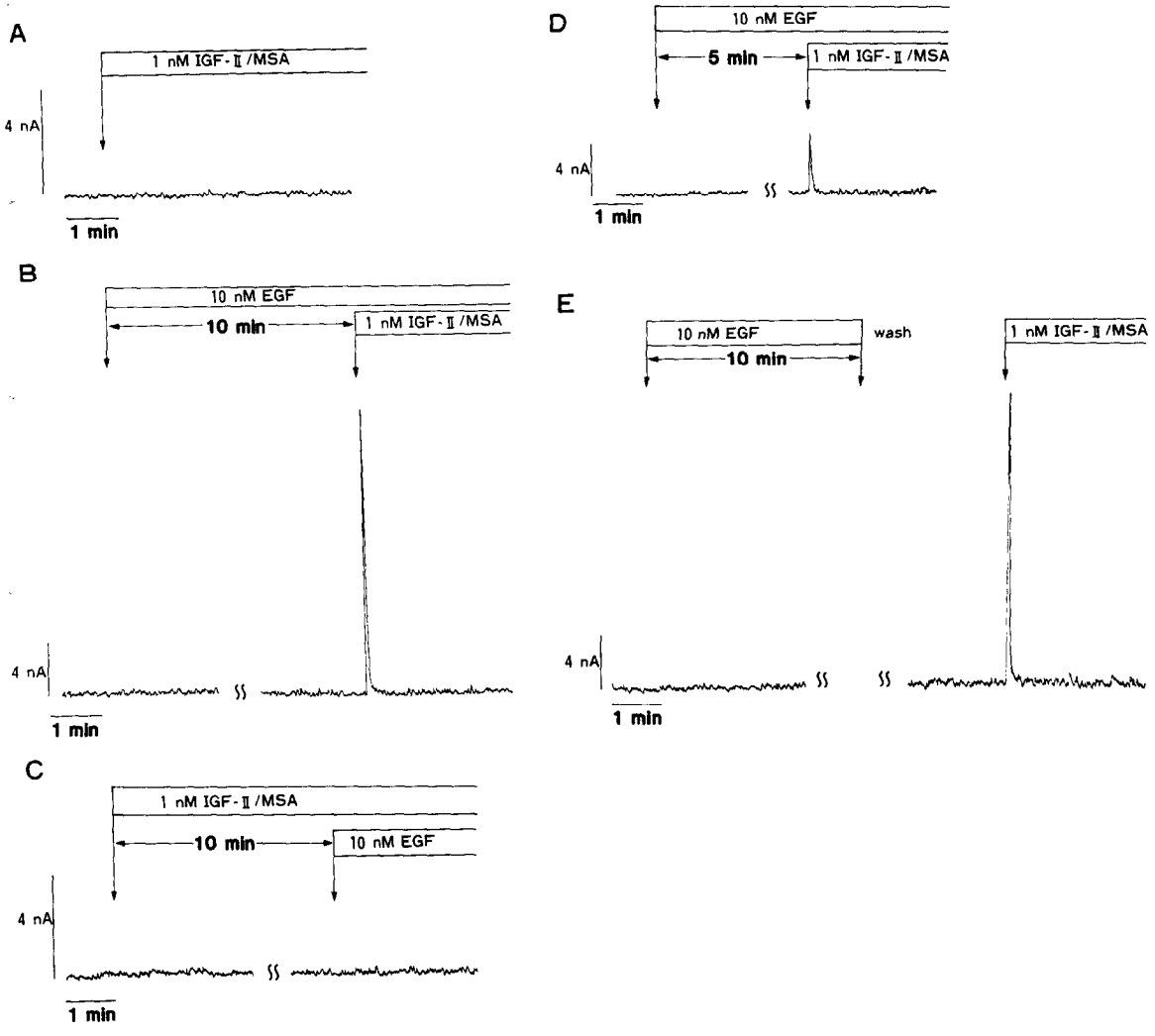


Fig. 1. Effect of EGF and IGF-II on $[Ca^{2+}]_c$ in PDGF-pretreated Balb/c 3T3 cells. Aequorin-loaded cells attached on microcarrier beads were incubated with 60 U/ml PDGF for 3 hr. Cells then were washed and incubated in DMEM containing 1.0 mM calcium. Each stimulator was added as indicated. Reactions proceed from left to right.

