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Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a M_r 80,000 protein in intact quiescent 3T3 cells

(Ca^{2+}-activated phospholipid-dependent protein kinase/platelet-derived growth factor/phorbol ester receptors/tumor promoters)

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ABSTRACT **Addition of biologically active phorbol esters to intact quiescent 3T3 mouse cells stimulates an extremely rapid (detectable within seconds) phosphorylation of a M_r 80,000 cellular protein (termed "80k"). Phorbol 12,13-dibutyrate enhances 80k phosphorylation in a dose-dependent manner; half-maximal effect is obtained at 32 nM. The possibility that this phosphorylation is related to the activation of Ca^{2+}-activated phospholipid-dependent protein kinase is suggested by the fact that phospholipid breakdown induced by exogenous treatment of the cells with phospholipase C from *Clostridium perfringens* or with platelet-derived growth factor, which is a potent activator of endogenous phospholipase C activity, also causes a rapid enhancement of 80k phosphorylation. Moreover, prolonged pretreatment of the cells with phorbol 12,13-dibutyrate, which leads to a marked decrease in the number of specific phorbol ester binding sites, prevents the phosphorylation of 80k stimulated by phorbol esters, phospholipase C, and platelet-derived growth factor. These findings provide evidence obtained with intact cells that implicate the stimulation of Ca^{2+}-activated phospholipid-dependent protein kinase in the action of phorbol esters and other growth factors.**

Tumor promoters are compounds that, although not carcinogenic by themselves, increase the incidence of tumors when applied repeatedly to animals that have received a subthreshold dose of a carcinogen (1). Their mechanism of action is of considerable interest, with attention focusing on their biological effects in various types of cultured cells. In confluent and quiescent mouse 3T3 cells, biologically active phorbol esters bind to a single class of high-affinity sites (2, 3) and rapidly stimulate ion fluxes (4), enhance nucleoside and hexose uptake (5, 6), decrease the affinity of the surface receptors for epidermal growth factor (7–9), induce ornithine decarboxylase activity (10), and subsequently stimulate reinitiation of DNA synthesis when added with other mitogens in serum-free medium (2, 3, 10, 11). Although tumor promoters and hormonal peptides such as vasopressin (12) and bombesin (13) share pathways of action (3, 10, 13), the mechanism whereby occupancy of the phorbol ester receptors leads to the elicitation of their biological responses remains unknown and of considerable importance to gain insight into the molecular events leading to tumor promotion.

A type of cyclic nucleotide-independent protein kinase has been discovered that is activated by association with membrane phospholipids in the presence of Ca^{2+} (14, 15); the activity of the membrane-associated enzyme is potently stimulated by unsaturated diacylglycerol (16). Recently, several reports indicate that biologically active phorbol esters can substitute for diacylglycerol in stimulating the partially purified enzyme incubated in the presence of phospholipids (17–20). Similar observations were made with extracts prepared from Swiss 3T3 cells (our unpublished results). Whether or not these observations *in vitro* are relevant to the mechanism whereby phorbol esters modulate cell proliferation in the intact cells remains difficult to assess because Ca^{2+}-activated phospholipid-dependent protein kinase interacts not only with phorbol esters but also with a variety of hydrophobic drugs added directly to the assay mixture (21, 22) and because little attention has been given to the early effects of phorbol esters on protein phosphorylation in intact, responsive cells. For these reasons, we focused our analysis on phorbol esters and protein phosphorylation in intact cells. Here we report that addition of biologically active phorbol esters to intact mouse and rat quiescent cells stimulates an extremely rapid (detectable within seconds) phosphorylation of a M_r 80,000 cellular protein (termed "80k"). We present evidence that supports the possibility that this phosphorylation reflects the activation of the Ca^{2+}-sensitive phospholipid-dependent protein kinase in intact fibroblastic cells.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3 cells (23) were maintained and grown to confluency in 33-mm Nunc Petri dishes as described (10, 13, 24).

