Endothelin-1 stimulates DNA synthesis and anchorage-independent growth of Rat-1 fibroblasts through a protein kinase C–dependent mechanism

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Stimulation of quiescent cultured fibroblasts with a variety of growth-promoting factors induces release of diacylglycerol (DG) and subsequent activation of protein kinase C (pkC), but the role of pkC in the induction of DNA synthesis and cell proliferation remains unclear. We have investigated the involvement of pkC in the response of Rat-1 fibroblasts to the newly described peptide endothelin-1 (Et-1), an agonist that is secreted by the vascular endothelium and that may play a role in the proliferative response of cells in the vessel wall. Addition of Et-1 to serum-deprived Rat-1 cells promoted DNA synthesis in the absence of additional factors and stimulated anchorage-independent growth in the presence of epidermal growth factor (EGF), indicating that Et-1 has many of the characteristics of a mitogen. The ability of Et-1 to stimulate both DNA synthesis and anchorage-independent growth was markedly reduced by the depletion of cellular pkC activity induced by prolonged exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA). In contrast, the ability of Et-1 to induce both second messenger production and transcription of *c-fos* and *c-jun* was largely independent of cellular pkC activity. Production of DG in response to Et-1 persisted for >12 h and may account for the ability of Et-1 to augment the G₁–S phase transition. Although these observations indicate that functional pkC is not an essential component of the proximal pathway leading to rapid changes in gene transcription and second messenger production in response to Et-1 treatment, the data suggest that activation of pkC is an essential component of the downstream events responsible for the stimulation of cell proliferation and anchorage-independent growth in Rat-1 cells exposed to Et-1.

Introduction

Induction of DNA synthesis in cultured cells by growth-stimulating factors is accompanied by

alterations in second messenger levels and by rapid transcriptional induction of specific genes (Berridge, 1987), but the involvement of these rapid events in the stimulation of the proliferative response remains unclear. Because commitment of the cells to DNA synthesis requires the presence of growth factors throughout the G₁ phase of the cell cycle (Pardee, 1989), it is apparent that at least some of the growth factor–induced signals must persist throughout most of the G₁ phase of the cell cycle. Little is currently known about the sustained signaling events involved in promoting the transition from the G₁ phase to S phase.

Activation of protein kinase C (pkC) has been implicated in the mitogenic response (Nishizuka, 1984; Bishop *et al.*, 1985; Claycomb and Moses, 1988). Although pkC is activated by a wide variety of growth factors through the release of diacylglycerol (DG) and Ca²⁺ (Nishizuka, 1984; Berridge, 1987), and overexpression of pkC in cultured cells results in altered growth characteristics (Housey *et al.*, 1988), the direct involvement of pkC activity in the mitogenic response to growth factors has been difficult to demonstrate. Addition of DGs to Swiss 3T3 cells has been demonstrated to induce DNA synthesis without pkC depletion (Issandou and Rozengurt, 1989), but such studies are complicated by the rapid degradation of exogenously added DG by cellular enzymes. Direct activation of pkC with tumor-promoting phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), stimulates mitogenesis in some cell types (Bishop *et al.*, 1985; Claycomb and Moses, 1988) but not in others (Kariya *et al.*, 1987). These studies are difficult to interpret because of the observation that TPA produces only a transient activation of pkC, and treatment with TPA for periods in excess of 5–8 h often results in the nearly complete depletion of pkC from cells (Rodriguez-Pena and Rozengurt, 1984; Ballester and Rosen, 1985). Because the length of the G₁ phase of the cell cycle in cells stimulated from a quiescent state is ~12 h (O'Keefe and Pledger, 1983; Pardee, 1989), it is unclear whether the observed stimulation of cell growth

after incubation with TPA indicates that a pkC-inducible event triggers entrance into the S phase, or whether the ultimate depletion of pkC activity by phorbol esters provides the activating stimulus. Therefore, positive responses to TPA treatment are very difficult to interpret. However, loss of a positive response after prolonged TPA treatment does suggest that activation of pkC was an important component of the observed response. This procedure has been utilized by other laboratories to demonstrate pkC-dependent and -independent pathways for mitogenic stimulation (Valge *et al.*, 1988; Mehmet *et al.*, 1989).

We have investigated the role of pkC in the stimulation of DNA synthesis and anchorage-independent growth of Rat-1 fibroblasts in response to the newly described peptide endothelin-1 (Et-1). Et-1 is the best characterized member of a family of peptides produced by endothelial cells and characterized by potent vasoconstrictive activity for smooth muscle cells (Yanagisawa *et al.*, 1988). More recently, Et-1 has been shown to have widespread stimulatory activity in a number of cell types for a variety of functions (Komura *et al.*, 1988; Takuwa *et al.*, 1989), including phosphatidylinositol turnover and gene transcription in the Rat-1 cell line (Muldoon *et al.*, 1989). Et-1 increases the incorporation of [3 H]-thymidine in vascular smooth muscle cells (Komura *et al.*, 1988) and in NIH-3T3 fibroblasts (Takuwa *et al.*, 1989), but the relationship of the second messenger changes induced by Et-1 to the stimulation of cell growth has not yet been identified.