low dose of IGF-II should induce changes in $[Ca^{2+}]_c$. Indeed, at a concentration as low as 100 pM, IGF-II increases $[Ca^{2+}]_c$ transiently (Fig. 2A) and 500 pM IGF-II induces a remarkable increase in $[Ca^{2+}]_c$ (Fig. 2B). To test an involvement of the IGF-I receptor, we have employed synthetic IGF-I as an agonist for the IGF-I receptor. When IGF-I is added to PDGF-pretreated cells primed with EGF, IGF-I at concentration up to 10 nM does

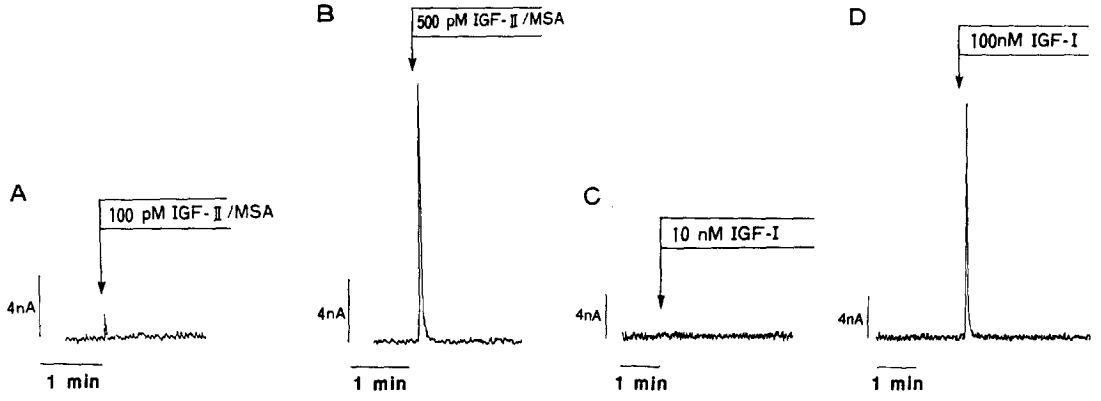


Fig. 2. Effect of IGF-II, IGF-I, and insulin on $[Ca^{2+}]_c$ in PDGF-pretreated Balb/c 3T3 cells primed with EGF. Aequorin-loaded cells pretreated with 60 U/ml PDGF for 3 hr were washed twice and then incubated in DMEM containing 10 nM EGF for 10 min. Cells were stimulated by 100 nM IGF-II (A), 500 pM IGF-II (B), 10 nM IGF-I (C), or 100 nM IGF-I (D) as indicated by the arrow.

