Transforming Growth Factor β and Epidermal Growth Factor Alter Calcium Influx and Phosphatidylinositol Turnover in Rat-1 Fibroblasts*

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Transforming growth factor type β (TGFβ) alters the cellular response to epidermal growth factor (EGF) for a variety of processes ranging from early transport activities and gene transcription to mitogenesis. In order to test the hypothesis that altered signal transduction mechanisms may mediate both the transforming effects of TGFβ and the modulation of EGF-stimulated processes by TGFβ, we have examined second messenger levels in response to growth factor treatment. The addition of EGF or prolonged treatment with TGFβ increased the rate of ⁴⁶Ca influx in serum-deprived, confluent Rat-1 cells, while the addition of EGF to TGFβ-pretreated cells produced an additive increase in Ca²⁺ influx. The stimulation of Ca²⁺ influx by TGFβ was only observed at incubation times greater than 1 h and was inhibited by inclusion of actinomycin D, suggesting that a newly synthesized gene product was required for the observed response to TGFβ. Both EGF and TGFβ displayed similar time and concentration dependencies for stimulation of Ca²⁺ influx and for accumulation of inositol trisphosphate (IP₃). The increase in IP₃ accumulation in response to either EGF or TGFβ required the presence of extracellular Ca²⁺, and the observed concentration dependencies were similar for the stimulation of phosphatidylinositol turnover and Ca²⁺ influx. The EGF- and TGFβ-stimulated increases in Ca²⁺ influx could be blocked by cobalt, cadmium, and [ethylenebis(oxyethylenenitrilo)] tetracetic acid, but not by specific Ca²⁺ channel blockers such as nifedipine or verapamil, suggesting that these growth factors do not act via L-type voltage-sensitive calcium channels. Those calcium blockers which inhibited Ca²⁺ influx also inhibited inositol phosphate release. These data, taken together, indicate that Ca²⁺ influx and inositol phosphate release are coupled in Rat-1 cells and suggest that influx of Ca²⁺ from the extracellular medium is responsible for the changes in IP₃ accumulation observed in response to both EGF and TGFβ.

Epidermal growth factor (EGF) provides a potent stimulus

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The abbreviations used are: EGF, epidermal growth factor; TGFβ, transforming growth factor β; IP₃, inositol trisphosphate; IPA, inositol tetrakisphosphate; PI, phosphatidylinositol; EGTA, [ethylenebis(oxyethylenenitrilo)] tetracetic acid; HPLC, high performance liquid chromatography; DMEM, Dulbecco’s Modified Eagle’s medium; EMEM, Eagle’s minimum essential medium.
in the presence of Cells were preincubated with the indicated concentration of LiCl for when they exceed the size of the symbols. Others have postulated that a Ca"-buffering artifact could explain the inability of EGF to increase intracellular Ca" levels in Ca"-free medium (19).

In the present paper we have examined the relationship between Ca" influx and the changes in PI metabolism observed following treatment with either EGF or TGFβ, in order to determine whether changes in Ca" influx in response to these growth factors are causally related to changes in PI metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—TGFβ was prepared from human blood platelets by the method of Assoian et al. (27) and was then purified to greater than 98% homogeneity by two rounds of reverse-phase HPLC in acetone/toluene, 0.1% trifluoroacetic acid, using a Du Pont Zorbax Bio Series Protein Plus analytical column. TGFβ eluted at 34% acetonitrile. EGF was prepared from mouse submaxillary glands by the method of Savage and Cohen (28) and was further purified to homogeneity as described by us (29).

**Inositol Phosphate Assay**—Rat-1 cells grown to confluency on 10-cm plates were serum deprived for 36 h in Dulbecco's modified Eagle's medium (DMEM) containing 2 μM/ml [3H]myo-inositol (American Radiolabeled Chemicals). Following removal of the labeling medium the cells were incubated for an additional 12 h in serum-free DMEM. For the experiments utilizing Cd", the cells were rinsed and incubated in phosphate-free EMEM for 4 h prior to the assay, in order to prevent the formation of cadmium phosphate precipitates. The assay was initiated by aspirating the DMEM and adding EMEM containing 10 μg/ml "Ca (Du Pont-New England Nuclear), in the presence or absence of growth factor. Unless otherwise indicated, the assay medium contained 1 mM unlabeled CaCl₂. The assay was terminated at the indicated times by aspirating the labeled medium and rinsing the cells with ice-cold phosphate-buffered saline containing 25 mM MgCl₂ to displace "Ca bound extracellularly. The cells were lysed in 0.5 M HCl and the "Ca content of the lysate was determined by liquid scintillation spectrometry. Specific "Ca influx was determined at each point by subtracting the nonspecific "Ca uptake observed in the presence of a 20-fold excess of CaCl₂. In contrast to the inositol phosphate release experiments described (8), "Ca influx experiments were performed in the absence of LiCl. Although addition of LiCl reduced both nonsimulated and growth factor-stimulated Ca" influx, TGFβ and EGF had comparable qualitative effects in the presence or absence of Li" (Fig. 1). "Ca" influx experiments were therefore performed in the absence of LiCl in order to observe the maximum level of stimulation.