We have examined both rapid and long-term effects of Et-1, utilizing the depletion of cellular pkC activity subsequent to prolonged treatment with TPA to examine the role of pkC in the induction of DNA synthesis and anchorage-independent growth, as well as the role of pkC in regulating the production of second messengers and stimulation of gene expression. Our results indicate that Et-1 was able to augment anchorage-independent growth in the presence of epidermal growth factor (EGF) and to induce DNA synthesis in Rat-1 cells in the absence of other growth factors. These actions of Et-1 were highly dependent on the presence of cellular pkC activity. In contrast, depletion of pkC activity did not abrogate the ability of Et-1 to stimulate either production of the second messengers DG and inositol trisphosphate (IP_3) or the induction of *c-fos* and *c-jun* transcription. These studies suggest that Et-1 operates via distinct pathways for the induction of short-term versus long-term events.

Results

Effect of Et-1 on anchorage-independent cell growth

Growth of cells in semisolid agarose is a marker for the transformed phenotype and can be induced experimentally by incubation of non-transformed cell lines with growth factors such as EGF and transforming growth factor β 1 (TGF β 1). We examined the ability of Et-1 to induce the anchorage-independent growth of Rat-1 cells in semisolid agarose both alone and in combination with EGF. Single cells were seeded into semisolid agarose in the presence of serum-supplemented Dulbecco's modified Eagle's medium (DME) and were maintained for 7 d before determination of colony size. In the absence of additional growth factors, no colonies containing more than four to eight cells were observed; the continued presence of TPA alone failed to stimulate anchorage-independent growth (Figure 1A). Culture of Rat-1 cells in the presence of TPA for as little as 18 h reduced measurable cytoplasmic and membrane-associated pkC activity below detectable levels (Table 1), and this reduction was sustained for at least 48 h (data not shown).

Addition of 10 ng/ml EGF to cells seeded in semisolid agarose induced the formation of

Figure 1. Effect of pkC depletion on the stimulation of anchorage-independent growth by growth factors. Rat-1 cells were seeded into semisolid agarose and colony size was measured after 7 d, as detailed in Methods. The cells were incubated with either 200 ng/ml TPA (striped bars) or the vehicle DMSO (open bars) throughout the 7-d growth period. (A) No peptide addition; (B) EGF 10 ng/ml; (C) Et-1 10^{-8} M; (D) Et-1 10^{-8} M plus EGF 10 ng/ml.

Table 1. Effect of TPA-treatment on Ca²⁺/phospholipid-dependent histone phosphorylation

Condition	Histone phosphorylation (cpm)			
	Cytoplasmic extract		Particulate fraction	
	DMSO 18 h	TPA 100 ng/ml 18 h	DMSO 24 h	TPA 100 ng/ml 24 h
EGTA	345 ± 108	321 ± 69	2070 ± 61	862 ± 33
0.5 mM CaCl ₂	1278 ± 83	400 ± 30	2563 ± 136	621 ± 44
	p < 0.001	N.S.	p < 0.001	p < 0.001

Rat-1 cells were serum deprived in the presence of 0.1% DMSO or 100 ng/ml TPA for 18 or 24 h as indicated. Cytoplasmic and particulate extracts were prepared separately, and histone phosphorylation was performed as described (Muldoon et al. 1987). After in vitro phosphorylation the extracts were run on sodium dodecyl sulfate-polyacrylamide gels, and the bands corresponding to histone H1s were excised and quantitated by liquid scintillation counting. Each value represents the mean ± SD of three (cytoplasmic) or four (particulate) independent phosphorylation reactions. Addition of CaCl₂ to the TPA-treated particulate fraction results in significant depression of histone phosphorylation. N.S., not significant.

large colonies, which were essentially undiminished in size by the inclusion of TPA in the medium (Figure 1B). Addition of Et-1 alone induced a negligible increase in the size of colonies, either in the presence or absence of TPA (Figure 1C). However, when EGF and Et-1 were added together, the resulting colonies were double the size observed after addition of EGF alone ($p < 0.001$; Figure 1D). When TPA was added to deplete cellular pkC levels, Et-1 treatment no longer augmented the size of EGF-stimulated colonies (Figure 1D).

These experiments have demonstrated that, although Et-1 was ineffective in stimulating anchorage-independent colony growth when applied independently, it could significantly increase the size of colonies obtained when added in the presence of EGF. Furthermore, although EGF-stimulated colony growth was unaffected by the inclusion of TPA, the depletion of cellular pkC subsequent to TPA treatment effectively eliminated the ability of Et-1 to augment EGF-stimulated colony growth.

Effect of pkC depletion on Et-1-induced DNA synthesis

In addition to a proliferative response, growth of cells in semisolid agarose involves a variety of cellular responses characteristic of neoplastic cells; stimulation of growth in monolayer culture may involve different activities. Increased thymidine incorporation in response to Et-1 has been demonstrated in smooth muscle cells (Komura et al., 1988), but the range of target cell types and the possible involvement of second messenger changes in the proliferative re-

sponse to Et-1 have not been investigated. We have used the CsCl density gradient method first described by Meyn (Meyn et al., 1973) to characterize the stimulation of DNA synthesis by Et-1 in serum-deprived Rat-1 fibroblasts and the effect of pkC-depletion on this process. The CsCl density gradient method has several advantages over methods that utilize uptake or incorporation of [³H]-thymidine. The reproducibility of the CsCl method is independent of artifacts caused by variations in cell number between samples, isotope labeling time, or alterations in agonist-induced nucleotide pools. Pulse-labeling with [³H]-thymidine produces measurements that are highly variable among experiments and that depend heavily on the presence of equal numbers of cells in each culture dish for obtaining reliable data within individual experiments. Moreover, the [³H]-thymidine method cannot be used to compare rates of DNA synthesis among different cell lines because of the differing rates of nucleoside transport among cell types. Because the measurement of DNA synthesis by the CsCl gradient method can be expressed in absolute values (percentage DNA replicated per unit time), this method avoids the relative measurements accompanying [³H]-thymidine pulse-labeling.

