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Cholera Toxin Potentiates TPA-Induced Mitogenesis and c-*fos* Expression in BALB/c-3T3-Derived Proadipocytes

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Abstract Treatment of quiescent density-arrested A31T6 proadipocytes with medium supplemented with either 12-O-tetradecanoylphorbol-13-acetate (TPA), insulin, or cholera toxin alone did not stimulate G_0/G_1 traverse and initiation of DNA synthesis. Combinations of either TPA and cholera toxin or insulin and cholera toxin caused a small stimulation of proliferation. Addition of medium supplemented with TPA and insulin caused a marked stimulation of cell cycle traverse which was significantly potentiated by the coaddition of cholera toxin. The actions of cholera toxin were mimicked by forskolin. Expression of c-*fos* was regulated in a manner that reflected the results of the mitogenic experiments. TPA caused a marked induction of expression, while only a small increase in transcript levels was seen after treatment with cholera toxin. Addition of a combination of cholera toxin and TPA caused a synergistic induction of c-*fos* expression. The model system described in this paper allows a detailed analysis of the regulation, by independent second messenger systems, of the transcription of a gene in a mitogenically relevant manner.

Cellular proliferation is regulated by a family of extracellular mitogens that act by binding to cell surface receptors and, by a process that is largely yet to be characterized, increasing the transcription of a set of growth-regulated genes [Lau and Nathans, 1991]. In nontransformed BALB/c-3T3 cells, a combination of mitogens is required for optimal mitogenesis. This was first demonstrated with the observation that neither platelet-derived growth factor (PDGF) nor platelet-poor plasma (PPP) alone are efficient mitogens, although a combination of PDGF and PPP causes a marked stimulation of DNA synthesis in quiescent, density-arrested cells [Pledger et al., 1978]. It was later shown that the comitogenic effects of PPP can be replaced with a combination of epidermal growth factor and insulin-like activity [Leof et al., 1982]. In NIH 3T3 cells, the addition of medium supplemented with PDGF alone causes maximal mitogenic stimulation. This appears to be due to the fact that PDGF uniquely stimulates multiple intermediate pathways in these cells, an effect mimicked only by combinations of other mitogens [Rozengurt, 1986]. Thus, it remains a general fact that cells are stimulated to traverse G_0/G_1 and initiate DNA synthesis only in the presence of appropriate combinations of intracellular signals which are generated, in most cases, by the presence of multiple mitogens.

One pathway that is critical to the generation of signals that regulate cell cycle traverse involves changes in phosphatidylinositol turnover, with a resulting increase in intracellular calcium and protein kinase C activity [Hall and Stiles, 1987; McCaffrey et al., 1987]. Activation of protein kinase C directly by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates mitogenesis in a number of cell types [Diamond et al., 1980]. In BALB/c-3T3 cells, however, the addition of medium supplemented only with TPA is not mitogenic. In order to stimulate optimal entry into S phase, the addition of a another mitogen such as insulin-like activity [Frantz et al., 1979; Selinfreund and Wharton, 1986; Smyth et al., 1990] or EGF [Frantz et al., 1978; Dicker and Rozengurt, 1978] together with TPA is also required.

The ability of mitogens such as PDGF or EGF to stimulate DNA synthesis in BALB/c-3T3 cells can be modulated by the intracellular concentra-

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tion of cyclic AMP. Cholera toxin directly potentiates the ability of PDGF to act synergistically with plasma components [Wharton et al., 1982]. In addition, elevations in cyclic AMP levels allow a marked increase in the sensitivity of several lines of cells to the mitogenic actions of EGF without modulating EGF receptor number or affinity [Olashaw et al., 1984]. In Swiss 3T3 cells, cyclic AMP analogs also act synergistically with a number of mitogens [Rozengurt, 1982]. Unlike the case in BALB/c-3T3 cells, however, in which elevations in cyclic AMP are not mitogenic in the absence of other mitogens, a marked stimulation of growth can be observed in Swiss 3T3 cells following the addition of only cholera toxin [Rozengurt et al., 1981]. A biochemical or molecular mechanism that accounts for either the general observation that elevations in cyclic AMP modulate mitogenic sensitivity in fibroblasts, or for the marked variation in the nature of the specific response of individual cell types, has not been described.

