Mitogenic signaling pathways of growth factors can be distinguished by the involvement of pertussis toxin-sensitive guanosine triphosphate-binding protein and of protein kinase C

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We have examined the possible involvements of pertussis toxin (PT)-sensitive guanosine triphosphate (GTP)-binding protein (G_p) and protein kinase C (PKC) in the mitogenic signaling pathways of various growth factors by the use of PT-pretreated and/or 12-O-tetradecanoyl phorbol-13-acetate (TPA)-pretreated mouse fibroblasts. Effects of PT pretreatment (inactivation of PT-sensitive G_p) and TPA pretreatment (depletion of PKC) on mitogen-induced DNA synthesis varied significantly and systematically in response to growth factors: mitogenic responses of cells to thrombin, bombesin, and bradykinin were almost completely abolished both in PT- and TPA-pretreated cells; responses to epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and vanadate were reduced to ~50% both in PT- and TPA-pretreated cells compared with native cells; response to basic fibroblast growth factor (bFGF) was not affected in PT-pretreated cells but was inhibited to some extent in TPA-pretreated cells. Thus, growth factors examined have been classified into three groups with regard to the involvements of PT-sensitive G_p and PKC in their signal transduction pathways. Binding of each growth factor to its receptor was not affected significantly by pretreatment of cells with PT or TPA. Inhibitory effects of PT and TPA

pretreatment on each mitogen-induced DNA synthesis were not additive, suggesting that the functions of PT-sensitive G_p and PKC lie on an identical signal transduction pathway. Although all three groups of mitogens activated PKC, signaling of each growth factor depends to a varying extent on the function of PKC. Our results indicate that a single peptide growth factor such as EGF, PDGF, or bFGF acts through multiple signaling pathways to induce cell proliferation.

Introduction

A variety of purified growth factors stimulates quiescent cells to reenter the cell cycle. Each mitogen binds to specific high-affinity cell-surface receptors and thereby initiates a sequence of events that culminates in DNA synthesis and mitosis. The links between occupation of the cell-surface receptors and nuclear events remain poorly understood; identification of transmembrane signaling pathways and the second messenger molecules that trigger DNA synthesis is of central importance in understanding the mode of action of growth factors.

So far, two major signal transduction pathways have been shown to be involved in cell growth stimulation: 1) protein-tyrosine phosphorylation, and 2) breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG). The former pathway plays an important role in the mitogenic action of growth factors such as epidermal growth factor (EGF), insulin-like growth factor I, and platelet-derived growth factor (PDGF); receptor molecules for these mitogens contain an inherent, growth factor-dependent protein tyrosine-kinase activity (Hunter and Cooper, 1985). On the other hand, mitogenic signal transduction of bombesin, bradykinin, and thrombin seems to depend mainly on the latter pathway; those growth factors activate phospholipase C (PLC) to hydrolyze PIP_2 and the resultant IP_3 mobilizes Ca^{2+} from the endoplasmic reticulum, whereas DG

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stimulates protein kinase C (PKC) (Nishizuka, 1984; Berridge and Irvine, 1989).

It has been suggested that breakdown of PIP_2 by PLC is mediated by one of a family of intracellular signal transducers, guanosine triphosphate (GTP)-binding proteins (Gilman, 1987). The GTP-binding proteins that activate PLC have been generically termed G_p ; one of them can be inactivated by the toxin of *Bordetella pertussis* (Ui, 1984; Nakamura and Ui, 1985). Thus, signaling pathways of growth factors may be distinguishable by the involvement of pertussis toxin (PT)-sensitive G_p . In fact, PT recently has been reported to inhibit the actions only of those mitogens whose main signal transduction pathway involves phosphoinositide hydrolysis (thrombin, bombesin, etc.), not those of another group of mitogens whose principal signal transduction pathway involves protein tyrosine phosphorylation (EGF, PDGF, etc.) (Letterio *et al.*, 1986; Chambard *et al.*, 1987). In this respect, however, there are several reports of PT inhibition of signaling by tyrosine kinase-containing receptor (Johnson *et al.*, 1986; Espinal, 1987; Church and Buick, 1988). PDGF and EGF can, in some cell systems, induce rapid activation of PLC (Berridge *et al.*, 1984; Pike and Eakes, 1987) and of PKC (Rozengurt *et al.*, 1983; Kazlauskas and Cooper, 1988). Matuoka *et al.* (1988) recently reported that microinjection of monoclonal antibody against PIP_2 abolished DNA synthesis induced not only by bombesin but also by PDGF, indicating that stimulated phosphoinositide hydrolysis (aside from whether or not PT-sensitive G_p is coupled) is a necessary event for DNA synthesis induced by PDGF. Thus, it is still obscure whether or not receptor tyrosine kinase-activating growth factors involve PT-sensitive G_p -mediated phosphoinositide hydrolysis, and the resultant PKC activation, in their mitogenic signaling pathways; the intracellular signal transduction pathways of those mitogens seem more complex than have been proposed previously (Chambard *et al.*, 1987).

Here we have examined in detail the possible involvement of PT-sensitive G_p —and further, of PKC—in the mitogenic signaling pathways of various growth factors by the use of PT-pretreated and/or TPA-pretreated mouse fibroblast cell lines (Swiss 3T3, Balb 3T3, and NR 6 cells). Inhibitory effects of the inactivation of PT-sensitive G_p and the depletion of PKC on mitogen-induced DNA synthesis have varied significantly and systematically in response to growth factors. Our results suggest that receptor tyrosine kinase-activating growth factors such as EGF

and PDGF transduce their mitogenic signals both by PT-sensitive G_p -/PKC-dependent and -independent pathways.

Results

Adenosine diphosphate (ADP) ribosylation of a $M_r = 40\,000$ protein by PT

When Swiss 3T3 cell membranes were incubated with $[^{32}\text{P}]\text{NAD}^+$ in the presence of activated PT, a protein with an apparent molecular weight of $\sim 40\,000$ was ADP ribosylated (Figure 1A); this protein may represent the α subunit of GTP-binding proteins (Ui, 1984). This ribosylation was inhibited in a dose-dependent fashion by preincubation of intact cells with PT, and $5\ \mu\text{g}/\text{ml}$ of PT was no longer able to catalyze incorporation of radioactivity into the $M_r = 40\,000$ protein in vitro after treatment of cells in vivo with toxin at a concentration of $>25\ \text{ng}/\text{ml}$. Essentially the same results were obtained with the membrane preparations from Balb/c and NR 6 cells (Figure 1, B and C).