**Bioassay of TGFβ Activity on Rat-1 Cells**—The biological responsiveness of Rat-1 cells to TGFβ was determined by measuring the acidification of the culture medium in cells exposed to TGFβ. Rat-1 cells plated on 35-mm dishes were grown in DMEM plus 10% calf serum, in the presence or absence of TGFβ. TGFβ concentrations ranged from 0.001 ng/ml to 50 ng/ml, and equivalent volumes of the acetonitrile/trifluoroacetic acid vehicle were added to all plates. After 3 days the medium was transferred to 12 × 75-mm glass tubes, and the pH of duplicate samples was determined.

**Measurement of Increases in Cell Number in Response to TGFβ**—Rat-1 cells were seeded in 35-mm dishes at an initial density of 10³ cells/plate, as determined by measurement of cell number using automated cell counter (Coulter Electronics, Hialeah, Fl). Triplicate dishes were harvested by trypsinization, and cell numbers were determined at intervals.

**RESULTS**

**Growth Effects of TGFβ on Rat-1 Cells**—Prolonged treatment of serum-deprived Rat-1 cells with TGFβ resulted in phenotypic changes typical of transformation, including acquisition of a transformed morphology, rapid aciddification of the culture medium, and stimulation of growth in confluent monolayers. The effect of TGFβ on growth of Rat-1 cell monolayers is shown in Fig. 2. When TGFβ was added to exponentially growing Rat-1 cells, there was an initial delay in the rate of proliferation. However, unlike the vehicle-treated Rat-1 cells which stopped proliferating and maintained a stable population density for at least 5 days, the TGFβ-treated cells continued to proliferate until they eventually detached from the culture dishes.

It was observed that, in addition to producing higher cell densities, TGFβ induced the acidification of the culture medium more rapidly than in control plates. Medium acidification has been reported for a large number of transformed cells and is generally considered to be a marker for the transformed phenotype (32). The ability of TGFβ to induce acidification of the medium over a wide range of concentrations is shown in Fig. 3. Thus, in contrast to the cytostatic effects of TGFβ
Effects of TGFB on Ca2+ Influx—Our previous work has demonstrated that the presence of extracellular Ca2+ is required for EGF-induced changes in intracellular Ca2+ in Rat-1 cells previously exposed to TGFB (8). Ca2+ influx was measured in Rat-1 cells following exposure to EGF and TGFB in order to correlate changes in Ca2+ fluxes with changes in inositol phosphate levels. Treatment of serum deprived, confluent Rat-1 cells with 10 ng/ml TGFB for 4 h increased the rate of 45Ca influx by 2-fold, from 2.57 ± 0.17 to 6.04 ± 0.14 pmol/min/10^6 cells (Fig. 4). Addition of 100 ng/ml EGF also produced an immediate increase in the rate of Ca2+ influx by more than 2-fold (to 8.12 ± 0.32 pmol/min/10^6 cells), while addition of EGF to TGFB-pretreated cells resulted in an additive increase in the rate of 45Ca influx (Fig. 4). Ca2+ influx in response to either growth factor alone, or to the combined factors, was approximately linear over the first 5 min of exposure to 45Ca. In the 45Ca uptake experiments, a concentration of 100 ng/ml EGF was used in order to optimize the initial rate measurements by increasing the rate of association of EGF with the EGF receptor.