A representative CsCl gradient from unstimulated cells compared with cells stimulated with 10 nM Et-1 for 30 h is shown in Figure 2A, demonstrating the replicated and unreplicated DNA peaks. Each point in Figure 2B represents a complete CsCl density gradient fractionation as from Figure 2A. For technical reasons it was not feasible to obtain replicate independent points within each experiment. However, each

Figure 2. Stimulation of DNA synthesis by Et-1. (A) CsCl gradient elution. Rat-1 cells prelabeled with [¹⁴C]-thymidine were serum deprived for 48 h before addition of BrUdr in the absence of agonist (○), or in the presence of 10⁻⁸ M Et-1 (●). The cells were harvested after 30 h, and the labeled DNA was separated by CsCl gradient and then eluted in 0.5-ml increments. BrUdr-substituted DNA banded at 1.75 g/ml, whereas unreplicated DNA banded at 1.70 g/ml. (B) Effect of pkC depletion on the stimulation of DNA synthesis by Et-1. Rat-1 cells prelabeled with [¹⁴C]-thymidine were serum deprived for 48 h in the presence of 200 ng/ml TPA (□, ●) or the vehicle DMSO (■, ○). BrUdr-substituted DNA was isolated at the indicated times, and the percent DNA replicated was determined by CsCl density gradient analysis as described in Methods. Each point represents an entire gradient as in A, and each experimental condition was repeated in duplicate or triplicate with qualitatively similar results. (■) Cells were treated with 10⁻⁸ M Et-1. (□) Cells were pretreated with 200 ng/ml TPA for 48 h before addition of 10⁻⁸ M Et-1. (●) Cells were pretreated with TPA for 48 h before addition of fresh TPA. (○) No additions.

experimental condition was repeated in at least three independent experiments with consistent results. We have previously demonstrated that the variability between replicate points is <1% of the total amount of DNA replicated (Magun and Bowden, 1979).

The time of onset and the maximal rates of DNA synthesis in response to Et-1 were examined in Rat-1 cells that had been serum-deprived for 48 h in either DME or in DME containing 200 ng/ml TPA. Addition of 10⁻⁸ M Et-1, a concentration that we had previously determined to produce maximal induction of phosphatidylinositol hydrolysis in Rat-1 cells (Muldoon *et al.*, 1989), produced an increase in DNA synthesis beginning 15 h after its addition. The maximal rate of DNA synthesis stimulated by Et-1 was 4.0% h⁻¹, as compared with 0.8% h⁻¹ in control cultures not stimulated by Et-1 (Figure 2B). By 30 h, 40% of the DNA had been replicated in cells stimulated by Et-1, compared with 13% of the DNA replicated in unstimulated cells.

Et-1 was much less effective as an inducer of DNA synthesis in pkC-depleted cells. When stimulated by Et-1, cells depleted of pkC began synthesizing DNA at an overall maximal rate of 0.8% h⁻¹, compared with 4.0% h⁻¹ for cells not pretreated with TPA (Figure 2B). The amount of DNA synthesized by 30 h after Et-1 addition was also markedly decreased in pkC-depleted cells, from 40% to 17%. These results suggest that depletion of pkC by long-term incubation in TPA

almost totally abolished the ability of Et-1 to induce DNA synthesis.

Effect of pkC depletion on Et-1 binding

The reduction in Et-1's ability to induce DNA synthesis in pkC-depleted cells could be explained by a reduction in the binding of Et-1 to the surfaces of pkC-depleted cells. To test this hypothesis, we analyzed the binding of Et-1 to cells that were either untreated or that had been exposed to TPA for 18 h, a time that is sufficient to abolish measurable Ca²⁺/phospholipid-dependent protein kinase activity (Table 1). Binding of [¹²⁵I]-Et-1 to high-affinity receptors on Rat-1 cells was demonstrated by dosage-dependent inhibition of [¹²⁵I]-Et-1 by unlabeled Et-1 (Figure 3A). The binding of [¹²⁵I]-Et-1 to cells remained essentially unchanged after exposure of cells to TPA (Figure 3B). These results suggested that the inability of TPA-treated cells to respond to the DNA-stimulatory effects of Et-1 could not be attributed to a diminished binding of Et-1 to Rat-1 cell surfaces.

Maintenance of elevated DG levels by Et-1

Previously we demonstrated that Et-1 induced a rapid and large increase in DG levels concomitant with an increase in inositol trisphosphate (IP₃) levels, indicative of elevated phosphatidylinositol hydrolysis (Muldoon *et al.*, 1989). Because of the known ability of endogenous DGs to stimulate cellular pkC activity, and in view of

Figure 3. Binding of [125 I]-Et-1 to Rat-1 cells. (A) Competition of [125 I]-Et-1 binding by Et-1. Rat-1 cells were exposed to 1 ng/ml [125 I]-Et-1 for 3 h at 4°C in the presence of the indicated concentration of unlabeled Et-1. (B) Effect of pkC depletion on Et-1 binding. Cells were pretreated for 18 h with DMSO (no addition) or with 100 ng/ml TPA (TPA 18 h). Specific [125 I]-Et-1 binding was determined as described in Methods. Each point represents the mean $\pm$ SD of triplicate measurements, and each experiment was performed in duplicate with similar results.

the time delay between the addition of Et-1 and the measurement of DNA synthesis, we examined the ability of Et-1 to sustain elevated levels of cellular DG over an extended period of time.