Figure 1. ADP ribosylation of a $M_r = 40\,000$ protein by PT. Quiescent Swiss 3T3 cells (A), Balb 3T3 cells (B), or NR 6 cells (C) were treated for 4 h with the indicated concentrations of PT. Membrane-rich fractions ($50\ \mu\text{g}$ protein) were incubated for 20 min at 37°C with $5\ \mu\text{g}/\text{ml}$ of activated PT and $1\ \mu\text{Ci}$ of $[^{32}\text{P}]\text{NAD}^+$ ($10\ \mu\text{M}$). The samples were then analyzed by SDS-PAGE and autoradiography as described in Materials and methods. Data are representative of two experiments that gave essentially the same results.

Figure 2. Down-regulation of PKC measured by Western blotting. Growth-arrested Swiss 3T3 cells (A), Balb 3T3 cells (B), or NR 6 cells (C) were treated with 200 ng/ml of TPA for 0 h (lane 1), 6 h (2), 12 h (3), 24 h (4), and 36 h (5). Total cell lysates (50 μ g protein) were resolved by SDS-PAGE, and PKC was visualized by Western blot analysis as described in Materials and methods. Lane St: purified bovine brain PKC. Arrow indicates immunoreactive PKC. Similar results were obtained in three independent experiments.

Down-regulation of protein kinase C

Lysates were prepared from Swiss 3T3, Balb 3T3 and NR 6 cells after treatment with 200 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA) for various lengths of time. Portions (50 μ g) of cell lysates were resolved on sodium dodecyl sulfate (SDS)/10%-polyacrylamide gels, transferred to nitrocellulose membrane, and probed with anti-PKC antibody. In each cell line, the antibody reacted primarily with a protein with apparent molecular weight of ~ 73 000. The $M_r = 73$ 000 protein comigrated with purified bovine brain PKC (Figure 2C, lane St.). By these criteria this protein was identified as PKC. In Swiss 3T3 cells, a marked decrease in the level of immunologically detectable PKC was apparent after TPA treatment for 6 h, and the decrease continued thereafter, becoming undetectable after 12–36 h. In Balb 3T3 and NR 6 cells, TPA treatment also caused the complete down-regulation of PKC (Figure 2). In NR 6 cells, however, after complete loss of PKC after TPA treatment for 12–24 h, it appeared to increase

gradually and became detectable again after 36 h.

Down-regulation of PKC in TPA-pretreated cells was also analyzed by the direct determination of total cellular PKC activity after extraction with NP-40 and the use of H1 histone as a substrate. The time courses of PKC down-regulation in the three cell lines differed slightly from each other, but the virtually complete loss of PKC activity was observed after 24–36 h treatment of Swiss 3T3 and Balb 3T3 cells, and 12–24 h treatment of NR 6 cells, with 200 ng/ml of TPA (Figure 3). As appears in Figure 3C, a substantially faster recovery of PKC activity was observed in NR 6 cells than in Swiss 3T3 and Balb 3T3 cells, consistent with the results obtained from the Western blot analysis of immunologically detectable PKC as described above (Figure 2C). Essentially the same results were obtained in three separate experiments.

Responses of Swiss 3T3, Balb 3T3, and NR 6 cells to stimulation with various mitogens

The addition of various mitogens to quiescent Swiss 3T3, Balb 3T3, and NR 6 cells led to DNA replication after a lag period of ~ 10 h, and the rate of DNA synthesis reached a maximal level 18–24 h after stimulation (data not shown; Kohno, 1985). Mitogenic response of cells to each growth-promoting agent was dose depen-

Figure 3. Down-regulation of PKC as measured by its activity. Quiescent Swiss 3T3 cells (A), Balb 3T3 cells (B), or NR 6 cells (C) were treated with 200 ng/ml of TPA for the indicated times. Total cellular PKC activity was measured after extracting it with NP-40 and using H1 histone as a substrate as described in Materials and methods. Percentages were calculated by comparison with corresponding untreated controls. One hundred percent PKC activity = 395 mU/mg protein (Swiss 3T3 cells), 800 mU/mg protein (Balb 3T3 cells), or 370 mU/mg protein (NR 6 cells). Data are the mean of duplicate determinations in a single experiment. Results shown are representative of three similar experiments.

Table 1. Inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis in Swiss 3T3 cells

Treatment	Native cells	PT-pretreated cells		TPA-pretreated cells		TPA/PT-pretreated cells	
	Labeled nuclei (%)	Labeled nuclei (%)	% of native cells	Labeled nuclei (%)	% of native cells	Labeled nuclei (%)	% of native cells
Control	0.3	0.1		0.1		0.1	
TPA (25 ng/ml)	9.7	9.2	(96.8)	0.1	(0)	0.1	(0)
EGF (10 ng/ml)	14.2	7.8	(55.4)	6.7	(47.5)	6.7	(47.5)
bFGF (5 ng/ml)	33.6	31.4	(94.3)	29.2	(87.4)	27.0	(80.8)
PDGF (10 ng/ml)	32.8	16.5	(50.5)	17.3	(52.9)	15.7	(48.0)
Vanadate (20 μ M)	12.3	3.0	(24.1)	3.6	(29.8)	2.8	(22.5)
Thrombin (1 U/ml)	9.7	0.3	(2.1)	0.5	(4.3)	0.3	(2.1)
Bombesin (20 nM)	11.2	0.9	(7.3)	0.6	(4.6)	0.7	(5.5)
Bradykinin (1 μ M)	11.7	1.2	(9.6)	0.7	(5.3)	0.6	(4.4)

Cultures of quiescent Swiss 3T3 cells, without (native) or with PT, TPA, or TPA and PT pretreatments, were exposed to mitogens, and BrdU-labeled nuclei were determined in duplicate cultures as described in Materials and methods. The percentage of labeled nuclei was determined in a total of >800 nuclei. The inhibitory effect of PT or/and TPA pretreatment on growth factor-induced DNA synthesis is indicated as a proportional percentage of each mitogen-stimulated DNA synthesis in PT- and TPA-pretreated cells to that in native cells (% of native cells). Results representative of 8–10 separate experiments are presented.

dent; those concentrations shown in Table 1 were maximally effective. Although responses of cells to each mitogen varied to some extent from one experiment to another, PDGF and basic fibroblast growth factor (bFGF) were always the most potent mitogens for all three fibroblast cell lines, stimulating 30–50% of growth-arrested cells to reinitiate DNA synthesis. EGF was also a good mitogen for Swiss 3T3 and Balb 3T3 cells but not for NR 6 cells, which lack EGF receptors (Pruss and Herschman, 1977). Treatment of Swiss 3T3 cells with either thrombin, bombesin, bradykinin, TPA, or vanadate (a potent inhibitor of phosphotyrosyl phosphatase) consistently resulted in a significant stimulation

of DNA synthesis. Thrombin, TPA, and vanadate were also mitogenic for Balb 3T3 and NR 6 cells; responses of these cells to bombesin and bradykinin, however, were weak and rather inconsistent, and thus we did not analyze those further (Tables 1–3).

Inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis

Effects of inactivation of PT-sensitive G_p on growth factor-stimulated DNA synthesis were analyzed after treating cells with 25 ng/ml of toxin for 4 h; under such conditions, PT-induced

Table 2. Inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis in Balb 3T3 cells

Treatment	Native cells	PT-pretreated cells		TPA-pretreated cells		TPA/PT-pretreated cells	
	Labeled nuclei (%)	Labeled nuclei (%)	% of native cells	Labeled nuclei (%)	% of native cells	Labeled nuclei (%)	% of native cells
Control	0.1	0.1		0.1		0.1	
TPA	11.7	9.8	(83.6)	0.1	(0)	0.1	(0)
EGF	20.6	11.2	(54.1)	9.2	(44.4)	8.8	(42.4)
bFGF	45.5	44.4	(97.6)	36.3	(79.7)	36.6	(80.4)
PDGF	31.3	16.0	(51.0)	13.1	(41.7)	13.4	(42.6)
Vanadate	8.0	2.0	(24.1)	2.3	(27.8)	1.7	(20.3)
Thrombin	4.7	0.1	(0)	0.2	(2.8)	0.1	(0)

The mitogenic response of quiescent Balb 3T3 cells, without (native cells) or with PT, TPA, or TPA and PT pretreatments, to various growth factors was analyzed as described in the legend of Table 1. Results representative of 8–11 separate experiments are presented.

Table 3. Inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis in NR 6 cells

Treatment	Native cells	PT-pretreated cells		TPA-pretreated cells		TPA/PT-pretreated cells	
	Labeled nuclei (%)	Labeled nuclei (%)	% of native cells	Labeled nuclei (%)	% of native cells	Labeled nuclei (%)	% of native cells
Control	0.2	0.1		0.3		0.2	
TPA	4.8	4.0	(84.8)	0.1	(0)	0.1	(0)
EGF	0.2	0.1		0.2		0.2	
bFGF	49.6	48.2	(97.4)	42.2	(84.8)	41.8	(84.2)
PDGF	35.3	17.4	(49.3)	18.6	(52.1)	16.5	(46.4)
Vanadate	14.6	4.0	(27.1)	6.5	(43.1)	3.4	(22.2)
Thrombin	8.4	0.2	(1.2)	0.1	(0)	0.1	(0)

The mitogenic response of quiescent NR 6 cells, without (native) or with PT, TPA, or TPA and PT pretreatments, to various growth factors was analyzed as described in the legend of Table 1. Results representative of 7–10 separate experiments are presented.

ADP ribosylation of the α -subunit of G_p in each cell was completed as described above (Figure 1). Effects of PT pretreatment on each mitogen-stimulated DNA synthesis were similar in all fibroblast cell lines (Swiss 3T3, Balb 3T3, and NR 6): responses to bFGF and TPA were not affected significantly; responses to EGF, PDGF, and vanadate were reduced to ~40–50% compared with native cells; mitogenic responses to thrombin, bombesin, and bradykinin were almost completely inhibited. Thus, growth factors were classified into three groups by the involvement of PT-sensitive G_p in their mitogenic signaling pathways.

Effects of PKC depletion on growth factor-stimulated DNA synthesis were analyzed after treating cells with 200 ng/ml of TPA for 24 h (Swiss 3T3 and Balb 3T3 cells) or 16 h (NR 6 cells). Under these conditions, down-regulation of PKC was completed in all three cell lines. To obtain the maximal response, stimulation of cells by growth factors continuously for ~12 h is required (data not shown; Westermarck and Heldin, 1985). The PKC level in TPA-pretreated cells remained undetectable or at most less than a few percent that of the control cells throughout the periods shown in Figures 2 and 3. The effects of a complete loss of PKC activity on each mitogen-stimulated DNA synthesis were identical in all three cell lines and separated into three types, similar to the effects of the PT pretreatments: response to bFGF was inhibited to a small extent; responses to EGF, PDGF, and vanadate were reduced to ~50% compared with native cells; mitogenic responses to thrombin, bombesin, and bradykinin were almost completely blocked. TPA-induced DNA synthesis was totally abolished in TPA-pretreated Swiss 3T3, Balb 3T3, and NR 6 cells,

confirming that PKC was totally depleted in those cells (because the receptor for TPA is believed to be PKC [Nishizuka, 1984]).

Thus, the inhibitory effects of PT and TPA pretreatment on each mitogen-stimulated DNA synthesis were very similar except that 1) bFGF-stimulated DNA synthesis was inhibited to some extent in TPA-pretreated cells but not in PT-pretreated cells, and 2) the mitogenic response to vanadate was significantly higher in TPA-pretreated cells than in PT-pretreated cells. It has been suggested that the function of PT-sensitive G_p is, through coupling to PLC, linked to the activation of PKC (Nishizuka, 1984; Nakamura and Ui, 1985). To examine such a probable link between PT-sensitive G_p and PKC, we analyzed whether or not the inhibitory effects of PT pretreatment and TPA pretreatment on each mitogen-induced DNA synthesis were additive. For this analysis growth-arrested cells were treated with 200 ng/ml of TPA for 24 h (Swiss 3T3 and Balb 3T3 cells) or 16 h (NR 6 cells), and 25 ng/ml of PT was also added during the final 4-h period of TPA pretreatment. Mitogenic responses of the cells to each growth factor were then determined. Mitogen-stimulated DNA synthesis levels in cells pretreated with both PT and TPA were about the same as in cells pretreated with either PT or TPA in all cases. Thus, the inhibitory effects of PT and TPA pretreatment were not additive, suggesting that the functions of PT-sensitive G_p and PKC lie on an identical signal transduction pathway.

Mitogenic responses of cells to each growth factor varied to some extent from one experiment to another. Nevertheless, the inhibitory effects of PT and TPA pretreatment on each mitogen-induced DNA synthesis were essentially identical in numerous experiments; the re-

sults of typical experiments in each cell system are shown in Tables 1–3. Results are also summarized in Figures 4–6; means $\pm$ SE of 7–11 different experiments, each determination with >800 nuclei using two coverslips/experimental condition, are shown.