The stimulation of Ca2+ influx by TGFB shown in Fig. 4 was measured following a preincubation in TGFB for 4 h, an exposure time previously determined to be maximal for TGFB stimulation of inositol phosphate release (8). We determined the temporal characteristics of the increase in Ca2+ influx seen in response to TGFB, and the effects of actinomycin D on that response (Fig. 5A). Serum-deprived confluent Rat-1 cells were incubated with 10 ng/ml TGFB, or the vehicle control, for the indicated times prior to determination of the rate of 45Ca influx. Incubation of cells with TGFB for periods under 1 h had no effect on Ca2+ influx. A significant increase in the rate of Ca2+ influx was observed by 2 h, and the rate of influx was maximal by 4 h. After 4 h the influx rate remained at a constant level, which was approximately 2-fold higher than control values (Fig. 5A). Coincubation with actinomycin D (10 μg/ml) blocked the TGFB-stimulated increase in 45Ca influx, indicating that an actively transcribed gene product was required for the stimulation of TGFB. Addition of actinomycin D to cells pretreated with TGFB for 4 h resulted in a rapid decline in the rate of Ca2+ influx, with a half-life of approximately 2 h (Fig. 5A). Incubation with actinomycin D, but not the ethanol vehicle, also produced a reduction of 45Ca influx in control cells. In order to demonstrate that the observed effects of actinomycin D were through blockade of a TGFB-induced gene product, rather than a nonspecific effect on the 45Ca influx mechanism, we examined the effects of actinomycin D on EGF-stimulated Ca2+ influx (Fig. 5B). While incubation with actinomycin D for 4 h completely blocked the TGFB-stimulated increase in Ca2+ influx, it had no effect on Ca2+ influx stimulated by EGF.

Correlation of Ca2+ Influx with Production of Inositol Phosphates—We next determined the dose-response characteristics for the TGFB-induced increase in Ca2+ influx and inositol phosphate accumulation (Fig. 6). The concentration of TGFB required to produce half-maximal stimulation of 45Ca influx was 1.5 ng/ml, while the half-maximal dose for inositol phosphate release was 2.0 ng/ml. Both dose-response measure-
uptake and IP3 release by EGF were not equivalent. The half-maximal dose of EGF, and the dose response was nearly linear up to the maximal dose tested, 4 ng/ml, and near maximal dose for stimulation of 45Ca influx was 100 ng/ml. The half-maximal dose of 6), the concentration dependencies for stimulation of Ca2+ accumulation of inositol phosphates by EGF (Fig. 7). In serum deprived cells, maximal inositol phosphate levels in the absence of EGF were IP3 = 82 ± 5 cpm, IP4 = 33 ± 5, n = 3. O, rate of Ca2+ influx, measured at 5 min. Basal Ca2+ influx = 16.1 ± 2.0 pmol/min/10^6 cells. Error bars have been included only when they exceed the size of the symbols.

Fig. 7. Concentration dependence for stimulation of Ca2+ influx and PI turnover by EGF. Serum-deprived Rat-1 cells were stimulated with the indicated concentrations of ultrapure EGF for a 5-min assay period. Accumulation of [3H]IP3 (A) and [3H]IP4 (B) was determined following a 20-min incubation with 100 mM LiCl. Basal inositol phosphate levels in cells treated with vehicle were: IP3 = 106 ± 11, IP4 = 19 ± 4, n = 3. O, rate of Ca2+ influx, measured at 5 min. Basal Ca2+ influx = 16.1 ± 2.0 pmol/min/10^6 cells. Error bars have been included only when they exceed the size of the symbols.

stimulation (approximately 300% control) was observed at 10 ng/ml EGF. Accumulation of IP4, was also stimulated approximately 2-fold by addition of EGF.

Whether considered in terms of Ca2+ influx or PI metabolism, the dose-response for TGFβ stimulation was unaffected by cotreatment with EGF. Similarly, addition of TGFβ had no effect on the dose-response characteristics for the stimulation of Ca2+ influx and PI metabolism by EGF (data not shown).

Dependence of Second Messenger Alterations on Extracellular [Ca2+]—We next examined the effects of varying extracellular [Ca2+] on the EGF- and TGFβ-stimulated responses, in order to determine if the entry of Ca2+ across the plasma membrane was essential for an increase in inositol phosphate release (Fig. 8). The rate of Ca2+ influx in response to either EGF or TGFβ was found to be highly dependent on the concentration of extracellular calcium. Maximal stimulation of the rate of Ca2+ influx in response to either EGF or TGFβ was obtained at physiological extracellular Ca2+ concentrations (1–2 mM). Only minimal Ca2+ influxes were observed at 0.1 mM extracellular Ca2+ (Fig. 8).