c-*fos* is a prototype of what is termed an "immediate early gene" in that it is rapidly and transiently induced following mitogenic stimulation of a variety of cell types, with the induction but not the decay of the message being independent of protein synthesis [reviewed in Ransone and Verma, 1990]. The product of the c-*fos* gene is a component of the AP-1 transcription factor [Rauscher et al., 1988] that appears to be absolutely required for traverse of the cell cycle in 3T3 cells [Holt et al., 1986; Nishikura and Murray, 1987]. The regulation of c-*fos* transcriptional activation can be very complex, with a variety of 5′ enhancer elements influencing either basal or induced levels of expression. Specific examples of c-*fos* regulatory elements whose activities are potentially modulated by mitogenic agents include the serum response element (SRE) [Treisman, 1985], a cyclic AMP response element (CRE) [Berkowitz et al., 1989], a *sis*/PDGF responsive element (SIF) [Hayes et al., 1987; Wagner et al., 1990], and an AP-1 site [Velcich and Ziff, 1990]. Investigations of the control of c-*fos* expression become even more complicated due to the fact that there is cell type-specific modulation of transcription [Muller et al., 1983; Bravo et al., 1987].

In this paper we describe the interactions between agents that stimulate protein kinase C and those that elevate cyclic AMP in both the stimulation of cell cycle traverse and the expression of c-*fos* in A31T6 cells. This cell line can be readily induced to differentiate into adipocytes [Sparks et al., 1986; Smyth and Wharton, 1992] and very rapidly becomes mitogenically nonresponsive after treatment with adipogenic agents [Smyth and Wharton, unpublished observations). A careful description of the mitogenic properties of these proadipocyte cells is a necessary requirement before an investigation into the lesions in mitogenically unresponsive cells can be undertaken. Studies of the regulation of c-*fos* expression are also relevant in these cells, since it is clear that this protooncogene plays an important role both in the regulation of cellular growth, as described above, as well as in the expression of adipogenic-specific genes [Distel et al., 1987].

MATERIALS AND METHODS

Cell Culture

BALB/c-3T3 cells (clone A31T6) were maintained in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS), 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2. Stock cultures were grown in 100 mm plates and subcultured every 3 days as described in Wharton and Smyth [1989]. Cells used in experiments were plated at an initial density of 4.8×10^4 cells per 35 mm dish or 1.4×10^5 cells per 60 mm dish. The cells were refed with fresh medium after 3 days and then used in an experiment after a further 5 or 6 day incubation.

Mitogenesis

Quiescent, density-arrested cells were treated for 24 h with fresh α-MEM containing 0.1% BSA together with the various supplements as described for each experiment. Preliminary experiments indicated that the peak of DNA synthesis occurred at this time point following addition of the agents used in this study. Twenty-five microliters of α-MEM containing 5 μCi ^{3}H-dT was then added directly to the medium of each plate. Following a further 70 min incubation the plates were cooled, washed twice with cold phosphate-buffered saline (PBS), and extracted with two rinses of cold 5% trichloroacetic acid (TCA) for 10 min each. The precipitated material was solubilized in 1 ml 0.1 N NaOH containing 0.1% SDS and the radioactivity was quantified in a liquid scintillation counter.

Cells prepared for microfluorimetry were removed from the plate by treatment with PBS

containing 0.125% trypsin and 0.5 mM EDTA. The cell slurry was added to α-MEM containing 10% FCS to neutralize the trypsin and centrifuged at 500g for 5 min. The cell pellet was resuspended in 1 ml serum free α-MEM and 4 ml 95% ethanol was slowly added while the cells were agitated. Following fixation for a minimum of 12 h at 4°C, the cells were stained with mithramycin according to the method of Crissman et al. [1976]. Aggregates were removed by filtration through a 44 μM nylon mesh and fluorescence was measured using a locally built flow cytometer [Holm and Cram, 1973]. An argon laser tuned to a wavelength of 457 nM was used to excite the DNA-dye complex, and emission above 520 nM was collected.