Addition of Et-1 to cultures of either serum-deprived or actively proliferating Rat-1 cells resulted in a several-fold increase in cellular DG within 5 min (Figure 4, A and B). In cells exposed to Et-1 for 24 h, cellular DG was maintained at levels at least twice that of cells not exposed to Et-1. Et-1 was a potent stimulus for release of DG, whether measured in cells proliferating in 10% serum (Figure 4B), or in cells made quiescent by incubation in serum-free medium (Figure 4A).

Effect of pkC depletion on second messenger levels

Depletion of cellular pkC by the extended exposure of cells to tumor-promoting phorbol esters has been employed frequently as a means for demonstrating the involvement of pkC activity in regulating cellular phenomena (Black-shear *et al.*, 1987; Earp *et al.*, 1988; Valge *et al.*, 1988; Corps *et al.*, 1989; Mehmet *et al.*, 1989). A likely explanation for abrogation of activities in pkC-depleted cells is that pkC is no longer available for activation after an agonist-induced increase in cellular DG. Alternatively, one might hypothesize that treatment with TPA has reduced the ability of the agonist to induce the formation of second messengers, which are essential for the activation of other kinases, such

as calmodulin-dependent protein kinases. We therefore tested the possibility that extended TPA treatment of Rat-1 cells diminished or abrogated the ability of Et-1 to increase the formation of IP₃ or DG, or to mobilize intracellular Ca²⁺ (Figure 5). pkC depletion produced a significant increase in the release of IP₃ in response to Et-1 (Figure 5A), but the Et-1-induced changes in maximal intracellular Ca²⁺ levels were unaltered by pkC depletion. The slower return to basal Ca²⁺ levels in TPA-treated cells has been clearly demonstrated by Corps *et al.* (1989) on a single-cell basis and may reflect the higher levels of IP₃. We found that TPA treatment slightly reduced the basal level of DG (Figure 5C) but that the magnitude of the stimulation by Et-1, EGF, or serum was unaffected by pkC depletion (Figure 5C). These data indicate that depletion of pkC did not reduce the ability of Et-1 to stimulate the formation of second messengers, and suggest that rapid activation of pkC subsequent to release of DGs may have an inhibitory feedback action in cells not treated with TPA.

Involvement of pkC in rapid gene induction by Et-1

The high levels of DG released in response to Et-1 stimulation suggested that at least some of the rapid gene responses to Et-1 may rely on activation of pkC and would thus be eliminated by depletion of cellular pkC. In view of the suggested involvement of the transcription factors *jun* and *fos* in stimulating secondary responses

Figure 4. Effect of Et-1 on DG production. (A) Et-1 time course in confluent serum-deprived cells. (B) Et-1 time course in actively proliferating cells in the presence of 10% serum. Rat-1 cells were harvested and lipids were isolated at the indicated times after addition of 10^{-8} M Et-1. Cellular DG levels were determined as described in Methods. DG levels in the absence of Et-1 were 45 ± 3 pmole/ 10^6 cells (A) and 117 ± 10 pmole/ 10^6 cells (B). Each bar represents the mean $\pm$ SD ($n = 5$), and each experiment was performed in triplicate with similar results.

to growth-promoting factors (Komura *et al.*, 1988; Nakabeppu *et al.*, 1988), it seemed likely that stimulation of these immediate early genes

by Et-1 may be compromised in pkC-depleted cells. We therefore examined *c-fos* and *c-jun* gene expression in response to Et-1 and determined the involvement of pkC in Et-1-induced gene induction.

The accumulation of *c-fos* and *c-jun* mRNA in response to Et-1 was determined by Northern analysis of total RNA from untreated cells or from cells treated with TPA for 48 h to deplete cellular pkC activity (Figure 6, A and B). Et-1 induced a marked elevation in *c-fos* RNA levels at 30 min, which declined to near control levels within 60 min (Figure 6A, No TPA). Et-1 also induced a marked stimulation of *c-fos* levels in cells depleted of pkC activity (Figure 6A, + TPA), although the magnitude of the response was reduced to $\sim 40\%$ of that seen in naive cells by chronic TPA treatment, as determined by densitometric analysis. The effectiveness of the pkC depletion after chronic TPA treatment was confirmed by the observed inability of freshly added TPA to induce *c-fos* RNA in pkC-depleted cells (data not shown). Accumulation of *c-jun* RNA was stimulated to a similar extent after Et-1 addition, whether or not the cells were pretreated with TPA. Et-1 induced a 7-fold and 5-fold induction of *c-jun* at 30 min and 1 h, respectively, in control cells and 8-fold and 10-fold at 30 min and 1 h in TPA-treated cells, as determined by densitometric analysis.