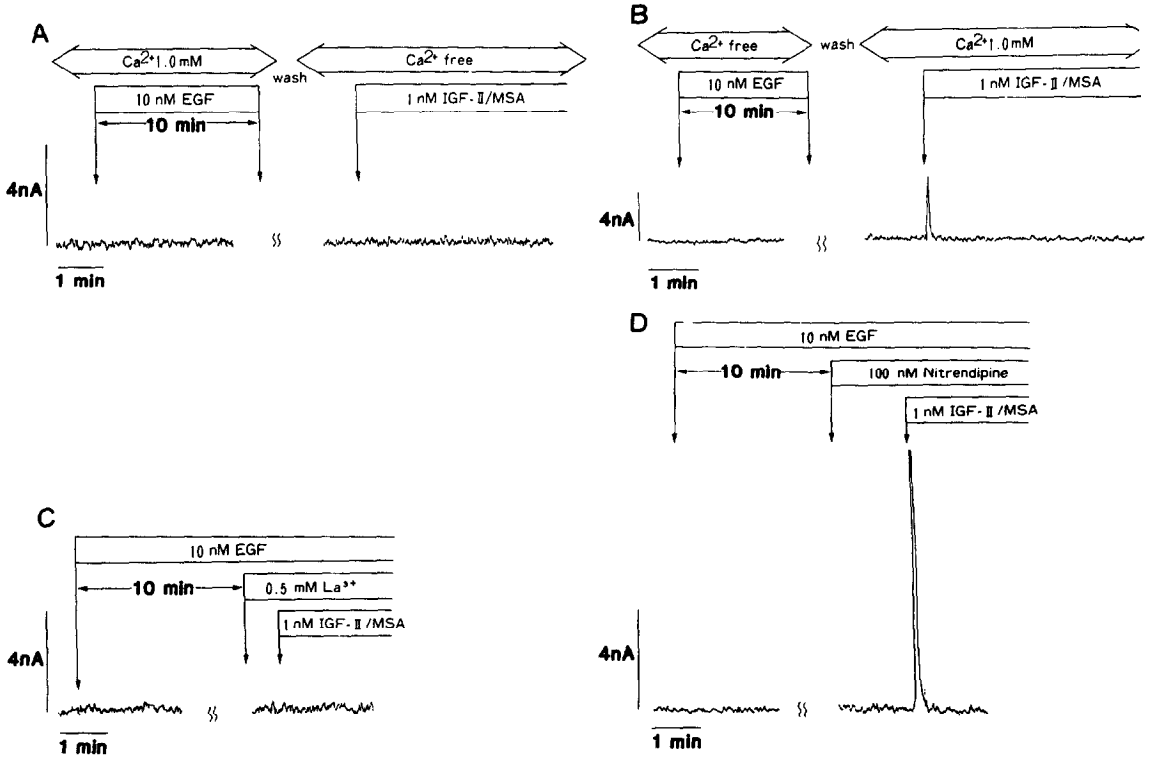


Fig. 3. Dependency of actions of IGF-II and EGF on extracellular calcium. Aequorin-loaded cells pretreated with 60 U/ml PDGF for 3 hr were washed twice with DMEM. (A): PDGF-pretreated cells were incubated in DMEM containing 1.0 mM calcium and were stimulated by 10 nM EGF. Ten minutes after EGF addition, medium was changed to calcium-free DMEM. Cells were stimulated by 1 nM IGF-II 1 min thereafter. (B): PDGF-pretreated cells were stimulated by 10 nM EGF in calcium-free DMEM. Ten minutes later,

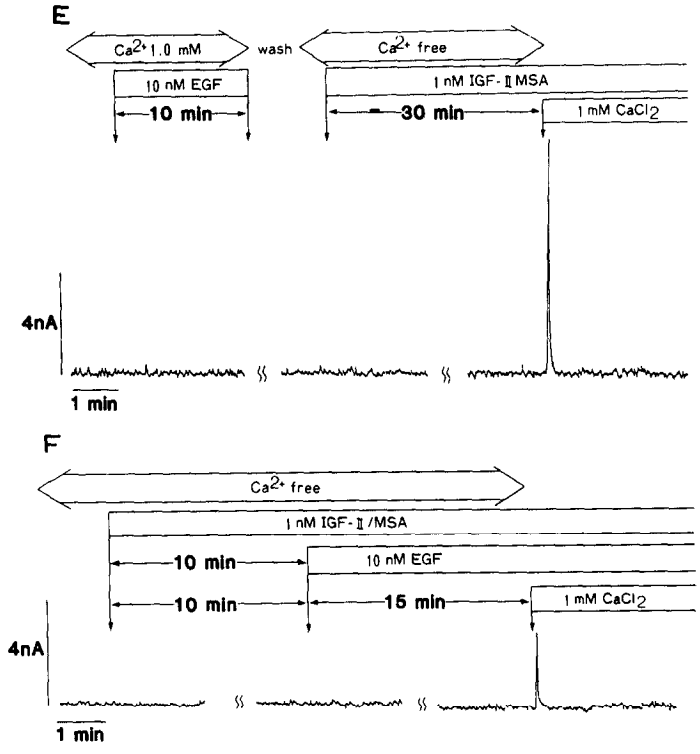


Fig. 3. cont.
the medium was changed to DMEM containing 1 mM calcium. IGF-II (1 nM) was added 1 min thereafter. (C): PDGF-treated cells were incubated in DMEM containing 1 mM calcium and were stimulated by 10 nM EGF. Ten minutes after EGF addition, cells were washed and incubated in phosphate-free DMEM containing 1 mM calcium and 0.5 mM lanthanum. IGF-II (1 nM) was added 1 min thereafter. (D): Cells were incubated in DMEM containing 1 mM calcium and were stimulated by 10 nM EGF. Nitrendipine (100 nM) was added 10 minutes after addition of EGF. (E): PDGF-pretreated cells were stimulated by 10 nM EGF. Ten minutes later, cells were washed and incubated in calcium free DMEM. One minute after changing the medium, 1 nM IGF-II was added. Thirty minutes later, extracellular calcium concentration was raised to 1 mM by adding calcium chloride. (F): PDGF-pretreated cells were incubated in calcium free DMEM containing 1 nM IGF-II and 10 nM EGF was added 10 minutes after IGF-II addition. Fifteen minutes later, extracellular calcium concentration was raised to 1 mM by adding calcium chloride.