RNA Extraction

Total cellular RNA was extracted by a modification of the single-step method of Chomczynski and Sacchi [1987]. Each monolayer of cells was rinsed with cold PBS and 350 μl solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 100 mM 2-mercaptoethanol] was added directly to the 100 mm dish. The lysate was scraped and transferred into autoclaved microfuge tubes, followed by successive additions of 35 μl 2 M sodium acetate (pH 4.0), 350 μl water-saturated phenol, and 70 μl chloroform:isoamyl alcohol (24:1), each followed by vortexing for 10 s. Samples were centrifuged at 10,000g for 20 min at 4°C, and RNA was precipitated from the aqueous phase by the addition of 2 volumes of isopropanol at room temperature. The RNA was resedimented by centrifugation at 10,000g, redissolved in 300 μl solution D, and reprecipitated with isopropanol. The RNA pellet obtained following centrifugation was resuspended in 70% ethanol, resedimented, and vacuum dried for 15 min. The final RNA pellet was resuspended in 25 μl RNase-free TE (pH 7.4) and 62.5 μl ice-cold ethanol was added. The RNA was then stored at −70°C. To recover the RNA, 2.5 μl RNase-free 3 M sodium acetate (pH 5.2) was added and the RNA was incubated at −20°C for 30 min. The tubes were centrifuged at 10,000g for 15 min and the pellet was air dried.

RNase Protection Assay

Murine c-*fos* antisense RNA probes were generated from pc-fos(mouse)-3 [Miller et al., 1984]. The BsshII to XhoI fragment including the first exon was isolated and inserted into SmaI/XhoI cut Bluescript II KS(−). The resulting plasmid, pBKSfos, was linearized with BamHI and transcribed with recombinant T3 polymerase to produce a 717 base antisense probe which protected a 293 nucleotide fragment of RNA. Antisense probe for cyclophilin was generated from p1B15 [Danielson et al. 1988] linearized with NcoI and transcribed using SP6 RNA polymerase. The resulting probe was 249 nucleotides long and protected a 221 nucleotide fragment of cyclophilin RNA.

RNase protection assays were carried out essentially according to the method of Zinn et al. [1983]. The total cellular RNA from one confluent 60 mm plate of cells (approximately 20 to 25 μg) was dissolved in 30 μl hybridization buffer containing 5×10^5 cpm of an antisense probe, heated to 85°C for 10 min, and incubated at 45°C overnight. Three hundred microliters digestion mix was added and the samples were incubated at 30°C for 60 min. One hundred micrograms proteinase K was added and samples were incubated for an additional 15 min at 37°C. Following a phenol extraction, the RNA was mixed with 2 μg yeast tRNA carrier, precipitated in ethanol, redissolved in formamide gel loading buffer, and the protected bands were fractionated on 5% acrylamide/7 M urea gels at 20–25 W such that the surface temperature of the gel was a constant 60°C. Dried gels were exposed to X-ray film for 5 to 14 h, such that unstimulated levels of message could be visualized without overexposing the induced lanes. Densitometry was performed on a Zenieh Soft-Laser Scanning Densitometer (Bio Med Instruments, Fullerton, CA) scaled to optimize the range of detection between the baseline and induced bands. After normalization of the signals to the cyclophilin signal, the induction of c-*fos* was calculated based on unstimulated levels having been assigned a value of 1.0.

Materials

Plastic tissue culture ware was from Corning Glass Works (Corning, NY). Medium and antibiotics were from Grand Island Biologicals (Grand Island, NY) and fetal calf serum was from HyClone (Logan, UT). RNase A and RNase T_1 was from Boehringer Mannheim (Indianapolis, IN), T_3 polymerase was from Stratagene (La Jolla, CA), and SP6 polymerase, RNasin, and RNase-free DNase were from Promega (Madison, WI). Cholera toxin was purchased from Sigma Chemical Co. (St. Louis, MO), and TPA was from