A similar dependence on extracellular Ca2+ was found for
Ca\textsuperscript{2+} Influx and Phosphatidylinositol Turnover

Ca\textsuperscript{2+} influx and phosphatidylinositol turnover were explored in the context of growth factor stimulation in Rat-1 cells. The production of IP_{3} in response to EGF and TGF\beta was determined as described under "Experimental Procedures." Each point represents the mean ± S.D., n = 3. Error bars have been included only when they exceed the size of the symbols.

In a parallel study, the effect of extracellular Ca\textsuperscript{2+} on Ca\textsuperscript{2+} influx was examined. Rat-1 cells grown to confluence in 24-well plates were serum-deprived overnight in DMEM. 4 h prior to the assay, the cells were rinsed and incubated in Ca\textsuperscript{2+}-free EMEM with CaCl\textsubscript{2} added to give final concentrations of Ca\textsuperscript{2+} from 0.1 to 3 mM. Cells assayed at 10 mM Ca\textsuperscript{2+} were preincubated with 3 mM Ca\textsuperscript{2+} to avoid precipitation of calcium phosphates during prolonged incubation with high Ca\textsuperscript{2+} concentrations. 10 ng/ml TGF\beta (\(\Delta, \Delta\)) or the TGF\beta vehicle (O, O) was added to the appropriate plates at this time. The assay was initiated by removing the incubation medium and adding EMEM containing 10 \(\mu\)Ci/ml \(^{45}\)Ca and the indicated concentration of CaCl\textsubscript{2}. The cells were treated as follows: O, no addition; \(\bullet\), EGF, 130 ng/ml; \(\Delta\), TGF\beta 10 ng/ml; \(\Delta\), TGF\beta 10 ng/ml plus EGF 100 ng/ml. \(^{45}\)Ca uptake was terminated after 5 min and "Ca\textsuperscript{2+} influx content was determined as described under "Experimental Procedures." Each point represents the mean ± S.D., n = 3. Error bars have been included only when they exceed the size of the symbols.

The effect of extracellular Ca\textsuperscript{2+} on stimulation of Ca\textsuperscript{2+} influx was studied. Rat-1 cells grown on 24-well plates were serum-deprived overnight in DMEM. 4 h prior to the assay the cells were rinsed and incubated in Ca\textsuperscript{2+}-free EMEM with CaCl\textsubscript{2} added to give final concentrations of Ca\textsuperscript{2+} from 0.1 to 3 mM. Cells assayed at 10 mM Ca\textsuperscript{2+} were preincubated with 3 mM Ca\textsuperscript{2+} to avoid precipitation of calcium phosphates during prolonged incubation with high Ca\textsuperscript{2+} concentrations. 10 ng/ml TGF\beta (\(\Delta, \Delta\)) or the TGF\beta vehicle (O, O) was added to the appropriate plates at this time. The assay was initiated by removing the incubation medium and adding EMEM containing 10 \(\mu\)Ci/ml \(^{45}\)Ca and the indicated concentration of CaCl\textsubscript{2}. The cells were treated as follows: O, no addition; \(\bullet\), EGF, 130 ng/ml; \(\Delta\), TGF\beta 10 ng/ml; \(\Delta\), TGF\beta 10 ng/ml plus EGF 100 ng/ml. \(^{45}\)Ca uptake was terminated after 5 min and "Ca\textsuperscript{2+} influx content was determined as described under "Experimental Procedures." Each point represents the mean ± S.D., n = 3. Error bars have been included only when they exceed the size of the symbols.

In contrast to specific inhibitors of voltage-sensitive Ca\textsuperscript{2+} channels, two nonspecific Ca\textsuperscript{2+} channel antagonists, Co\textsuperscript{2+} and Cd\textsuperscript{2+}, were highly effective inhibitors of both the Ca\textsuperscript{2+} influx and inositol phosphate release stimulated by EGF and TGF\beta (Table I). Inclusion of 3 mM Co\textsuperscript{2+}, which was previously shown to completely block the increase in intracellular free Ca\textsuperscript{2+} in response to combined EGF and TGF\beta treatment (8), also reduced the growth factor-stimulated Ca\textsuperscript{2+} influx to the levels seen in unstimulated cells (Fig. 10A). IP_{3} accumulation in response to EGF and TGF\beta was also inhibited by Ca\textsuperscript{2+} (Fig. 10B), and the dose-response characteristics for inhibition of Ca\textsuperscript{2+} influx and IP_{3} accumulation were similar (data not shown). The presence of 1 mM Ca\textsuperscript{2+} in phosphate-free medium was also sufficient to completely inhibit the growth

Ca\textsuperscript{2+} influx was determined as described under "Experimental Procedures." Each point represents the mean ± S.D., n = 3. Error bars have been included only when they exceed the size of the symbols.