Effect of PT and TPA pretreatment on the binding of growth factors

To examine the possibility that suppressed mitogenic responses of PT- and TPA-pretreated cells to growth factors are due to the inhibition of binding of ligands to their receptors, we measured the binding of ^{125}I -labeled growth factors to PT- or TPA-pretreated Swiss 3T3 cells. EGF, bFGF, and bradykinin were chosen as the representatives of growth factors classified into three groups as described above. As shown in Figure 7, specific binding profiles of EGF and bFGF were similar among cells with or without PT or TPA treatment, although those of bFGF did not follow the typical saturation manner. Specific binding of bradykinin was partially inhibited in TPA-pretreated cells, but not in PT-pretreated cells; both treatments almost completely abolished the mitogenic response of cells to bradykinin (Figure 4). Thus, decreased mitogenic responses of cells to EGF and bradykinin by PT and TPA pretreatment were unlikely to be the results of inhibition of binding of these ligands to their receptors, but indeed due to the inactivation of PT-sensitive G_p and to the depletion of PKC. Although TPA treatment has been reported to cause the rapid desensitization of EGF receptors in many cell types—presumably by decreasing the binding affinity of receptor to the ligand (Brown *et al.*, 1979)—the above results clearly showed that specific EGF receptor binding returned to the initial levels after 24 h of TPA pretreatment.

Effect of PT pretreatment on mitogen-stimulated PKC activity

The functional link between PT-sensitive G_p and PKC was examined more directly by analysis of the effect of PT pretreatment on the activation of PKC, as measured by increased phosphorylation of the $M_r = 80\,000$ acidic cellular protein, which is widely believed to be a major and specific *in vivo* substrate for PKC in a variety of cells, including fibroblasts (Rozengurt *et al.*, 1983; Rodriguez-Pena and Rozengurt, 1986). Thus, growth-arrested Swiss 3T3 cells were labeled with ^{32}P orthophosphate, stimulated for 10 min with mitogens, then analyzed for total cellular phosphoproteins by two-dimensional

Figure 4. Summary of the inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis in Swiss 3T3 cells. Inhibitory effects of PT pretreatment (□), TPA pretreatment (▨), and TPA and PT pretreatments (■) on each mitogen-stimulated DNA synthesis in Swiss 3T3 cells, indicated as the percentages of native cells (Table 1), are shown. Although the mitogenic response of Swiss 3T3 cells to each growth factor varied to some extent from one experiment to another, inhibitory effects of PT and/or TPA pretreatment were essentially identical. Variations in response of native cells to each mitogen in the experiments are shown with the numbers of the experiments in parentheses: TPA, 9.3–14.6% (10); EGF, 10.1–15.8% (8); bFGF, 20.6–35.1% (10); PDGF, 31.4–42.9% (10); vanadate 9.2–22.0% (8); thrombin, 4.2–9.7% (8); bombesin, 9.3–20.8% (8); bradykinin, 5.0–13.6% (8). Results represent the average $\pm$ SE of 8–10 separate experiments.

gel electrophoresis. As shown in Figure 8, markedly increased phosphorylation of $M_r = 80\,000$ protein was observed in bombesin-, PDGF-, and bFGF-stimulated cells. EGF also stimulated the phosphorylation of $M_r = 80\,000$ protein, although to a lesser extent compared with PDGF (data not shown; M. Kohno, Y. Chantani, N. Nishizawa, E. Tanaka, and H. Nomoto, manuscript in preparation). PT pretreatment almost completely inhibited the bombesin-stimulated increase of $M_r = 80\,000$ protein phosphorylation as well as of DNA synthesis, indicating an absolute link between the function of PT-sensitive G_p and PKC in the mitogenic signaling pathway of bombesin. PDGF-stimulated phosphorylation of $M_r = 80\,000$ protein apparently decreased in PT-pretreated cells. The relative intensity of PDGF-stimulated $M_r = 80\,000$ protein phosphorylation in PT-pretreated cells compared with native cells, quantified by laser densitometry of the autoradio-

Figure 5. Summary of the inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis in Balb 3T3 cells. Inhibitory effects of PT pretreatment (□), TPA pretreatment (▨), and TPA and PT pretreatments (■) on each mitogen-stimulated DNA synthesis in Balb 3T3 cells are summarized as in Figure 4. Variations in responses of native cells to each mitogen in the experiments and the numbers of them are as follows: TPA, 7.4–16.3% (11); EGF, 11.2–23.8% (8); bFGF, 41.5–55.2% (11); PDGF, 24.0–49.5% (10); vanadate, 5.3–10.0% (8); thrombin, 3.8–5.5% (8). Results represent the average $\pm$ SE of 8–11 separate experiments.

graphs, was 50%–60% in three separate experiments. PT pretreatment did not significantly affect the bFGF-stimulated phosphorylation of $M_r = 80\ 000$ protein as it did not affect the bFGF-induced DNA synthesis. The above results imply multiple pathways of mitogen-induced PKC activation; bFGF (totally) and PDGF (partly) activated PKC by PT-sensitive G_p -independent mechanism(s).

Effect of PT pretreatment on mitogen-induced phosphoinositide hydrolysis

As described above, a variety of growth factors induced rapid activation of PKC, implying that those mitogens stimulated the formation of DG in the cells. It has long been known that polyphosphoinositide is an important source of DG; breakdown of PIP_2 by PLC results in the formation of IP_3 and DG (Berridge and Irvine, 1989), and involvement of PT-sensitive G_p in the mitogen activation of PLC has also been sug-

gested (Nakamura and Ui, 1985). To get insight into the molecular mechanism of DG formation, we analyzed the mitogen-induced phosphoinositide hydrolysis by determining the IP_3 levels of mitogen-treated Swiss 3T3 cells with or without PT pretreatment. Because the time courses of IP_3 accumulation differed according to growth factor (Y. Okano, M. Kohno and Y. Nozawa, unpublished results), IP_3 levels were determined in Swiss 3T3 cells stimulated with each mitogen for 10 and 90 s. As shown in Figure 9, bombesin and bradykinin caused the rapid accumulation of IP_3 , which was completely inhibited by pretreatment of cells with PT. PDGF also stimulated IP_3 production with, however, a rather delayed time course, which was not inhibited significantly in PT-pretreated cells. Similar results have recently been reported (Huang and Ives,

Figure 6. Summary of the inhibitory effects of PT and TPA pretreatment on growth factor-stimulated DNA synthesis in NR 6 cells. Inhibitory effects of PT pretreatment (□), TPA pretreatment (▨), and TPA and PT pretreatments (■) on each mitogen-stimulated DNA synthesis in NR 6 cells are summarized as in Figure 4. Variations in responses of native cells to each mitogen in the experiments and the numbers of them are as follows: TPA, 4.8–7.0% (9); bFGF, 30.5–54.3% (9); PDGF, 34.4–52.2% (10); vanadate, 10.3–16.9% (7); thrombin, 6.4–14.6% (8). Results represent the average $\pm$ SE of 7–10 separate experiments.