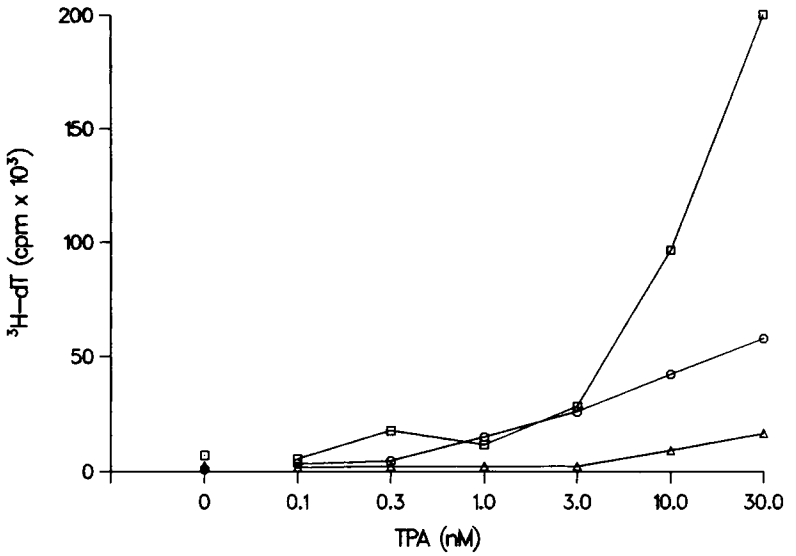

Fig. 1. Potentiation of TPA-induced DNA synthesis by cholera toxin or insulin. Quiescent, density-arrested cultures of A31T6 cells were treated with fresh medium containing 0.1% BSA supplemented with the indicated concentrations of TPA either alone (triangles), or together with 1 μg/ml cholera toxin (circles), or 1 μM insulin (squares). Following a 24 h incubation, the rate of thymidine incorporation was determined as described in Materials and Methods.

Chemicals for Cancer Research (Eden Prairie, MN).

RESULTS

Characterization of the Mitogenic Effects of Combinations of TPA, Insulin, and Cholera Toxin

Figure 1 shows the stimulation of DNA synthesis in A31T6 cells following the addition of various concentrations of TPA either alone or in the presence of 1 μg/ml cholera toxin or 1 μM insulin. Twenty-four hours after addition of purified mitogens to density-arrested cells, DNA synthesis was measured by the incorporation of ^{3}H-thymidine into acid-insoluble material during a 70 min pulse. The addition of fresh medium containing insulin alone caused approximately a 2-fold increase in thymidine incorporation, while addition of TPA alone caused a dose-dependent increase in DNA synthesis, with a maximal 6-fold increase measured following treatment with medium supplemented with 30 nM TPA. A dramatic synergism was seen when medium containing TPA plus insulin was added, with a 77-fold increase in thymidine incorporation seen in cells treated with 30 nM TPA and 1 μM insulin. This is similar to the synergistic effects of insulin and TPA in these cells seen earlier [Smyth et al., 1990]. The addition of only cholera toxin to quiescent cells did not stimulate DNA synthesis. A combination of cholera toxin and TPA also caused synergistic increases in thymidine incorporation, although the effects were not as dramatic as those seen when insulin and TPA were added together. A maximum 22-fold increase in DNA synthesis was seen at combinations of 30 nM TPA and 1 μg/ml cholera toxin.

Stimulation of DNA synthesis following the addition of 30 nM TPA or 1 μg/ml cholera toxin in the presence of increasing concentrations of insulin are shown in Figure 2. As seen earlier, the addition of cholera toxin alone caused no increase in thymidine incorporation, while TPA in the absence of insulin caused approximately a 4-fold increase in DNA synthesis compared to cells stimulated with medium alone. The co-addition of increasing concentrations of insulin with either TPA or cholera toxin caused a dose-dependent increase in thymidine incorporation, although at each concentration of insulin a greater effect was seen in cells treated with TPA than in those treated with cholera toxin. In the presence of 1 μM insulin, there was a 37-fold increase in thymidine incorporation in cells that were treated with TPA, while there was a 16-fold increase in incorporation in cells treated with cholera toxin.

The data in Figure 3 show that in the presence of 1 μM insulin, low concentrations of cholera toxin and TPA acted synergistically to