Table I

Effect of Ca\textsuperscript{2+} channel blockers on Ca\textsuperscript{2+} influx

| Assay condition | Ca\textsuperscript{2+} influx (pmol/min/10\textsuperscript{6} cells) |
|-----------------|---------------------------------------------------------------|
| Control cells, no inhibitor added | 7.44 ± 0.27 |
| EGF, 10 ng/ml | 12.16 ± 0.23 |
| High K\textsuperscript{+} | 11.00 ± 0.20 |
| Nifedipine, 5 \(\mu\)M | 15.85 ± 0.49 |
| High K\textsuperscript{+} | 9.29 ± 0.17 |
| Verapamil, 10 \(\mu\)M | 7.47 ± 0.07 |
| EGF, 10 ng/ml | 13.41 ± 0.26 |
| High K\textsuperscript{+} | 10.62 ± 0.07 |
| TGF\beta, 4-h preincubation, followed by EGF | 15.54 ± 0.58 |
| High K\textsuperscript{+} | 5.89 ± 0.29 |

In the presence of 1 mM Ca\textsuperscript{2+} in phosphate-free medium was also sufficient to completely inhibit the growth factor-stimulated Ca\textsuperscript{2+} influx.
modulation of growth factor-stimulated $Ca^{2+}$ influx and PI turnover by $Co^{2+}$ and $Cd^{2+}$. Serum-derived Rat-1 cells were treated as described below. Cells treated with TGFβ were incubated in the presence of 10 ng/ml TGFβ for 4 h prior to the determination of either $Ca^{2+}$ influx or IP₃ accumulation. Those cells were not exposed to TGFβ were exposed to the TGFβ vehicle alone for the 4-h preincubation period. Each point represents the mean ± S.D. for $n = 5$ (Ca²⁺ influx) or $n = 3$ (IP₃ accumulation). $Ca^{2+}$ influx was measured over a 5-min period as described under “Experimental Procedures.” The $Ca^{2+}$-labeling medium also included EGF, TGFβ, $Co^{2+}$ or $Cd^{2+}$, as detailed below. The effect of $Co^{2+}$ or $Cd^{2+}$ on $Ca^{2+}$ influx was determined in cells treated as described below, proceeding from left to right for panels A and C: unstirn, no stimulus was added. Control $Ca^{2+}$ influx rates were 2.35 ± 0.21 pmol/min/10⁶ cells for panel A, and 1.25 ± 0.13 pmol/min/10⁶ cells for panel C. $Ca^{2+}$ or $Cd^{2+}$, cells were preincubated either in 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ for 5 min prior to replacement of the medium by fresh medium containing $Ca^{2+}$ plus either $Co^{2+}$ or $Cd^{2+}$. EGF, EGF (100 ng/ml) was added simultaneously with $Ca^{2+}, EGF + Co^{2+}$ (panel A) or $Cd^{2+}$ (panel C), cells were preincubated in the presence of 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ for 5 min prior to addition of EEMEM containing $Ca^{2+}$, EGF (100 ng/ml) and either $Co^{2+}$ or $Cd^{2+}$. TGFβ, cells were exposed to TGFβ for 4 h prior to addition of fresh EEMEM containing $Ca^{2+}$ and TGFβ. TGFβ + $Co^{2+}$ (panel A) or $Cd^{2+}$ (panel C) to cells pretreated for 4 h in the presence of TGFβ was added either 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ for 5 min. Fresh EEMEM containing $Ca^{2+}$, TGFβ, and either $Co^{2+}$ or $Cd^{2+}$ was then added. EGF + TGFβ, to cells pretreated with TGFβ for 4 h was added fresh EEMEM containing $Ca^{2+}$ and 100 ng/ml EGF. EGF + TGFβ + $Co^{2+}$ (panel A) or $Cd^{2+}$ (panel C), cells to cells pretreated with TGFβ for 4 h was added either 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ for 5 min. The medium was then replaced with fresh EEMEM containing $Ca^{2+}$, EGF (100 ng/ml), TGFβ, and either $Co^{2+}$ or $Cd^{2+}$. IP₃ accumulation in serum-deprived Rat-1 cells was determined as described under "Experimental Procedures." All cells were treated with 100 mM Li⁺ for 20 min prior to harvesting. Also added to the culture medium at either 5 or 10 min prior to harvesting were EGF, $Co^{2+}$, or $Cd^{2+}$, as detailed below. The effect of $Co^{2+}$ or $Cd^{2+}$ on induction of $IP₃$ accumulation was determined in cells treated as described below, proceeding from left to right for panels B and D: unstirn, no stimulus was added. Control IP₃ accumulations were 115 ± 5 cpm for panel B and 36 ± 3 cpm for panel D. $Co^{2+}$ or $Cd^{2+}$, either 3 mM $Co^{2+}$ (panel B) or 1 mM $Cd^{2+}$ (panel D) was added to the culture medium 10 min prior to harvesting of the cells. EGF, EGF (100 ng/ml) was added 5 min prior to harvesting. EGF + $Co^{2+}$ (panel B) or $Cd^{2+}$ (panel D), 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ were added 10 min prior to harvesting. EGF (100 ng/ml) was added 5 min prior to harvesting. TGFβ, cells were exposed to TGFβ for 4 h prior to harvesting of the cells. TGFβ + $Co^{2+}$ (panel B) or $Cd^{2+}$ (panel D), to cells pretreated with TGFβ for 4 h was added either 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ for 10 min prior to harvesting. EGF + TGFβ, cells were pretreated with TGFβ for 4 h. 5 min prior to harvesting EGF (100 ng/ml) was added. EGF + TGFβ + $Co^{2+}$ (panel B) or $Cd^{2+}$ (panel D), cells were pretreated with TGFβ for 4 h. 3 mM $Co^{2+}$ or 1 mM $Cd^{2+}$ were added 10 min prior to harvesting and EGF (100 ng/ml) was added 5 min prior to harvesting.