Labeling of Cells with $^{32}P_i$. The cultures were washed twice with Dulbecco's modified Eagle's medium without phosphate and the cells were incubated with this medium containing carrier-free $^{32}P_i$ at 200 μCi/ml (1 Ci = 3.7×10^{10} Bq) at 37°C for 3 hr to label the endogenous ATP pool. Then, phorbol esters or other agents were added for various times. The reaction was stopped by removing the medium and rapidly washing the cultures twice with ice-cold Tris/saline solution (0.15 M NaCl/20 mM Tris·HCl, pH 7.5). The cells were immediately extracted with 5% trichloroacetic acid at 4°C for 20 min. The acid-soluble materials were removed; the precipitated protein was washed twice with Tris/saline and dissolved with 100 μl of solution A [3% $NaDodSO_4$/5% (vol/vol) glycerol/10 mM Tris·HCl, pH 7.8/2% (vol/vol) 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride] heated at 100°C. After 2 min, the samples were transferred to tubes and placed in a boiling water bath for 20 min prior to resolution by gel electrophoresis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was performed using a 5–15% acrylamide gradient and 0.1% $NaDodSO_4$ by the method of Laemmli (25). After electrophoresis the slabs were stained with Coomassie brilliant

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Abbreviations: 80k, M_r 80,000 protein; PBt_2, phorbol 12,13-dibutyrate; PDGF, platelet-derived growth factor.

blue R250 [0.01% in 40% methanol/7.5% acetic acid (vol/vol)], destained in 40% methanol/7.5% acetic acid, and dried down onto paper for radioautography with Fuji x-ray film (Fuji Photo Film, Kyoto, Japan).

After developing, autoradiograms were scanned with a Joyce–Loebl double-beam densitometer and the incorporation of $^{32}P_i$ into a specific protein band was quantitated by measuring the peak area (Hewlett–Packard digitizer) above background in densitometry tracings. In order to correct for small changes in the total isotope present in each sample, the peak area was divided by the sum of the areas of other peaks not affected by the test agents. The result of this division is taken as the $^{32}P_i$ incorporated expressed in arbitrary units and the values are presented as percent of the control.

Materials. Phorbol 12,13-dibutyrate (PBt_2), phospholipase C from *Clostridium perfringens* (type XII), phospholipase D from peanut (type III), phospholipase A_2 from bee venom or from porcine pancreas, and bovine insulin were from Sigma. Platelet-derived growth factor (PDGF) was partially purified (700-fold) by Sulfadex (sulfated Sephadex G-50) gel chromatography and heating and kindly supplied by P. Stroobant. Carrier-free $^{32}PO_4$ was from the Radiochemical Centre.

RESULTS

Phorbol Esters Stimulate Phosphorylation of a M_r 80,000 Protein. Quiescent cultures of Swiss 3T3 cells were labeled with $^{32}P_i$ in Dulbecco's modified Eagle's medium without P_i for 3 hr and then some of the cultures received PBt_2 at 200 nM for 60 sec. The pattern of phosphorylated proteins was examined on $NaDodSO_4$/polyacrylamide slab gel electrophoresis and the protein bands containing $^{32}P_i$ were revealed by autoradiography of the stained dried gel. Fig. 1*A* shows that addition of PBt_2 markedly increased the phosphorylation of a band migrating with an apparent molecular weight of 80,000 (80k). Integration of densitometer tracings of the autoradiograph shown in Fig. 1*A* reveals that the phosphorylation of 80k is markedly enhanced (Fig. 1*B*). In seven independent experiments performed as described in Fig. 1, addition of PBt_2 resulted in a 3.86 ± 0.21-fold (mean ± SEM) increased phosphorylation of 80k. The increased phosphorylation of 80k caused by PBt_2 is not due to changes in the specific activity of the cellular ATP pool because most phosphoproteins show no change after PBt_2 addition for 60 sec and because PBt_2 increased 80k phosphorylation in Swiss 3T3 cells labeled for 4 or 6 hr with $^{32}P_i$ before the addition of this ligand (results not shown). When the gels were treated with alkali (26) most of the radioactivity corresponding to 80k is eliminated, suggesting that 80k is phosphorylated at serine rather than at tyrosine residues.