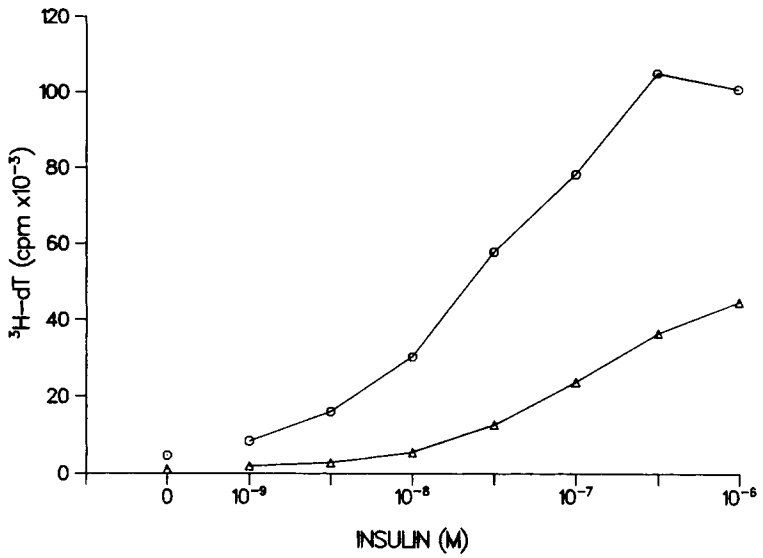

Fig. 2. Dose-dependent effects of insulin on the stimulation of DNA synthesis in A31T6 cells. Quiescent, density-arrested cultures of A31T6 cells were treated with medium containing 0.1% BSA, the indicated concentrations of insulin together with either 1 μg/ml cholera toxin (triangles), or 30 nM TPA (circles). The rate of thymidine incorporation was determined 24 h later as described in Materials and Methods.

stimulate thymidine incorporation in density-arrested A31T6 cells. Concentrations of TPA ranging from 0.1 to 3.0 nM caused less than a 3-fold increase in thymidine incorporation when added in the presence of 1 μM insulin. Addition of either 30 ng/ml or 100 ng/ml cholera toxin together with insulin caused a 5- and 11-fold increase in thymidine incorporation, respectively. Co-addition of cholera toxin and TPA in the presence of insulin caused a dramatic synergistic effect. For example, treatment of cells with 3.0 nM TPA and 30 ng/ml cholera toxin caused a 25-fold increase in thymidine incorporation, as compared to the 6-fold induction that would have been predicted if the effects were additive. Similar synergistic effects were seen when TPA and forskolin, another activator of adenylate cyclase, were added together with insulin (data not shown).

The synergistic stimulation of DNA synthesis by TPA and cholera toxin, measured by increases in thymidine incorporation, was reflected by an increase in the percentage of cells moving out of G_1, measured by flow cytometry (Fig. 4). Less than 1% of the cells in cultures treated either with medium supplemented with 1 μM insulin alone (panel A), 3 nM TPA plus insulin (panel B), or 10 ng/ml cholera toxin plus insulin (panel C) were in S phase. In contrast, when cultures were treated with a combination of TPA, cholera toxin, and insulin, over 15% of the cells had been stimulated to enter S (panel D).

Effects of TPA and Cholera Toxin on the Induction of c-*fos* in A31T6 Cells

To correlate the effects of mitogens on DNA synthesis with increases in the expression of known growth-regulated genes, levels of the induction of the immediate early gene c-*fos* were measured. An autoradiograph of protected c-*fos* bands is shown in Figure 5, while densitometric quantitation normalized to cyclophillin is shown in Figure 6. When quiescent cells were treated with medium supplemented with 30 nM TPA the level of c-*fos* mRNA rose to a level of 7-fold over control values by 30 min, and returned to baseline by 90 min. In contrast, when 10 μg/ml cycloheximide was added together with TPA, c-*fos* mRNA continued to increase over the time course of the experiment, reaching levels of 15-fold over control by 90 min. This effect of TPA either alone or in the presence of inhibitors of protein synthesis on *fos* RNA levels in A31T6 cells is similar to that seen in several other murine fibroblasts [reviewed by Ransone and Verma, 1990].

The effects of TPA and cholera toxin on c-*fos* mRNA levels are shown in Figure 7. The agents were added in the presence of cycloheximide to prevent a decrease in message levels due to instability of the RNA. The addition of TPA