Figure 7. Effects of PT and TPA pretreatment on the binding of growth factors. Quiescent Swiss 3T3 cells were pretreated with pertussis toxin ($\times$), TPA ($\blacktriangle$), or untreated ($\circ$), then binding of ^{125}I -labeled growth factors was measured as described in Materials and methods. Specific binding of EGF (A), bFGF (B), and bradykinin (C) are shown; each value represents the mean of duplicate determinations of a representative experiment. Similar results were obtained in three independent experiments.

1989). In contrast, EGF and bFGF did not stimulate the production of IP_3 for up to 5 min, although these mitogens apparently induced rapid activation of PKC, as shown in Figure 8. These results indicate the existence of multiple pathways of mitogen-stimulated DG formation: breakdown of PIP_2 by a PT-sensitive G_p -dependent mechanism (as in the case of bombesin and bradykinin), breakdown of PIP_2 by a PT-sensitive G_p -independent mechanism (as in the case of PDGF), and hydrolysis of other phospholipids besides PIP_2 (as in the case of bFGF, etc.).

Discussion

In the present study we have shown that peptide growth factors can be classified into three groups with regard to the involvements of PT-sensitive G_p and of PKC in their mitogenic signaling pathways. The first group contains bombesin, bradykinin, and thrombin; mitogenic signal transduction of these growth factors depends totally on the functions of PT-sensitive G_p and PKC. The second group contains EGF and PDGF; the mitogenic signal of these growth factors is transduced both by PT-sensitive G_p -/PKC-dependent and -independent mechanisms. The third group contains bFGF, the mitogenic signal of which is transduced by a PT-sensitive G_p -independent mechanism; some of its mitogenic effect, however, is elicited through a PKC-involving pathway. This classification is based on the observation that inhibitory effects

of PT pretreatment (inactivation of PT-sensitive G_p) and/or TPA pretreatment (PKC depletion) on mitogen-induced DNA synthesis varied significantly and systematically with growth factors; effects of each pretreatment on various growth factor-stimulated DNA syntheses were identical in the three mouse fibroblast cell lines analyzed (Balb 3T3, Swiss 3T3, and NR 6 cells). Essentially the same inhibitory effect of PT pretreatment on each growth factor-induced DNA synthesis has been observed with human diploid foreskin fibroblasts (FS-4); depletion of PKC has been incomplete under our standard TPA-pretreatment condition (200 ng/ml, 24–36 h), and thus the involvement of PKC in the mitogenic signal transduction pathway of each growth factor in FS-4 cells is unclear.

Although all three groups of mitogens activate PKC (Figure 8), mechanisms of DG formation vary with growth factors. The first group of classified growth factors (bombesin, etc.) apparently stimulates the breakdown of PIP_2 by a PT-sensitive G_p -dependent mechanism to form IP_3 and DG, and the resultant PKC activation plays an essential role in transducing the mitogenic signal of these types of growth factors. In contrast, mechanisms of DG formation stimulated by the other two groups of growth factors seem complex. PDGF does stimulate phosphoinositide hydrolysis, however, by a novel mechanism that does not include the function of PT-sensitive G_p ; both DNA synthesis and PKC activation induced by PDGF are reduced to $\sim 50\%$ by pretreating cells with PT. EGF does not stimulate the formation of IP_3 , although mitogenic signal transduction of EGF depends partially on the functions of PT-sensitive G_p and PKC. The above results—in combination with the partial inhibition of PDGF-stimulated DNA synthesis in PKC-depleted cells, as well as in both PT-sensitive G_p -inactivated and PKC-depleted cells—suggest that PDGF and EGF stimulate the hydrolysis of phospholipids besides PIP_2 by a PT-sensitive G_p -dependent mechanism and that the DG thus formed activates PKC, which is directly involved in the mitogenic signal transduction pathways of PDGF and EGF. PKC activated by PIP_2 -derived DG (by a PT-sensitive G_p -independent mechanism) may not be involved in the mitogenic response of cells to PDGF. These results also imply the existence of several types of functionally different PKC. PKC has recently been reported to be a large family with multiple species (reviewed in Nishizuka, 1988). It may be that each DG, derived from different phospholipids by various mechanisms, activates different species of PKC

Figure 8. Effect of PT pretreatment on mitogen-stimulated PKC activity as determined by the increased phosphorylation of $M_r = 80\ 000$ protein. Growth-arrested Swiss 3T3 cells, with (B, D, F, and H) or without (A, C, E, and G) PT pretreatment, were labeled with [32 P]Pi, then treated for 10 min with no addition (A and B), bombesin (C and D), PDGF (E and F), and bFGF (G and H). Labeled phosphoproteins were analyzed by two-dimensional gel electrophoresis as described in Materials and methods. The arrow indicates the position of $M_r = 80\ 000$ protein. Similar results were obtained in three independent experiments.

or, alternatively a different state of the same species of PKC: preferential down-regulation of any specific species of PKC in cells is impos-

sible, and thus we cannot address such an interesting question at present. There is no evidence of increased phosphoinositide hydrolysis

Figure 9. Effect of PT pretreatment on mitogen-induced phosphoinositide hydrolysis. Growth-arrested Swiss 3T3 cells, with (▨) or without (□) PT pretreatment, were stimulated with various mitogens for 10 s (left) or 90 s (right), then IP_3 (1,4,5-isomer) was measured as described in Materials and methods. Percentages were calculated by comparison with untreated controls; 100% IP_3 = 9.04 pmol/ 10^5 cells (10 s), or 7.01 pmol/ 10^5 cells (90 s). Data are the mean $\pm$ SE for triplicate determinations in a single experiment. Results shown are representative of two similar experiments. Bomb, bombesin; BK, bradykinin.

in bFGF-stimulated cells; bFGF, however, induces the rapid activation of PKC, and PT pretreatment does not attenuate the response (Figure 8). These results suggest that bFGF stimulates DG formation through the hydrolysis of phospholipid(s) other than PIP_2 by a PT-sensitive G_p -independent mechanism. All the above results thus indicate multiple pathways and multiple sources of DG formation in mitogen-stimulated cells. To confirm these possibilities, it will be necessary to analyze the DG levels as well as the fatty acid composition of DG in mitogen-stimulated cells. In this respect, there is increasing evidence that a variety of growth factors (EGF, PDGF, etc.) stimulates the hydrolysis of phosphatidylcholine by phospholipase C and D to yield DG and phosphatidic acid (Bocckino *et al.*, 1985; reviewed in Exton, 1990).