not cause any increase in $[Ca^{2+}]_c$ (Fig. 2C). However, when 100 nM IGF-II is added, $[Ca^{2+}]_c$ increases rapidly (Fig. 2D). These results together with the fact that 100 nM insulin has no effect on $[Ca^{2+}]_c$ (data not shown) suggest, though not conclusively, that the IGF-II receptor is a possible site of action of IGF-II in Balb/c 3T3 cells.

The transient rise in $[Ca^{2+}]_c$ induced by IGF-II in PDGF-treated cells depends totally on extracellular calcium: when PDGF-pretreated cells are incubated with 10 nM EGF in the presence of calcium for 10 min and the medium is changed to calcium-free DMEM, 1 nM IGF-II, added 1 min thereafter, does not cause any increase in $[Ca^{2+}]_c$ (Fig. 3A). EGF is capable of potentiating subsequent IGF-II action even if EGF is added in the absence of extracellular calcium. When PDGF-pretreated cells incubated in calcium-free medium, are stimulated by EGF and extracellular calcium then is restored by changing the medium, subsequent IGF-II action is detected (Fig. 3B). Thus, extracellular calcium is required for the action of IGF-II on $[Ca^{2+}]_c$ but not for the EGF-mediated potentiation process. To our knowledge, there has been no report showing that binding of IGF-II is blocked by removal of extracellular calcium. Hence, the dependency of IGF-II action on extracellular calcium suggests that IGF-II may increase transmembrane calcium influx. This notion is supported by an observation shown in Fig. 3C. When PDGF-treated cells primed with EGF are treated with IGF-II in the presence of lanthanum, the effect of IGF-II on $[Ca^{2+}]_c$ is abolished. In contrast, 100 nM nitrendipine, a voltage-dependent calcium channel blocker, does not affect IGF-II-induced increase in $[Ca^{2+}]_c$ (Fig. 3D). Although IGF-II-mediated changes in $[Ca^{2+}]_c$ are only transient, IGF-II-mediated calcium influx appears to be continuous. When PDGF-treated cells are primed with EGF in the presence of calcium and the medium is then changed to calcium-free DMEM, addition of IGF-II does not induce any increase in $[Ca^{2+}]_c$ (Fig. 3E). However, when extracellular calcium is restored by adding calcium chloride 30 min after IGF-II addition, $[Ca^{2+}]_c$ increases rapidly (Fig. 3E). Note that restoration of extracellular calcium causes a negligible change in aequorin luminescence in the absence of IGF-II (data not shown). Assuming

that the binding of IGF-II is not affected by extracellular calcium, these results indicated that IGF-II induces sustained stimulation of calcium influx. Reasons for the transient nature of the elevation of $[Ca^{2+}]_c$ in the presence of continuous calcium influx may be the following. First, since aequorin detects a small increase in $[Ca^{2+}]_c$ localized in a small area, subplasma membrane domain, diffusion into cytosol, and/or extrusion of calcium outside the cells (11) lead to a rapid reduction of aequorin luminescence. Second, if calcium influx is pulsatile rather than continuous as described in the study employing single cell recording in aequorin-loaded hepatocyte (14), an absence of synchronization makes it difficult to detect aequorin luminescence. The latter may explain why no change in $[Ca^{2+}]_c$ is observed when PDGF-pretreated cells first are treated with IGF-II and then EGF is added subsequently (Fig. 1C). Presumably, individual cells become responsive to IGF-II after slightly different intervals so that responses are not synchronized. The results shown in Fig 3F verify this notion. When PDGF-pretreated cells are stimulated by the successive addition of IGF-II and EGF in the absence of extracellular calcium and calcium is added 10 min later, $[Ca^{2+}]_c$ increases immediately, although the magnitude of the response is smaller. Thus, when calcium influx is synchronized, an increase in aequorin luminescence is observed.