**FIG. 10.** Modulation of growth factor-stimulated $Ca^{2+}$ influx and PI turnover by $Co^{2+}$ and $Cd^{2+}$. Phosphate-free EEMEM was employed to prevent the formation of cadmium-phosphate precipitates. TGFβ was significantly less effective in phosphate-free incubation medium than in normal DMEM (1 mM phosphate). Neither $Co^{2+}$ nor $Cd^{2+}$ significantly depressed the rate of $Ca^{2+}$ influx in unstimulated cells.

Further evidence for a direct correlation between $Ca^{2+}$ influx and PI metabolism was obtained when Rat-1 cells were exposed to the $Ca^{2+}$ ionophore A23187. A 1 ng/ml dose of A23187 was sufficient to increase $Ca^{2+}$ influx by 70%, and a 120% increase in IP₃ accumulation was observed concomitantly (Table II).

**DISCUSSION**

Previously, we have demonstrated that TGFβ and EGF modulated both PI metabolism and intracellular $Ca^{2+}$ levels in Rat-1 cells (8). Treatment with TGFβ stimulated an increase in IP₃ and PIP₂ accumulation which was maintained at an elevated level for several hours. Stimulation of control...
phospholipase C mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate to physiological levels (11, 33, 34). Activated protein-mediated activation of phospholipase C is often very similar Ca²⁺ concentration dependence; and Ca²⁺ influx appears to be essential for the maintenance but not for the initiation of sustained processes such as salivary secretion (35) and pancreatic enzyme secretion (36). Studies performed in nonsecretory cells demonstrate that intracellular Ca²⁺ in many cell types shows a biphasic response to Ca²⁺-mobilizing growth factors (11, 37). The initial increase does not require extracellular Ca²⁺ but instead involves IP₃-mediated mobilization of intracellular Ca²⁺. In contrast, extracellular Ca²⁺ is required for the delayed and more prolonged secondary phase of Ca²⁺ elevation. In this scheme the hydrolysis of IP₃ is the initiating trigger which leads to the mobilization of intracellular Ca²⁺, and entry of Ca²⁺ across the plasma membrane is a later process, activated by a poorly defined mechanism. However, in those systems in which PI turnover and Ca²⁺ influx can be distinctly separated, IP₃ release is not always the initiating event. Mämla (38) has reported that activation of Ca²⁺ influx in response to EGF is independent of PI turnover in A431 cells, and is therefore not a consequence of IP₃ hydrolysis.