As discussed above, PKC is now known to be a large family with at least seven subspecies (α , β_1 , β_2 , γ , δ , ϵ , and ζ) (Nishizuka, 1988). These PKC subspecies have subtle individual enzymological characteristics and may have distinct functions in the processing and modulation of a variety of cell responses to external stimuli. Most cell types contain more than one subspecies of PKC; enzymatic and immunochemical analyses have shown that α -PKC is most commonly distributed in all tissues and cell types thus far examined. In the present study, down-

regulation of PKC in TPA-pretreated cells was analyzed by the immunochemical method with anti-PKC antibody, which detected α , β_1 , and β_2 subspecies (Figure 2), and also by the direct determination of PKC activity, which depended on the presence of Ca^{2+} , phosphatidylserine, and DG (Figure 3). PKC analyzed by these procedures apparently included α , β_1 , β_2 , and γ but not δ , ϵ , and ζ subspecies. Although NIH 3T3 mouse fibroblasts have been reported to contain only a single species, α , of PKC (McCaffrey *et al.*, 1987), we cannot at present rule out the possibility that δ , ϵ , and ζ PKC remained in the TPA-pretreated three lines of mouse fibroblasts analyzed here. Whether or not these PKC isozymes played roles in the mitogenic signal transduction of growth factors such as PDGF and EGF remains to be elucidated.

Mitogenic responses not only to PDGF, EGF, and bFGF, but also to vanadate, are attenuated in PKC-depleted cells, indicating that signal transduction pathways of these mitogens involve the function of PKC. Receptor molecules for EGF and PDGF (Hunter and Cooper, 1985) and, recently, for bFGF (Lee *et al.*, 1989) have been reported to possess tyrosine kinase activity, upon which mitogenic signal transductions of at least EGF and PDGF totally depend. Vanadate is a potent inhibitor of phosphotyrosyl protein phosphatase (Leis and Kaplan, 1982; Swarup *et al.*, 1982). It thus seems very likely that the levels of tyrosine phosphorylation are increased in those mitogen-stimulated cells. By which mechanism(s) is the increased tyrosine phosphorylation linked to the activation of PKC? The most plausible hypothesis is that the activated receptor tyrosine kinases induce, either directly or indirectly, the phosphorylation of some elements that are involved in the PKC-activating pathway. In this regard, PDGF-, EGF-, and vanadate-induced DNA synthesis, not only in PKC-depleted cells but also in PT-sensitive G_p -inactivated cells, is reduced to $\sim 50\%$ compared with native cells (Figures 4–6). The inhibitory effects of PT and TPA pretreatment are not additive, indicating that the function of PT-sensitive G_p is linked to the activation of PKC. These results thus suggest that PDGF and EGF stimulate tyrosine phosphorylation of the component(s) whose function(s) lie(s) upstream of PT-sensitive G_p on their mitogenic signaling pathways. In accordance with this possibility, the β subunit of G protein has been found to be phosphorylated by EGF receptors in vitro (Valentine-Braun *et al.*, 1986). Furthermore, PDGF- and EGF-stimulated tyrosine phosphorylation of GTPase-activating protein (GAP) has also been

reported very recently (Molloy *et al.*, 1989; Ellis *et al.*, 1990; Kaplan *et al.*, 1990). Whereas bFGF-stimulated DNA synthesis, as well as EGF- and PDGF-stimulated DNA synthesis (which is most evident in Balb 3T3 cells), is inhibited to a higher extent in TPA-pretreated cells than in PT-pretreated cells, other mechanisms are implied in that PKC is activated independently of the function of PT-sensitive G_p as described above or, alternatively, that these mitogens also stimulate the phosphorylation of the element(s) that stand(s) downstream of PT-sensitive G_p . In this respect, increased tyrosine phosphorylation of the enzymes that are involved in phospholipid metabolism—such as phospholipase C- γ (PLC- γ) (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989) and 3' phosphatidylinositol kinase (PI-3 kinase) (Kaplan *et al.*, 1987)—in EGF- and PDGF-stimulated cells has recently been reported. Our data do not yet allow discrimination between these possibilities. They indicate the existence of multiple points of convergence between protein-tyrosine phosphorylation and PKC-activating pathway in the mitogenic signal transduction pathway of receptor tyrosine kinase-activating growth factors. The physiological significance, as well as the precise mechanism, of the mitogen-stimulated tyrosine phosphorylation of G-protein β subunit, GAP, PLC- γ , and PI-3 kinase is presently unknown. Much work remains to answer those questions.

An important question concerns the mechanism of partial inhibition of EGF-, PDGF-, and bFGF-induced DNA synthesis in PT- or TPA-pretreated cells. DNA synthesis in each cell is an all-or-none response. ADP ribosylation of the G_p α -subunit in PT-pretreated cells and PKC-depletion in TPA-pretreated cells are complete in our experimental conditions (Figures 1–3), indicating that some of the EGF-, PDGF-, or bFGF-stimulated cells enter into S phase without the functions of PT-sensitive G_p and/or PKC, whereas some of the EGF-, PDGF-, or bFGF-stimulated cells failed to initiate DNA synthesis without them. In this respect, Rozengurt (1986) has recently been proposed that, in normal cells responding to a single mitogen, there are multiple intracellular pathways leading to protein phosphorylation, cytoplasmic alkalinization, increased gene expression, etc. (some of them may involve the function of PT-sensitive G_p and/or PKC, whereas others do not), the sum of which leads to cell division and proliferation. Different cell responses to the same mitogen described in this report might thus be explained by the heterogeneity of the cell population with respect to the minimal signal required to trigger

entry into S phase. This could be the difference, for example, in the growth-arrested states of the cells with regard to the functions of PT-sensitive G_p and/or PKC required for the G_0/G_1 -S transition. All our analyses have been performed on populations of cells but not on single cells, and it is certainly difficult at present to know the precise mechanism of the partial inhibition of some mitogen-induced DNA synthesis in PT- or TPA-pretreated cells. Similar incomplete inhibition of some growth factor-induced DNA synthesis (Hildebrandt *et al.*, 1986), S6 kinase activation (Blenis and Erikson, 1986; Šuša *et al.*, 1989), and *c-myc* induction (Kaibuchi *et al.*, 1986) in PT-pretreated or TPA-pretreated cells has been reported. About 50% inhibition of EGF- and PDGF-stimulated DNA synthesis in PT- or TPA-pretreated cells compared with native cells may suggest that PT-sensitive G_p -/PKC-dependent and -independent pathways play almost equally important roles in the mitogenic signal transduction of EGF and PDGF, whereas ~20% inhibition of bFGF-stimulated DNA synthesis in PKC-depleted cells compared with native cells may imply that mitogenic signal transduction of bFGF depends more on PKC-independent than on PKC-dependent pathways.