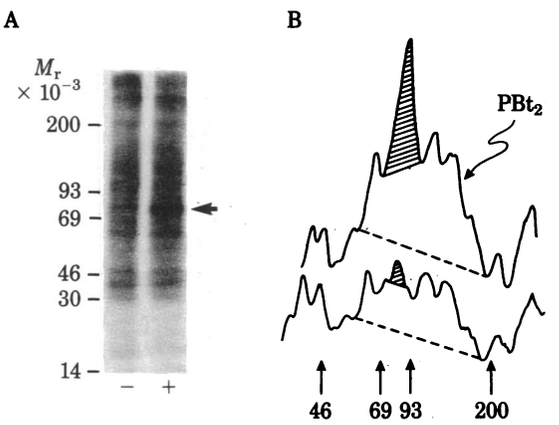

FIG. 1. Effect of PBt_2 on protein phosphorylation in intact Swiss 3T3 cells. Quiescent cultures of these cells were washed and labeled with $^{32}P_i$ for 3 hr and exposed to PBt_2 at 200 nM or to an equivalent volume of solvent for 60 sec. Then, the reaction was rapidly terminated by adding 5% trichloroacetic acid. (*A*) Autoradiographs of dried $NaDodSO_4$ slab gels. The arrow indicates the position of 80k. Lane −, control; lane +, PBt_2 added. (*B*) Densitometer tracing of the autoradiograph shown in *A*.

The time course of relative increase in 80k phosphorylation induced by PBt_2 is shown in Fig. 2*A*. An enhancement in 80k phosphorylation can be detected as early as 15 sec after PBt_2 addition; maximal stimulation can be seen 60 sec after PBt_2 addition, and then $^{32}P_i$ incorporation into 80k persists elevated for 5 min. In other experiments, the increased phosphorylation of 80k persisted for at least 1 hr after the addition of PBt_2 (not shown). PBt_2 also stimulates the phosphorylation of at least two other proteins migrating with M_r = 87,000 and M_r = 33,000 after a lag of 5 min and 15 min, respectively (not shown). A similar pattern was obtained in four independent experiments. These results indicate that the stimulation of 80k phosphorylation is one of the earliest effects elicited by phorbol esters in intact 3T3 cells.

As shown in Fig. 2*B*, PBt_2 stimulates the phosphorylation of 80k in a dose-dependent manner; the half-maximal effect was obtained at a concentration of PBt_2 of 32 nM (16 ng/ml). Stimulation of 80k phosphorylation is also observed when phorbol 12-tetradecanoate 13-acetate is used instead of PBt_2, but it is not obtained when 3T3 cells are exposed to the biologically inactive analogues phorbol 12-tetradecanoate 13-acetate 4-*O*-methyl ether or phorbol (all tested at 100 ng/ml).

As shown in Fig. 3, phosphorylated 80k is readily extracted by a buffer containing 0.5% Triton X-100. This result suggests that 80k is a membrane or cytosolic protein rather than part of the detergent-resistant cytoskeleton (27, 28).

The rapid stimulation of 80k phosphorylation is not confined to Swiss 3T3 cells. Addition of PBt_2 to quiescent cultures of mouse embryo fibroblasts (second passage) or of rat-1 cells labeled with $^{32}P_i$ caused a rapid stimulation of a protein band migrating with an apparent molecular weight of 80,000 (Fig. 4).

PDGF and Exogenous Phospholipase C Stimulate 80k Phosphorylation. *In vivo*, Ca^{2+}-activated, phospholipid-dependent protein kinase is regulated by diacylglycerol generated by breakdown of membrane phospholipids catalyzed by an en-