To confirm that modulation of *c-fos* and *c-jun* RNA levels by Et-1 occurred at the level of transcription, we performed nuclear run-on analyses of gene transcription in naive and TPA-treated Rat-1 cells (Figure 7). Et-1 stimulated a marked increase in both *c-jun* and *c-fos* transcription within 10 min in both untreated (No TPA) and pkC-depleted cells (+ TPA). Basal transcription rates were slightly elevated in TPA-treated cells, perhaps through release of a basal level of inhibition mediated by pkC. The Et-1 gene was not expressed in Rat-1 cells, as determined by lack of hybridization to the pEt4 human Et-1 cDNA, and no hybridization to the pBR322 plasmid control was observed. Transcription of the cyclophilin internal control was unaltered by Et-1 treatment. These data indicate that the induction of gene responses to Et-1 was not dependent on the presence of pkC activity.

Discussion

Depletion of pkC activity from cells after prolonged exposure to tumor-promoting phorbol esters has been a useful tool in determining the involvement of pkC activity in the responses to physiological stimuli (Blackshear *et al.*, 1987; Earp *et al.*, 1988; Valge *et al.*, 1988; Corps *et*

Figure 5. Second messenger production in pkC-depleted cells. (A) Effect of pkC depletion on IP₃ release. Rat-1 cells were treated with 500 ng/ml TPA (□) or the vehicle DMSO (■) for 24 h before the experiment. Cells were stimulated with 10⁻⁸ M Et-1 and harvested at the indicated times. IP₃ levels were determined by elution of [³H]-IP₃ from Dowex-1 columns as described in Methods. Each point indicates the mean ± SD of triplicate samples, and the experiment was performed in duplicate with similar results. (B) Effect of pkC-depletion on Ca²⁺ mobilization. Rat-1 cells were treated with 500 ng/ml TPA (dashed line) or the vehicle DMSO (solid line) for 24 h before loading with fura-2. 10⁻⁸ M Et-1 was added at the time indicated by the arrow, and the response was monitored continuously as described in Methods. (C) Effect of pkC depletion on DG production. Rat-1 cells were treated for 24 h with DMSO (No TPA) or 500 ng/ml TPA (pkC depleted). Cellular lipids were isolated in unstimulated cells, designated control, and in cells stimulated for 5 min with 10 ng/ml EGF, 10⁻⁸ M Et-1, or 10% serum, as indicated below each bar. Cellular DG levels were determined as described in Methods. Each point represents the mean ± SD (n = 5), and each experiment was performed in duplicate with similar results.

al., 1989; Mehmet *et al.*, 1989). This procedure is superior to the use of inhibitors such as the isoquinolinesulfonamides or sphingosine and its derivatives, as the latter compounds may be cytotoxic and also exhibit a lack of specificity (Hidaka *et al.*, 1984; Jefferson and Schulman, 1988). Prolonged treatment of cells with high concentrations of TPA has been shown to remove most or all measurable pkC activity from the cells (Rodriguez-Pena and Rozengurt, 1984). The existence of multiple isozymes of pkC may complicate the interpretation of pkC-depletion experiments, as the substrate specificity and susceptibility to TPA treatment may vary among isozymes. Nevertheless, the Rat-1 cells used in the present experiments lack measurable pkC activity after TPA treatment, as determined by the Ca²⁺- and phospholipid-dependent phosphorylation of histones by cytoplasmic and particulate cell extracts. We have demonstrated

that pkC depletion is essentially complete within 18 h after addition of 100 ng/ml TPA to Rat-1 cells, similar to our previous results with a Rat-1 derivative (Rodland *et al.*, 1988). This procedure can therefore be used to determine the role of pkC in the response to Et-1. A similar protocol has been utilized to demonstrate the existence of pkC-dependent and -independent pathways for the stimulation of mRNA expression and [³H]-thymidine incorporation by T-cell mitogens (Valge *et al.*, 1988) and for the stimulation of mitogenesis by bombesin in Swiss 3T3 cells (Mehmet *et al.*, 1989).

Involvement of pkC in the stimulation of anchorage-independent growth by Et-1

One of the characteristics of the growth of non-transformed cells in culture is their dependence on a solid growth substrate. Growth of cells in

Figure 6. Effect of pkC depletion on Et-1 stimulation of *c-fos* and *c-jun* RNA accumulation. (A) *c-fos* and cyclophilin expression in response to Et-1. (B) *c-jun* expression in response to Et-1. Rat-1 cells serum-deprived for 48 h in the presence of 500 ng/ml TPA (+ TPA) or the vehicle DMSO (No TPA) were treated with 10^{-8} M Et-1 for the indicated times, and total cellular RNA was isolated and size-fractionated on 1.2% agarose/formaldehyde gels. The RNA was hybridized with 32 P-labeled *c-fos* (A) or *c-jun* (B) and autoradiography was performed. The *c-fos* blot was also probed with the internal control cyclophilin.

an anchorage-independent manner is a marker for the transformed phenotype and has been shown to be a general indicator of tumorigenicity (Roberts *et al.*, 1983; Moses *et al.*, 1984; Dion *et al.*, 1988). The transformed phenotype also can be induced experimentally in non-transformed fibroblasts by the transforming growth factors and by EGF (Roberts *et al.*, 1983; Moses *et al.*, 1984). The ability of Et-1 to induce anchorage-independent growth has not been previously investigated. However, most growth-promoting peptides do not induce colony formation in semisolid agarose, and it is a distinguishing characteristic of the transforming growth factors that they are capable of inducing such growth (Roberts *et al.*, 1983; Moses *et al.*, 1984, 1985).