Our results indicate that the mitogenic signaling pathway of EGF involves, at least in part, the function of PT-sensitive G_p and PKC. As discussed above, EGF seems to stimulate the hydrolysis of phospholipids besides PIP_2 by a PT-sensitive G_p -dependent mechanism, and the DG thus formed activates PKC. Inability of EGF to stimulate phosphatidylinositol turnover (Figure 9) is consistent with previously published results (L'Allemain and Pouyssegur, 1986). However, different observations have been reported as to whether PT-sensitive G_p and PKC are involved in the signal transduction pathway of EGF. For instance, EGF-induced mitogenicity has been reported to be both not inhibited (Chambard *et al.*, 1987) and inhibited (Church and Buick, 1988) by PT. EGF-induced activation of PKC has been reported (Kazlauskas and Cooper, 1988) but has not been observed by others (Rozengurt *et al.*, 1983; Isacke *et al.*, 1986). These inconsistencies could be attributed, in part, to different cell types. In this respect, Wright *et al.* (1988) recently used a sensitive assay to show clearly that total DG content increases after EGF treatment in Chinese hamster embryo fibroblasts. Thus, although the matter remains controversial, evidence is accumulating that EGF elicits some of its mitogenic effect through PKC.

Because possible involvements of EGF receptor tyrosine kinase in the signal transduction of some mitogens such as PDGF (Davis and Czech, 1985) and thrombin (Kohno and Pouyssegur, 1986) have been suggested, we have analyzed the mitogenic response of various growth factors also in NR 6 cells, a variant line of Swiss 3T3 cells lacking EGF receptors. NR 6 and Swiss 3T3 cells, however, responded to all mitogens except EGF in essentially the same manner, indicating that EGF receptors do not play a role in their signal transduction pathways. In this respect, we have recently obtained evidence indicating that mitogenic signaling pathway of tumor necrosis factor includes EGF receptor-tyrosine kinase activity (E. Tanaka, Y. Chatani, M. Tsujimoto, and M. Kohno, unpublished results).

In conclusion, we have demonstrated in this report that the mitogenic signaling pathways of various growth factors have been affected differently by PT and/or TPA pretreatment of cells. We propose that peptide growth factors can be classified into at least three groups with regard to the involvements of PT-sensitive G_p and PKC in their mitogenic signaling pathways.

Materials and methods

Cell culture

Swiss albino mouse fibroblasts (Swiss 3T3 [American Type Culture Collection, CCL92]) and Balb/c mouse fibroblasts (Balb 3T3 Clone A31-1-1 [JCRB 0601]) were obtained through the Japanese Cancer Research Resources Bank. Balb 3T3 cells were cultured to isolate clonal lines; one clone (Balb 3T3-K7) from such cultures was used throughout this study. NR 6 cells, a variant line of Swiss 3T3 cells lacking EGF receptors (Pruss and Herschman, 1977), were obtained through Dr. M.B. Sporn (National Institutes of Health, Bethesda, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). For experimental use, confluent cultures of the cells in DMEM containing 10% FCS were rendered quiescent by incubating for 36–48 h in serum-free medium: DMEM containing 2 mg/ml of bovine serum albumin (BSA, Boehringer Mannheim, Mannheim, FRG), 10 μ g/ml of soybean lipid (Boehringer Mannheim), 1 μ g/ml of insulin, 2 μ g/ml of transferrin, 20 nM Na_2SeO_3 and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4. Then growth factors were added (Kohno, 1985; Kohno *et al.*, 1990).

Measurement of growth stimulation

DNA synthesis was measured 18 h after exposure of the cells to mitogens by the addition to the cultures of 5-bromo-2'-deoxyuridine (BrdU) (cells were inoculated onto glass cover slips) followed by incubation for 6 h. After incorporation, the percentage of BrdU-labeled nuclei was determined with a cell proliferation kit (Amersham, Arlington Heights, IL) (Kohno *et al.*, 1990). The percentage of labeled nuclei was determined in a total of >800 nuclei using two cover slips per experimental condition.

ADP ribosylation

Cells being rendered quiescent in serum-free medium were incubated in varying concentrations of PT (generously provided by Prof. M. Ui, University of Tokyo) for the final 4-h period. Each plate was rinsed three times with cold phosphate-buffered saline (PBS) and once with buffer A containing 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 10 mM NaCl, 5 mM MgCl_2 , 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were scraped from the dish into buffer A and broken by homogenizing with a tight-fitting glass-glass Dounce homogenizer (20 strokes). After removing the nuclei and unbroken cells by centrifugation at $400 \times g$ for 10 min, the supernatant was centrifuged at $100\,000 \times g$ for 30 min (Hitachi RP55 rotor, 42 000 rpm). The final pellet (membrane-rich fraction) was resuspended in buffer containing 25 mM Tris-HCl, pH 7.4, and 2.5 mM MgCl_2 , and protein was determined using the BCA protein assay reagent (Pierce, Rockford, IL) with BSA as a standard.

ADP ribosylation was performed as described (Katada and Ui, 1982; Ribeiro-Neto *et al.*, 1985) with minor modifications: 50 μ g of total protein from the membrane-rich fraction was diluted with an equal volume of ribosylation buffer (final concentration, 25 mM Tris-HCl, pH 7.4, 10 mM thymidine, 1 mM adenosine triphosphate [ATP], 0.1 mM GTP, 2.5 mM MgCl_2 , 1 mM EDTA, and 5 mM dithiothreitol [DTT]). PT was activated by incubation with 50 mM DTT in 25 mM Tris-HCl, pH 7.4, at 37°C for 30 min and added to a final concentration of 5 μ g/ml. [^{32}P]NAD $^+$ (1 μ Ci/sample, New England Nuclear, Boston, MA) was added with cold NAD $^+$ to a final concentration of 10 μ M and the reaction mixture incubated at 37°C for 20 min. ADP ribosylation reaction was terminated by trichloroacetic acid (TCA) precipitation, and the samples were resolved by SDS-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed.