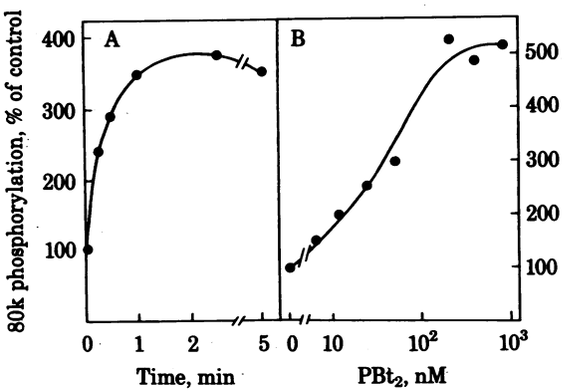

FIG. 2. Time course of 80k phosphorylation induced by addition of PBt_2 to quiescent Swiss 3T3 cells. (*A*) The cultures were washed and labeled with $^{32}P_i$ for 3 hr. Then PBt_2 at 200 nM was added and the incubation was terminated at various times. Addition of an equivalent volume of solvent (0.15 M NaCl/0.005% acetone) did not affect the basal level of 80k phosphorylation in short (10- to 60-sec) or long (up to 60-min) incubations. (*B*) Dose response for the enhancement of 80k phosphorylation by PBt_2 in quiescent cultures of Swiss 3T3 cells. The cultures were washed and labeled with $^{32}P_i$ for 3 hr. Then PBt_2, at various concentrations, was added for 5 min. The samples were analyzed for $^{32}P_i$ incorporation into 80k.

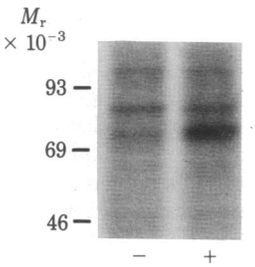

FIG. 3. Solubilization of phosphorylated 80k by Triton X-100. Quiescent cultures of Swiss 3T3 cells were labeled with $^{32}P_i$ for 3 hr and exposed to PBt_2 at 200 nM (lane +) or solvent (lane −) for 2 min. Then the cultures were extracted for 5 min at 22°C with a solution containing 100 mM 1,4-piperazinediethanesulfonic acid (Pipes) at pH 6.9, 1 mM EGTA, 10 mM NaF, 1 mM N^{α}-*p*-tosyl-L-arginine methyl ester and 0.5% Triton X-100. The soluble material was centrifuged (3 min at 5,000 rpm at 4°C in a Sorvall centrifuge with the SS-34 rotor). The supernatants were mixed with 1/10 vol of 10× solution A and then the phosphorylated proteins were examined on $NaDodSO_4$/polyacrylamide slab gel electrophoresis.

dogenous phospholipase C (16, 17). To determine whether 80k phosphorylation is related to the activation of this protein kinase in intact 3T3 cells, we tested whether agents known to induce breakdown of cellular phospholipids and to generate diacylglycerol in the membrane also stimulate 80k phosphorylation. Recently, PDGF (29) has been shown to be a potent activator of endogenous phospholipase C (30, 31), and activation leads to a marked increase in diacylglycerol in 3T3 cells (31). As shown in Fig. 5, PDGF, at concentrations that rapidly stimulate [^{3}H]inositol incorporation into the cells under our experimental conditions (results not shown), causes a striking time-dependent increase in 80k phosphorylation. Maximal effect is observed 5 min after the addition of PDGF. In other experiments, we found that PDGF stimulates 80k phosphorylation in a dose-dependent manner. In contrast, insulin (1–10 μg/ml) or EGF (5 ng/ml), neither of which stimulates endogenous phospholipase C activity in 3T3 cells (refs. 31 and 32 and unpublished results), failed to increase 80k phosphorylation even after 15 min of incubation, at which time both peptides enhance the phosphorylation of a M_r 33,000 protein (results not shown). Further, a variety of agents that cause large increases in the level of cAMP and stimulate DNA synthesis in 3T3 cells, including prostaglandin E_1 (33), adenosine agonists (34), and cholera toxin (35), did not affect 80k phosphorylation.

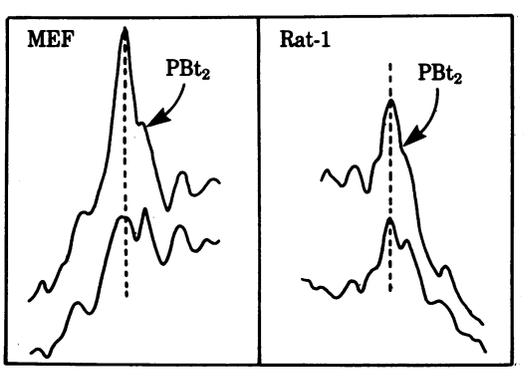

FIG. 4. PBt_2 stimulates 80k phosphorylation in secondary cultures of mouse embryo fibroblasts (left) and rat-1 cell line (right). Quiescent cultures of both cell types were washed and labeled with $^{32}P_i$ for 3 hr. Then they received either 200 nM PBt_2 or an appropriate volume of solvent. The reaction was terminated 2 min after the additions. The figure shows densitometer tracings of autoradiographs of dried $NaDodSO_4$ slab gels of the region around the 80k component, which is indicated by the vertical broken line.