The Rat-1 cells used in these experiments failed to form colonies in semisolid agarose in the absence of EGF, indicating that the abundance of factors present in 10% serum are insufficient to induce or maintain the transformed phenotype in these cells. Growth of Rat-1 cells in semisolid agarose was not significantly stimulated by the addition of Et-1 alone, but in the presence of EGF Et-1 promoted a marked increase in the size of colonies growing in semisolid agarose. The formation of colonies in response to EGF was independent of the level of pkC activity in the cells, as demonstrated by the

unaltered size of colonies in the continued presence of EGF and TPA. However, the ability of Et-1 to augment the size of colonies in the presence of EGF was completely abolished by pkC depletion. These results suggest that a pkC-mediated process stimulated by Et-1 increased the ability of EGF to stimulate anchorage-independent growth.

The use of tumor-promoting phorbol esters as an agonist to demonstrate the involvement of pkC in delayed activities such as DNA synthesis and anchorage-independent growth has been complicated by the destruction of pkC that occurs during extended phorbol ester treatment (Rodriguez-Pena and Rozengurt, 1984; Ballester and Rosen, 1985). Therefore, studies that demonstrate stimulation or inhibition of soft agar growth in response to TPA (Dion *et al.*, 1988) are difficult to interpret. Housey *et al.* (1988) generated a number of fibroblast cell lines that overproduced pkC, and found an increased tendency toward colony formation in semisolid agarose, both in the basal state and when stimulated by TPA. These results indicate that pkC can play an important role in growth control, but they do not address the role of pkC in the stimulation of anchorage-independent growth by specific growth factors. Our data indicate that there are pkC-dependent and -independent pathways for the stimulation of growth in semi-

Figure 7. Effect of pkC depletion on Et-1-stimulated gene transcription. Rat-1 cells serum-deprived for 48 h in the presence of 500 ng/ml TPA (+ TPA) or the vehicle DMSO (No TPA) were not further treated (no addition) or were treated with 10^{-8} M Et-1 for 10 min. Nuclei were isolated and initiated transcripts were elongated to generate 32 P-labeled RNA, which were then hybridized against the indicated DNAs blotted onto Gene Screen + membranes.

solid agarose by polypeptides. These pathways are separable, as we have demonstrated that EGF remains an effective agonist for anchorage-independent growth in pkC-depleted cells; however, the pathways are coordinated, in that the increase in colony size after stimulation of the pkC-dependent pathway by Et-1 requires concomitant activation of a pkC-independent pathway with EGF.

Involvement of pkC in the stimulation of DNA synthesis by Et-1

Although Et-1 has been shown to increase thymidine uptake and incorporation in both vascular smooth muscle cells (Komura *et al.*, 1988) and Swiss 3T3 mouse fibroblasts (Takuwa *et al.*, 1989), the relationship between Et-1-induced second messengers and DNA synthesis has not been investigated. In the experiments reported here, Et-1 stimulated a significant increase in DNA synthesis in Rat-1 cells. The total amount of DNA replicated in response to Et-1 reached only 40%, and the maximal rates of DNA synthesis observed after Et-1 stimulation were ~50% of those achieved by EGF, which acts as a complete mitogen in Rat-1 cells (Matrisian *et al.*, 1981). These results suggest that, although activation of pkC can stimulate DNA

synthesis and anchorage-independent growth to a limited extent, stimulation of pkC activity alone was insufficient to induce a complete DNA synthetic response in this cell type.

The observation that the ability of Et-1 to stimulate DNA synthesis required the presence of functional pkC suggests that activation of pkC plays an important role in the long-term response to Et-1. This response may be mediated by the dramatic increase in DG levels produced by Et-1 exposure in Rat-1 cells. Et-1 increased DG levels significantly more than did 10% serum, and the elevation of DG was maintained over an extended period of time both in serum-deprived cells and in actively proliferating cells. Et-1 would thus be expected to activate pkC throughout the G₁ phase of the cycle by the continuous generation of DG. The inability of Et-1 to stimulate DNA synthesis in pkC-depleted cells suggests that the long-term elevation of pkC activity by Et-1 plays a vital role in the mitogenic response to this agonist.

Alterations in second messenger levels and gene expression are not affected by pkC depletion

TPA is thought to act specifically by activating cellular pkC and has not been demonstrated to affect the activity of other enzymes (Housey *et al.*, 1988). In view of the broad range of cellular activities regulated by DG production and activation of pkC, we investigated whether depletion of pkC by TPA altered the induction of intracellular second messengers by Et-1. We found that stimulation of PI turnover by Et-1 was not diminished and was even elevated in pkC-depleted cells. The augmentation of inositol phosphate release might be a consequence of the loss of pkC-mediated feedback inhibition, which normally acts to limit IP₃ production (Berridge, 1987). Changes in intracellular Ca²⁺ and DG induced by Et-1 were similarly unaffected by TPA treatment. The presence of pkC was therefore not essential for the generation of these second messengers. These data are in agreement with recent work (Corps *et al.*, 1989) demonstrating that mobilization of intracellular Ca²⁺ in response to peptide agonists was unaltered by pkC depletion.