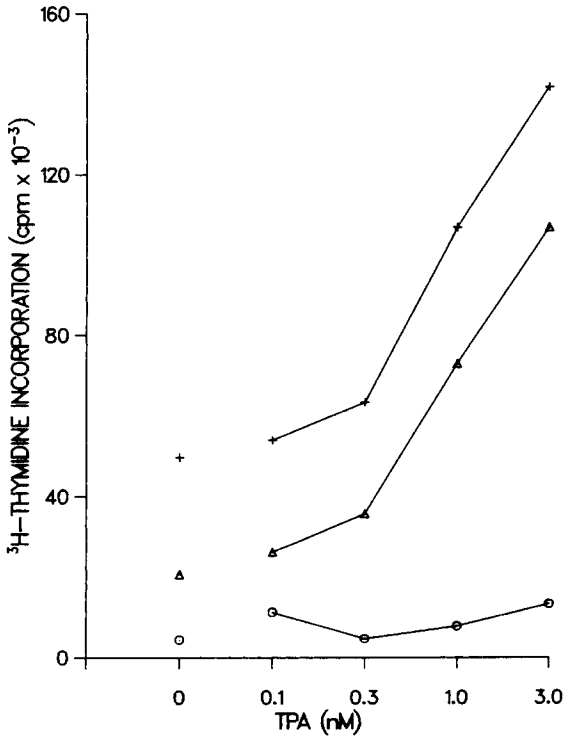

Fig. 3. Potentiation of TPA-induced mitogenesis by cholera toxin. Density-arrested cultures of A31T6 cells were treated with medium containing 0.1% BSA, 1 μM insulin, the indicated concentrations of TPA either alone (circles), or together with 30 ng/ml (triangles) or 100 ng/ml (plus signs) cholera toxin. Following a further 24 h incubation, the rate of thymidine incorporation was determined.

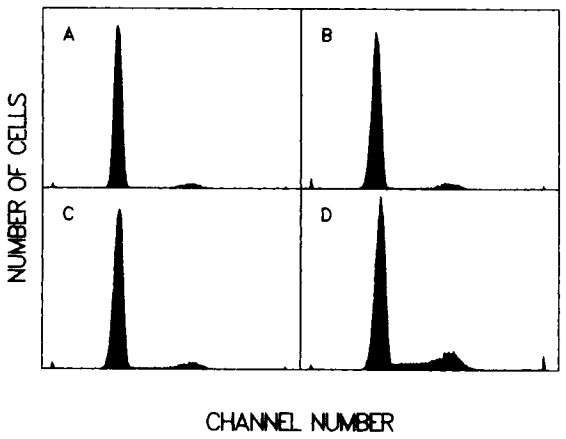

Fig. 4. Cytometric analysis of stimulation of DNA synthesis in A31T6 cells. Quiescent density-arrested cultures were stimulated for 24 h either with medium containing 0.1% BSA together with 1 μM insulin alone (**panel A**), insulin plus 3 nM TPA (**panel B**), insulin plus 10 ng/ml cholera toxin (**panel C**), or a combination of insulin, TPA, and cholera toxin (**panel D**). Cells were then removed from the plate with trypsin, fixed in ethanol, and their DNA content was analyzed with a flow cytometer. The graphs represent digitized fluorescence representing DNA content on the abscissa, and the number of cells using a relative scale on the ordinate.

alone caused a dose-dependent increase in *fos* mRNA, with approximately a 15-fold increase seen in the presence of 10 nM TPA. Addition of 10 ng/ml cholera toxin alone caused only a 3-fold increase in message. At each concentration of TPA, co-addition of cholera toxin cause a synergistic induction of c-*fos* message, with a combination of 10 ng/ml cholera toxin and 10 nM TPA producing approximately a 40-fold increase in message levels.

DISCUSSION

In this paper we have described the interactions between discrete classes of mitogens that stimulate cell cycle traverse in A31T6 cells which, unlike the parent BALB/c-3T3 cells, can differentiate into adipocytes. As is typically the case with density-arrested murine fibroblasts, the addition of medium supplemented with only a single mitogen was unable to cause large numbers of cells to traverse G_0/G_1 and initiate DNA synthesis. Addition of combinations of either insulin and cholera toxin or TPA and cholera toxin stimulated a moderate amount of growth. A combination of TPA and insulin caused a marked stimulation of DNA synthesis in density-arrested cells, as we observed earlier [Smyth et al., 1990]. Cholera toxin further potentiated the stimulation of growth seen in the presence of TPA and insulin. These results are similar to the potentiation by cholera toxin of PDGF-induced mitogenesis in the presence of PPP previously reported in the parent BALB/c-3T3 cells [Wharton et al., 1982].