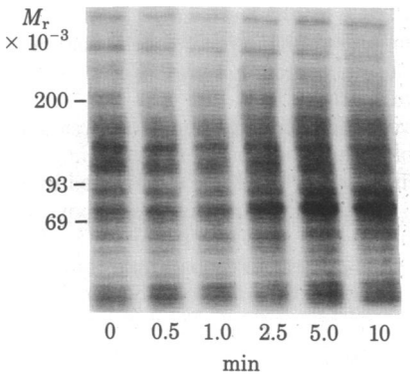

FIG. 5. Autoradiogram of a polyacrylamide gel showing ^{32}P incorporation into 80k protein from intact 3T3 cells treated with PDGF for various times. Quiescent cultures of Swiss 3T3 cells were labeled with $^{32}P_i$ for 3 hr. Then, partially purified PDGF was added and the incubation was terminated at various times.

A different approach to test further a relationship between diacylglycerol generation and 80k phosphorylation (which is not dependent upon receptor-mediated phospholipid breakdown) is provided by the use of exogenous phospholipase C from *C. perfringens*, which causes a rapid degradation of phosphatidylcholine and increases the content of diacylglycerol in the membrane of cultured cells (36, 37). We verified that phospholipase C causes a dose-dependent release of radioactivity from [^{3}H]choline-labeled Swiss 3T3 cells (results not shown). Accordingly, we tested whether phospholipase C could stimulate $^{32}P_i$ incorporation into 80k in intact quiescent cultures of Swiss 3T3 cells that were labeled with $^{32}P_i$ for 3 hr prior to the enzymatic treatment. The results depicted in Fig. 6 show that treatment of 3T3 cells with phospholipase C from *C. perfringens* (0.5 and 1 unit/ml) stimulated $^{32}P_i$ incorporation into 80k in a dose-dependent manner. We found that phospholipase C enhances 80k phosphorylation in a time-dependent manner; maximal effect was observed after a 5-min incubation. In con-

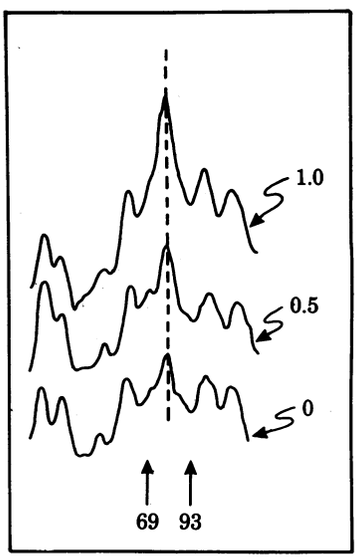

FIG. 6. Treatment of intact 3T3 cells with phospholipase C stimulates $^{32}P_i$ incorporation into 80k. Quiescent cultures of Swiss 3T3 cells were labeled with $^{32}P_i$ and exposed to phospholipase C from *C. perfringens* (0.5 or 1.0 unit/ml) for 10 min. The figure shows densitometer tracings of autoradiographs of dried $NaDodSO_4$ slab gels of the region around the 80k component that is indicated by the vertical broken line.