The ability of Et-1 to alter second messenger levels in pkC-depleted cells is consistent with our previous finding that the ability of Et-1 to induce expression of the VL30 gene in a derivative of the Rat-1 cell line is not affected by pkC depletion (Muldoon *et al.*, 1989). In contrast to the lack of stimulation of *c-fos* by bombesin in pkC-depleted Swiss 3T3 cells (Mehmet *et al.*,

1989), we found that the induction of both *c-fos* and *c-jun* by Et-1 was not abolished by pkC-depletion. Therefore, the failure of Et-1 to induce anchorage-independent growth and stimulation of DNA synthesis in TPA-treated cells could not be explained by reduced levels of the trans-activating factors *c-fos* and *c-jun*. These results indicate that the abrogation of Et-1's ability to induce DNA synthesis after extended TPA treatment resulted neither from a reduction in second messenger levels nor from a failure of the rapid transcriptional activation of nuclear protooncogenes in response to Et-1. These data further suggest that, although the presence of pkC activity was not mandatory for rapid gene responses to Et-1 stimulation, the continuous presence of pkC throughout most or all of the G₁ phase of the cell cycle was required for the ability of Et-1 to stimulate an entrance into S phase.

The present studies provide insight into the role of pkC activity in the mitogenic response to growth factors. The finding that different growth factors rely on pkC activity to different extents for the stimulation of cell growth emphasizes the point that multiple pathways for the stimulation of mitogenesis exist. Alterations in either the availability of pkC or the ability of growth factors and peptide agonists to activate pkC could have wide-ranging effects on cell growth. Carcinogenesis is a multistage process that depends, at least in part, on the coordinated functioning of distinct, growth factor-specific pathways in the regulation of cell growth. The coordinated growth responses of growth factors such as EGF, which utilize tyrosine kinases as receptors, and of agonists such as Et-1, which maximally stimulate phospholipase C activity, may be relevant to the process of tumor promotion and progression.

The Et-1 peptide is produced by endothelial cells, and its production is modulated by factors that affect endothelial cell function or growth (Yanagisawa *et al.*, 1988). Because of Et-1's dual activity as a potent vasoconstrictor and as a mitogen for fibroblasts and possibly smooth muscle cells, Et-1 can be expected to have a variety of effects on the cells of the vessel wall. Normal levels of Et-1 expression may have a role in maintaining vessel homeostasis, whereas elevated or inappropriate expression of Et-1 may produce changes characteristic of the disease phenotype. It will be important to examine the coordinated activity of Et-1 and other agonists, which may induce long-term effects through the activation of pkC, in combination with tyrosine kinase-activating growth factors,

or in relation to the initiation or maintenance of cardiovascular disease.

Methods

Materials

Et-1 (human/porcine) was obtained from Peptides International (Louisville, KY) and was dissolved in H₂O and stored at -70°C before use. EGF was prepared from mouse submaxillary gland and purified to homogeneity by high-performance liquid chromatography as described (Matrisian *et al.*, 1982). TPA was obtained from Sigma and was suspended in dimethyl sulfoxide (DMSO).

Cell culture

Rat-1 cells were routinely grown in DME containing 10% fetal bovine serum (Hyclone) at 37°C in a humidified 5% CO₂/95% air atmosphere.

Growth of cells in semisolid agarose

Rat-1 cells were dispersed as single cells in 0.2% agarose in DME and distributed at 7500 cells/well to six-well plates containing bottom agarose. Bottom agarose consisted of 0.5% agarose in DME containing 10% calf serum and the factors indicated in the figure legends. Cell growth proceeded for 7 d before determination of colony size. For each experimental condition, the diameters of 100 colonies were measured in two dimensions using a Wilovert microscope. Colonies formed in the absence of added factors contained one to eight cells and had a mean diameter of 33 μ m.

CsCl gradient analysis of DNA synthesis

Subconfluent Rat-1 cells grown in six-well plates were pre-labeled for 48 h in 2-[¹⁴C]-thymidine (0.1 μ Ci/ml, Dupont/NEN) and brought to quiescence by serum deprivation for 48 h in the presence of either 200 ng/ml TPA or the vehicle DMSO. The medium was then changed to fresh serum-free DME containing 50 μ g/ml 5'-bromodeoxyuridine (BrUdr), and 0.1 μ g/ml 5'-fluorodeoxyuridine as described by Meyn *et al.* (Meyn *et al.*, 1973). This medium also contained DMSO or TPA, in the presence or absence of Et-1. Cellular DNA was extracted at the indicated times, and BrUdr-substituted DNA (density 1.75 g/ml) was separated from parental DNA (density 1.70 g/ml) by centrifugation on neutral CsCl gradients. Centrifugation proceeded for 48 h at 33 000 rpm in a type SW40.3 rotor at 22°C. The percentage of DNA replicated was determined by the percentage of BrUdr-substituted DNA.

PkC assay

Rat-1 cells grown to confluence on 10-cm plates were serum-deprived for 18 h in DME supplemented with either 100 ng/ml TPA, 500 ng/ml TPA, or 0.1% DMSO. Cells were scraped from the plates with a rubber policeman and pelleted at 2000 rpm. Cytoplasmic and particulate extracts were prepared as described (Muldoon *et al.*, 1987). Phosphorylation of exogenous histone III_s proceeded for 3 min at 30°C, in the presence of 5 μ g of cytosolic extract, 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 5 mM MgSO₄, 0.05 mg/ml phosphatidylserine, and 50 μ M ATP (200 cpm/pmole), in the presence of either 1 mM EGTA or 0.5 mM CaCl₂ as indicated. Phosphorylated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described (Muldoon *et al.*, 1987), and phosphorylated histones were excised from dried gels and counted by liquid scintillation.