There has not previously been a report of the stimulation of growth in the parent 3T3 line with TPA and cholera toxin. In addition, the results of stimulation with a combination of cholera toxin and insulin are different than reported in BALB/c-3T3 cells where it was found that cholera toxin was not mitogenic unless either PDGF [Wharton et al., 1982], or EGF [Olashaw et al., 1984] was also present. The cellular mechanisms that underlie changes in sensitivity to mitogenic stimulation are not known, although descriptions of such processes would certainly provide insights into critical regulatory steps. In this case, it would be very interesting to determine if alterations in sensitivity to the mitogenic effects of cholera toxin are related to the increased ability of A31T6 cells to

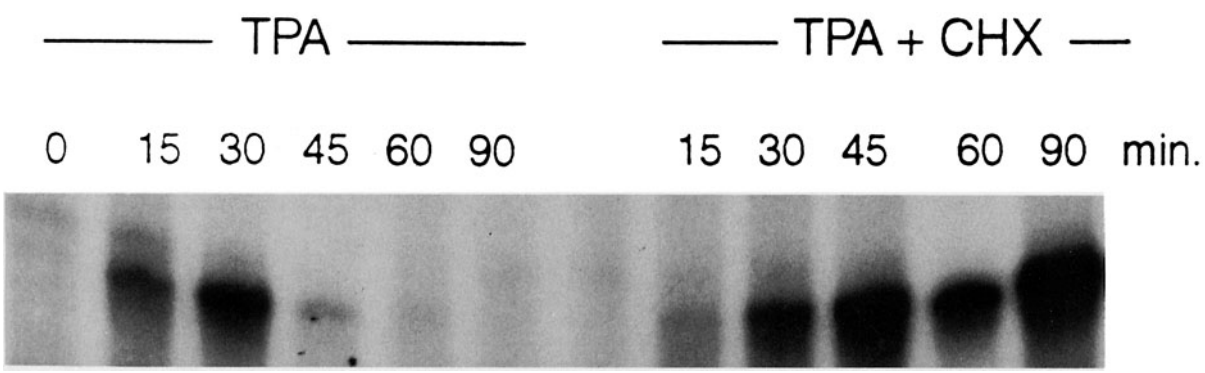

Fig. 5. Autoradiograph of c-*fos* protected bands. RNA was extracted from cells treated with either TPA or TPA plus cycloheximide for the indicated times, and RNase protection assays were performed as described in Materials and Methods.

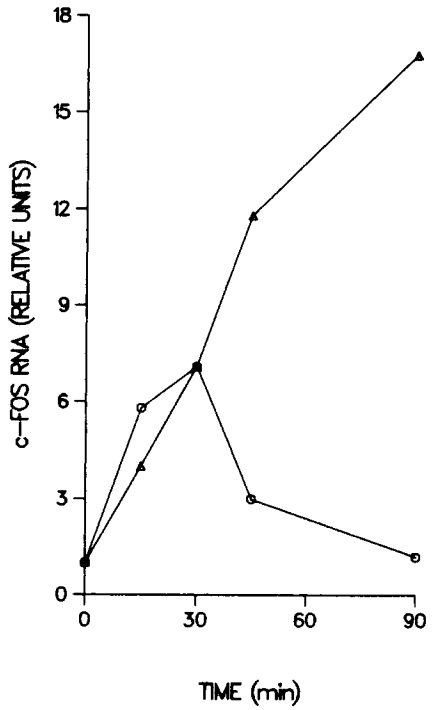

Fig. 6. TPA-induction of c-*fos* mRNA in A31T6 cells. Quiescent cultures were treated with medium containing 0.1% BSA and 30 nM TPA either alone (circles) or together with 10 μg/ml cycloheximide (triangles). At the indicated times, RNA was extracted, and c-*fos* mRNA was quantified using an RNase protection assay. The ordinate represents normalized c-*fos* RNA levels determined by a densitometer with the unstimulated cells assigned a value of 1.0.