Receptor-operated Ca²⁺ channels have been postulated in several cell systems (59) but have been directly demonstrated in only a few instances. Patch clamp recordings indicate that ATP can directly gate Ca²⁺-permeable channels in smooth muscle (40). This receptor-activated Ca²⁺ channel operated independently of second messengers and required only that ATP bind to its receptor. Other mechanisms for growth factor-stimulated increases in Ca²⁺ permeability have been suggested, involving hydrolysis of PI₄P and subsequent opening of plasma membrane Ca²⁺ channels by either IP₃ (41), or IP₄ (42), or by Ca²⁺ released from intracellular stores in response to IP₃ or IP₄ (43). Our data indicate that accumulation of IP₃ and IP₄ was not responsible for the increase in Ca²⁺ influx, as LiCl-induced accumulation of IP₃ and IP₄ was not accompanied by an increase in Ca²⁺ influx. Since concentrations of Li⁺ which allow observation of growth factor-stimulated IP₃ accumulation caused a decrease in Ca²⁺ influx (Fig. 1), the data presented in Figs. 4-10 argue that EGF and TGFβ directly modulate Ca²⁺ influx, independent of changes in PI metabolism. However, these data cannot exclude the possibility that alternate second messenger systems may act to amplify or modulate the growth factor-stimulated changes in Ca²⁺ influx.

Direct activation of phospholipase C by elevated intracellular [Ca²⁺] has been postulated in other cell systems, based on the observation that extracellular Ca²⁺ is required for changes in intracellular Ca²⁺ levels in several cell types (33). These reports have been largely discounted (33), on the basis that use of EGTA to reduce extracellular Ca²⁺ levels would subsequently lower intracellular [Ca²⁺] below the level at which coupling by G-proteins is effective (34). Similarly, the observation that Ca²⁺ ionophores can stimulate IP₃ release has been attributed to the nonphysiologically high levels of Ca²⁺ brought into the cell by ionophores. Our data indicate that even a moderate reduction in the concentration of extracellular Ca²⁺, without the addition of EGTA, is sufficient to markedly reduce the Ca²⁺ influx and accumulation of IP₃ seen in response to stimulation by TGFβ and EGF. Similarly, increasing the rate of Ca²⁺ influx even moderately with the ionophore A23187 is sufficient to double the release of IP₃.

These data suggest that phospholipase C is highly sensitive to the rate of Ca²⁺ influx.

It is clear that phospholipase C is a highly Ca²⁺-dependent enzyme. Early reports indicated that in vitro activation requires very high, nonphysiological concentrations of Ca²⁺ (34). Ryu et al. (44) recently purified a PI-specific phospholipase C. This enzyme shows a steep calcium dependence in the physiological range between 10⁻⁷ to 10⁻⁴ M Ca²⁺. An attractive hypothesis for growth factor action is that increased Ca²⁺ influx through the growth factor-operated channels increases the local concentration of Ca²⁺ sufficiently to increase phospholipase C activity.
phospholipase C activity. The activation of phospholipase C results in second messenger generation and amplification of the Ca²⁺ signal. Further evidence of this hypothesis is provided by our observations that IP₃ accumulation and the increased intracellular [Ca²⁺] stimulated by EGF and TGFβ are synergistic (8), while the stimulation of Ca²⁺ influx by the combined growth factors is only additive. This suggests that the initial influx of Ca²⁺ may act to trigger a cascade of Ca²⁺-sensitive events, resulting in stimulation of PI metabolism and mobilization of intracellular Ca²⁺, and, possibly, modulation of cell morphology and growth.

The observation that simultaneous treatment with EGF and TGFβ produces an additive increase in Ca²⁺ influx suggests that EGF and TGFβ may interact with distinct populations of Ca²⁺ channels, or may influence distinct channel-activating mechanisms. The differing sensitivity of the EGF and TGFβ effects to actinomycin D supports this conclusion and indicates that TGFβ may exert its influence by promoting the transcription of a gene product which either subsequently activates mechanisms. Further evidence of this hypothesis is provided by our observations that cellular [Ca²⁺] stimulated by EGF and TGFβ are synergistic (8), while the stimulation of Ca²⁺ influx by the combined growth factors is only additive. This suggests that the initial influx of Ca²⁺ may act to trigger a cascade of Ca²⁺-sensitive events, resulting in stimulation of PI metabolism and mobilization of intracellular Ca²⁺, and, possibly, modulation of cell morphology and growth.

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