¹²⁵I-Et-1 binding assay

Five micrograms of Et-1 were iodinated using the iodobead method as described by the manufacturer (Pierce Chemical). Final specific activity was 0.2 μ Ci/ng Et-1. Cells were plated onto 24-well dishes so that at the time of the assay there were $\sim 10^6$ cells in exponential growth phase per dish. Cells were pretreated with 100 ng/ml TPA or the DMSO vehicle for 18 h before the assay. Plates were rinsed twice with serum-free DME at room temperature, after which 1 ml of binding medium (DME with 1 mg/ml bovine serum albumin and 25 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethane sulfonic acid, pH 7.4) containing 1 ng/ml [¹²⁵I]-Et-1 was added. Binding proceeded for 3 h at 4°C, and was terminated by rinsing six times with ice-cold Hanks' balanced salt solution. Total cell-associated radioactivity was determined by solubilizing the cells in 1 ml 0.5 N NaOH and counting in a Tracor gamma counter. Specific [¹²⁵I]-Et-1 binding was determined by subtracting nonspecific binding in the presence of 200-fold excess unlabeled Et-1.

DG mass measurements

Rat-1 cells plated in 10-cm dishes were assayed either in the exponential growth phase (Figure 6B only) or were grown to confluence and serum-deprived for 24 h before the experiment. Cellular DG levels were determined essentially as described by Priess *et al.* (1986) utilizing bacterial diacylglycerol kinase (Lipidex) to convert cellular DG to ³²P-phosphatidic acid. The identity of labeled phosphatidic acid was confirmed by thin-layer chromatography analysis (data not shown), and DG levels were quantitated as previously described (Muldoon *et al.*, 1989).

Inositol phosphate assay

Rat-1 cells were grown to confluency on 10-cm plates and serum-deprived for 36 h in DME containing 2 μ Ci/ml [³H]myo-inositol (Dupont/NEN). After removal of the labeling medium, the cells were incubated for an additional 4 h in serum-free DME. Before harvesting, all cells were exposed to 100 mM LiCl for 20 min, and then water-soluble inositol phosphates were extracted as described (Muldoon *et al.*, 1989). IP₃ was eluted from Dowex-1 ion exchange columns with 0.75 M ammonium formate/0.1 M formic acid and quantified by liquid scintillation counting.

Measurement of intracellular Ca²⁺ using fura-2

Rat-1 cells grown on 25-mm-diam glass coverslips were serum-deprived overnight in DME before the experiment, in the presence of 100 ng/ml TPA or the DMSO vehicle. Cells were loaded with fura-2 (1 μ M for 30 min), and fluorescence measurements were performed using a Fluoroplex III microspectrofluorometer (Tracor Northern) as described Muldoon *et al.*, 1989). Each trace represents measurements recorded from a single field of ~ 30 cells and is representative of at least three similar traces.

Northern analysis of *c-fos* and *c-jun* mRNA

Rat-1 cells grown to confluence on 10-cm plates were serum-deprived for 48 h in the presence of 500 ng/ml TPA or the vehicle DMSO. After treatment with 10 nM Et-1 for the indicated times, cells were dissolved in 4 M guanidine thiocyanate/100 mM Tris pH 7.5/0.1% 2-mercaptoethanol. RNA was precipitated by addition of 4 M LiCl and further purified as described (Fourney *et al.*, 1989). Twelve micrograms of total RNA was fractionated on 1.2% agarose/formaldehyde gels and blotted onto Nytran membranes. The

hybridization probes were prepared by random hexamer extension of *c-fos* (7×10^8 dpm/ μ g) and *c-jun* (5.5×10^8 dpm/ μ g). To confirm uniform loading of RNA, the blots were stripped and reprobed with cyclophilin (plasmid pSP61B15, supplied by J. Douglas) labeled to 3×10^8 dpm/ μ g using an SP6 riboprobe kit. Autoradiography was conducted for 1–3 d at -70°C with intensifying screens. Quantitation of the fold induction of *c-fos* and *c-jun* was performed by densitometric analysis of multiple autoradiographic exposures representing a linear response range, utilizing a Hoefer densitometer and Bioquant digitizer.

Transcriptional run-on analysis

Rat-1 cells grown to confluence on 10-cm plates were serum-deprived for 48 h in the presence of 500 ng/ml TPA or the vehicle DMSO. After treatment with 10 nM Et-1 for the indicated times, cells were lysed with 0.3% NP40, and nuclei were isolated as described (Machida *et al.*, 1988). Initiated transcripts were elongated in the presence of ³²P-CTP, and labeled transcripts were hybridized against specific DNA blotted on Nytran membranes. *c-fos*, pBR322, and pEt4 (obtained from M. Yanagisawa) were cut with *Eco*RI, *c-jun* was linearized with *Hind*III, and cyclophilin was linearized with *Pst* I. Autoradiography was conducted for 1–3 d at -70°C with intensifying screens.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Jean M. Aschenbach, Thanh-Hoai V. Dinh, Markus L. Forsythe, and Herve Enslin.

This work was supported by NIH grants CA-39360, CA-47404, and CA-08444 and by a grant from Chugai Pharmaceuticals, Japan.

Received: January 11, 1990.

Revised and accepted: February 26, 1990.

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