Down-regulation of PKC

Cells being rendered quiescent in serum-free medium were treated with 200 ng/ml of TPA for various lengths of time at the final period of growth arrest. Each plate was rinsed three times with PBS. Cells were removed with a rubber policeman, pelleted at 5000 rpm for 30 s in a microcentrifuge, and lysed in 10 mM Tris-HCl buffer, pH 7.0, containing 1% SDS, 10 μ g/ml of leupeptin, 1 mM PMSF, and 1% aprotinin (Sigma, St. Louis, MO). SDS sample buffer (25 μ l, 4 times the normal concentration, containing 250 mM DTT as a reducing agent) was added to 100 μ l of cell lysate, and samples were boiled for 5 min. Small portions of cell lysate to which SDS sample buffer had not been added were used for protein determination. Samples containing 50 μ g of cellular proteins were resolved by SDS-PAGE on 10% polyacrylamide gels, after which proteins were transferred electrophoretically to a nitrocellulose filter and stained with anti-PKC, clone MC5, which detected α , β_1 , and β_2 subspecies of PKC (Amersham), followed by ^{125}I -labeled protein A (Amersham) as described (Towbin *et al.*, 1979). Autoradiography was performed on X-ray film with an intensifying screen at -80°C .

PKC activity was determined as described (Thomas *et al.*, 1987). Briefly, control and TPA-treated cells, washed and pelleted as described above, were resuspended in buffer B (containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 10 μ g/ml of leupeptin, and 2 mM PMSF), then sonicated briefly on ice. Total cellular PKC activity was extracted with 1% NP-40 in buffer B. Soluble extracts were applied onto DEAE Sephacel columns to remove the endogenous

components and excess detergent that interfere with the assay. PKC activity, eluted with buffer containing 0.15 M NaCl, was determined with H1 histone as a substrate. The reaction mixture (250 μ l) contained 20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 10 μ M [32 P]ATP (Amersham), 0.5% H1 histone, 0.1 mM CaCl_2 , 2 μ g phosphatidylserine, 0.2 μ g diolein, and the enzyme preparation. Phosphatidylserine and diolein were taken in a tube and organic solvents were evaporated. Lipid dispersion was accomplished by brief sonication. The difference between the phosphorylation activity determined in the presence of 0.5 mM EGTA (basal activity) and in the presence of CaCl_2 , phosphatidylserine, and diolein was calculated as being PKC activity. One unit of PKC was defined as the amount of enzyme catalyzing incorporation of 1 nmol of phosphate/min from ATP into histone H1.

Iodination of growth factors

Iodination of EGF, bFGF, and (Tyr⁸)bradykinin (Peptide Institute, Osaka, Japan) was performed by the modified chloramine T method (Haigler, 1983); the specific activities of the ^{125}I -labeled growth factors were 191 000 cpm/ng for EGF, 149 000 cpm/ng for bFGF, and 47 600 cpm/ng for bradykinin.

Binding assay for ^{125}I -labeled growth factors

Binding of ^{125}I -labeled growth factors to Swiss 3T3 cell monolayers was measured as described previously (Amano *et al.*, 1988). In brief, Swiss 3T3 cells seeded in 24-well tissue culture trays were growth-arrested and treated with 25 ng/ml of PT for 4 h or with 200 ng/ml of TPA for 24 h, as described above. Finally, the medium was removed, cells were rinsed once with DMEM, and 0.5 ml DMEM containing 50 mM HEPES-NaOH buffer, pH 7.4, was added. After the cells were on ice for 30 min, we added either 100-fold concentrations of cold growth factors in 0.2% BSA or only 0.2% BSA to each set of duplicate wells and then added ^{125}I -labeled growth factors to final concentrations of 0.3–100 ng/ml for EGF, 0.1–30 ng/ml for bFGF, and 0.01–3 μ g/ml for bradykinin. Cells were kept on ice for 2 h, washed three times with PBS, resolved with 0.5 ml 0.5-M NaOH, and counted for radioactivity with a Beckman gamma counter (type 5550, Birmingham, AL). The number of cells recovered from each assay was estimated by counting nonradioactive duplicates, which were treated in the same way as the other samples, and the results were expressed as cpm/ 10^5 cells. Specific binding was calculated by subtracting the radioactivity with cold growth factors (nonspecific binding) from that without cold growth factors (total binding).

Analysis of $M_r = 80\,000$ protein phosphorylation

Mitogen-induced $M_r = 80\,000$ protein phosphorylation in Swiss 3T3 cells was analyzed as described previously (Kohno, 1985; Kohno *et al.*, 1990). In brief, growth-arrested cells were labeled with 1 mCi/ml of [32 P]Pi (ICN Biochemicals, Costa Mesa, CA) for 16 h; PT (25 ng/ml) was added for the final 4-h period. Growth factors were then added and the cells were incubated for a further 10 min. Lysates of $\sim 5 \times 10^4$ cells were analyzed by two-dimensional gel electrophoresis and autoradiography. The relative intensity of the spot of the $M_r = 80\,000$ protein was quantified by scanning the resultant autoradiographs on an LKB laser densitometer; for this procedure, we used lower autoradiographic exposures than those shown in Figure 8.

Radioimmunoassay of inositol IP_3

Swiss 3T3 cells seeded in 12-well tissue culture trays were growth-arrested and treated with 25 ng/ml of PT for 4 h. The medium was removed and then cells were incubated for 10 min in 400 μ l of serum-free medium containing 10 mM LiCl. Various mitogens in 100 μ l of the above medium were added to the cells for 10 or 90 s, then the reaction was terminated by adding 180 μ l of 10% perchloric acid. After neutralizing by addition of 10N-KOH, samples were centrifuged to remove KClO_4 and IP_3 was determined in the supernatants using an IP_3 assay kit (Amersham), which specifically detects the 1,4,5-isomer, as described (Fu *et al.*, 1988). The number of cells recovered from each assay was estimated by counting duplicate samples treated in the same way as the other samples, and IP_3 content was expressed as pmol/ 10^5 cells.

Materials

EGF purified from mouse submaxillary glands and bFGF from bovine pituitary glands were purchased from Toyobo (Osaka, Japan). Recombinant human PDGF (B-B homodimer) was from Amersham. TPA, thrombin (human), bombesin, bradykinin, insulin, transferrin (human), phosphatidylserine, and diolein were from Sigma. Other chemicals and reagents were of the purest grade available.

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