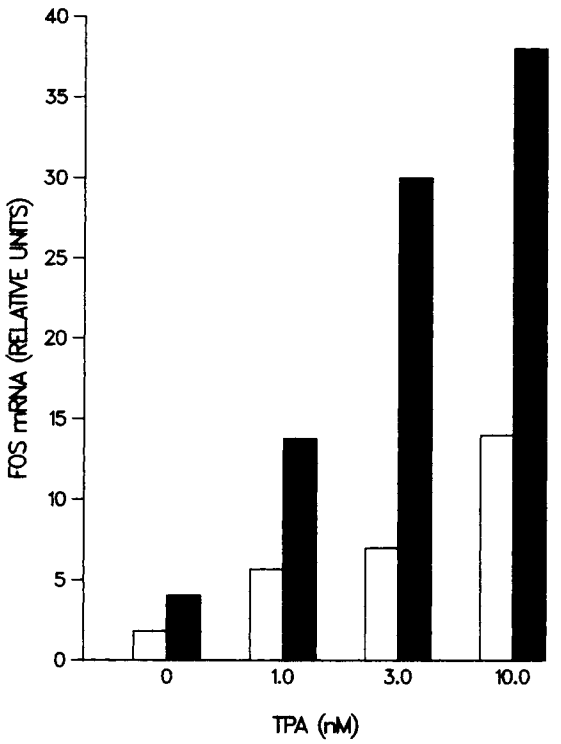

Fig. 7. Synergistic stimulation of c-*fos* mRNA levels by a combination of TPA and cholera toxin. Quiescent cultures were treated for 90 min with medium containing 0.1% BSA, 10 μg/ml cycloheximide, and the indicated concentration of TPA either alone (open squares) or together with 10 ng/ml cholera toxin (solid squares). The results are presented as described in the legend to Figure 6.

differentiate into adipocytes and, simultaneously, become mitogenically non-responsive. Independent of mechanistic questions, however, these results confirm that the actions of specific mitogens need to be determined in individual cell lines, even when they were obtained by subcloning from a very well characterized parent cell.

Expression of the immediate early gene, c-*fos*, was also synergistically induced by a combination of TPA and cholera toxin. Since the synergism was observed in the presence of cycloheximide, thus preventing message turnover [Wilson and Treisman, 1988], it is valid to assume that

the increase in transcript levels represents an increase in transcription rate. The molecular basis for this increase in transcription is, however, presently unknown. It is possible that in the presence of elevated concentrations of cyclic AMP, a given concentration of TPA causes an enhanced stimulation of protein kinase C. This would be analogous to cholera toxin allowing an enhanced stimulation of inositol phosphate formation by EGF [Olashaw and Pledger, 1988]. It is also possible that upstream regulatory regions in the c-*fos* gene are modulated synergistically by separate signals generated by cholera toxin and TPA. The c-*fos* enhancer region contains elements that are regulated both by TPA [Fisch et al., 1987] and by cyclic AMP [Berkowitz et al., 1989]. The requirements for involvement of specific regions of the promoter in the induction of c-*fos* RNA is, however, critically dependent on cell type [Siegfried and Ziff, 1989]. We are presently conducting experiments to identify the nature of the mechanisms that are responsible for the synergistic induction.

Ran et al. [1986] reported that while EGF and cholera toxin interacted to induce c-*fos* mRNA in BALB/c-3T3 cells, the addition of cholera toxin did not potentiate a TPA-mediated induction. These results, however, are not incompatible with the data presented in this paper. Unlike the A31T6 cells, in which cholera toxin and TPA act synergistically to induce mitogenesis and c-*fos* expression, cholera toxin does not potentiate TPA-induced mitogenesis in the 3T3 cells used by Ran et al. (J. Campisi, personal communication). Therefore, the differences in the regulation of c-*fos* expression in the two cell lines reflect differences in mitogenic regulation.

Stimulation of fibroblasts with PDGF results in the activation of multiple second messenger systems either independently or interactively [Williams, 1989], including an activation of tyrosine kinase activity [Nishimura et al., 1982] and a stimulation of phosphoinositol turnover with a subsequent increase in protein kinase C activity [Berridge et al., 1984]. Activation of various classes of PDGF receptors by different isoforms of PDGF has been shown to mediate increases in c-*fos* expression by independent combinations of these second messenger systems [Salhany et al., 1992]. Given this complexity of cellular responses, it is interesting to compare activation of second messenger systems with mitogenic responsiveness. Addition of medium supplemented with PDGF and insulin, conditions under which both the PDGF-induced tyrosine kinase and protein kinase C pathways are activated, is not mitogenic in either the parent BALB/c-3T3 cells [Leof et al., 1982] or in A31T6 cells [Wharton and Smyth, unpublished observation). However, addition of medium containing only TPA and insulin, resulting in the activation of the protein kinase C pathway independent of the receptor tyrosine kinase activity, results in a marked stimulation of proliferation. It is possible that the protein kinase C pathway activated by PDGF represents the positive arm of the mitogenic cascade, while tyrosine kinase induced events are negative modulators of proliferation. This is an interesting proposal in light of the recent observation that 2-amino purine, a tyrosine kinase inhibitor, releases cells from a number of growth arrest states [Andreassen and Margolis, 1992].

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