There are three major observations in the present study. First, one of the progression factors, IGF-II, increases cytoplasmic-free calcium concentration. Second, the action of IGF-II on $[Ca^{2+}]_c$ is cell cycle specific. While EGF and IGF-II, either alone or in combination, do not affect $[Ca^{2+}]_c$ in quiescent cells, the combination of these two agents generates a calcium signal in PDGF-treated competent cells. Stiles, Pledger, and their colleagues postulated that PDGF treatment renders Go-arrested

Balb/c 3T3 cells to become competent, as characterized by an acquired sensibility to growth-promoting progression factors such as PPP or a combination of EGF and IGFs (3). Since PDGF-treated cells but not Go-arrested cells enter the S phase in response to a progression factor (3), and since EGF is required in the initial period of the progression phase while IGFs are needed throughout the progression phase (2), the $[Ca^{2+}]_c$ response to EGF and IGF-II may be a critical feature of competent cells. Third, EGF primes subsequent IGF-II action on $[Ca^{2+}]_c$. Although PDGF treatment is shown to reduce bindings of EGF to its receptor (15), priming with EGF is indispensable for IGF-II action. Several mechanisms are possible for the EGF action. First, EGF modifies binding of IGF-II so that IGF-II effectively generates its signal in the presence of EGF. This seems unlikely since higher doses of IGF-II do not increase $[Ca^{2+}]_c$ in EGF-unprimed cells (data not shown). Second, EGF amplifies IGF-II-induced calcium influx by acting directly on the calcium gating apparatus. An alternate possibility is that EGF modifies the transducing mechanism between the IGF-II receptor and the calcium gating apparatus. A candidate for such a transducing mechanism is a GTP-binding protein in the plasma membrane. In this regard, Murayama and Ui have shown that a pertussis toxin-sensitive GTP-binding protein may participate in the transducing binding of an extracellular signal to the calcium gating apparatus in Swiss 3T3 fibroblasts (16). Additionally, it is noteworthy that a certain class of GTP-binding proteins is shown to be phosphorylated by EGF with a resultant increase in its own GTP-binding activity (17). Whatever the nature of the action of EGF is, this is the first recorded calcium signal generated by a member of somatomedins, IGF-II.

At present, the significance in cell proliferation of IGF-II induced calcium influx is uncertain. When PDGF-pretreated

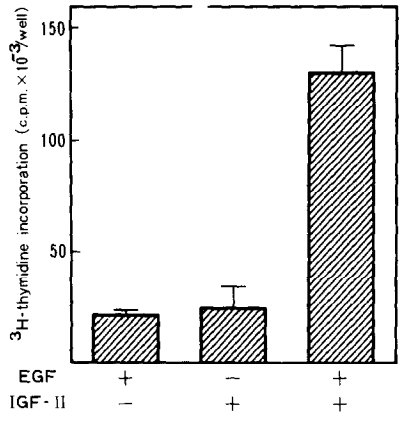


Fig. 4. Effect of EGF and/or IGF-II on [$^3$H]thymidine incorporation in PDGF-pretreated competent Balb/c 3T3 cells. Quiescent cells were treated with 60 U/ml PDGF for 3 hr. Cells were washed one time with DMEM containing 28 mM $\beta$-mercaptoethanol, washed two more times with DMEM and incubated for 10 minutes in DMEM in the presence and absence of 10 nM EGF. Cells were washed three times with DMEM and then incubated for 24 hr in DMEM containing 1 $\mu$Ci/ml [$^3$H]thymidine in the presence and absence of 1 nM IGF-II. Values are the means ± SD for four determinations. Results are representative of three experiments with similar results.

Balb/c 3T3 cells are stimulated by 1 nM IGF-II alone, [$^3$H]thymidine incorporation increases only slightly (Fig. 4). Likewise, a brief treatment with 10 nM EGF alone has a minimal effect on [$^3$H]thymidine incorporation in PDGF-treated cells. However, when PDGF-treated competent cells are stimulated by IGF-II after a brief treatment with EGF, [$^3$H]thymidine incorporation increases markedly. These observations are consistent with the notion that EGF and IGF-II act synergistically to stimulate DNA synthesis in PDGF-treated Balb/c 3T3 cells (2, 3). The fact that both calcium influx and DNA synthesis induced by IGF-II are greatly enhanced by priming with EGF in competent cells indicates that IGF-II-induced calcium influx may be causally related to the promotion of cell proliferation.

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