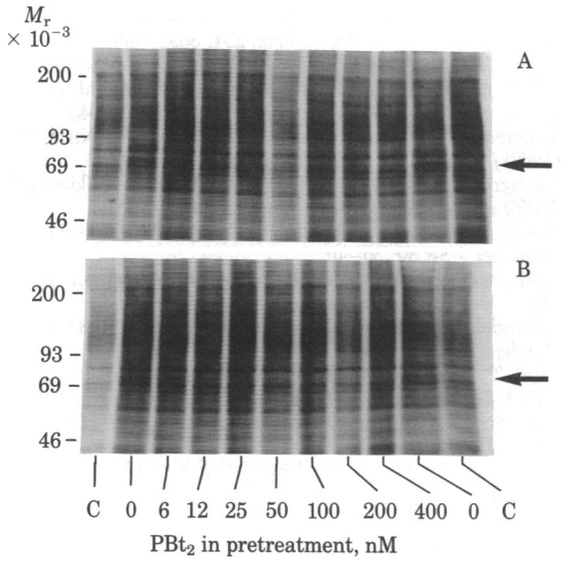

FIG. 7. Effect of pretreatment with various doses of PBt_2 on the ability of subsequent addition of PBt_2 (*A*) or phospholipase C (*B*) for enhancing 80k phosphorylation. Quiescent cultures of Swiss 3T3 cells were incubated for 24 hr in the absence (0) or presence of different concentrations of PBt_2, as indicated. Then the cultures were washed, labeled with $^{32}P_i$ for 3 hr, and exposed to PBt_2 at 200 nM (*A*) or to phospholipase C from *C. perfringens* at 1.2 units/ml (*B*). Both treatments were terminated after 10 min. The figure shows autoradiographs of dried $NaDodSO_4$ slab gels of the region around the 80k protein (indicated by the arrow). Also notice that treatment with PBt_2 for 10 min enhances the phosphorylation of an 87k protein. Lanes C represent the samples of cultures that were not pretreated with PBt_2 and received the respective solvent during the 10-min experimental incubation.

trast, phospholipase A_2 (from either bee venom or porcine pancreas) or phospholipase D (from peanut), tested in the range of 1–10 units/ml, failed to enhance 80k phosphorylation (results not shown).

Down-Modulation of PBt_2 Receptors Prevents Enhancement of 80k Phosphorylation by PBt_2, Phospholipase C, or PDGF. PBt_2 binds to a single class of high-affinity binding sites in Swiss 3T3 cells (2, 3). These sites are progressively decreased or lost after prolonged treatment with PBt_2 (2, 3, 38). To determine whether the stimulation of 80k phosphorylation induced by PBt_2 is mediated by the phorbol ester receptors, quiescent cultures of Swiss 3T3 cells were exposed to PBt_2 at various concentrations for 24 hr. Then the cultures were washed, labeled with $^{32}P_i$, and challenged with PBt_2 at 200 nM. As can be seen in Fig. 7*A*, PBt_2 pretreatment causes a dose-dependent decrease in the stimulation of 80k phosphorylation; PBt_2 at concentrations higher than 100 nM during the pretreatment completely blocked the stimulation of 80k phosphorylation by subsequent addition of PBt_2. Thus, prolonged treatment of 3T3 cells with PBt_2 prevents the stimulation of 80k phosphorylation by the homologous ligand.

If the phorbol ester receptor and the Ca^{2+}-activated phospholipid-dependent protein kinase are closely related or in the same molecule (17, 18, 39), down-modulation of the former by prolonged treatment with PBt_2 should prevent the enhancement of 80k phosphorylation by exogenous phospholipase C or PDGF. As shown in Fig. 7*B*, pretreatment with PBt_2 at various concentrations results in a dose-dependent decrease in the enhancement of 80k phosphorylation produced by phospholipase C. Similar findings were obtained in cells pretreated with PBt_2 and challenged with PDGF (results not shown). These observations are consistent with the possibility that the PBt_2 receptors and the activity that enhances 80k phosphorylation might reside in the same molecule, which is down-modulated by PBt_2.

DISCUSSION

The findings presented here demonstrate that addition of biologically active phorbol esters to intact, quiescent 3T3 cells rapidly enhances the phosphorylation of a M_r 80,000 protein. The effect can be detected as early as 15 sec after the addition of the compounds and constitutes one of the earliest responses elicited by phorbol esters in fibroblastic cells. The enhancement of 80k phosphorylation by PBt_2 is dose dependent; half-maximal effect is obtained at 32 nM, a value in close agreement to that required to induce half-maximal stimulation of early events and of DNA synthesis in 3T3 cells (2, 3) and to the apparent dissociation constant for $[^3H]PBt_2$ and its receptor in this cell type (2, 3, 38). Rapid enhancement of 80k phosphorylation by PBt_2 can be also observed in quiescent cultures of rat-1 cells and of secondary mouse embryo fibroblasts. The findings clearly implicate protein phosphorylation in mediating some of the biological effects of phorbol esters in cultured cells.

Recently, several reports indicate that phorbol esters bind and stimulate *in vitro* a soluble Ca^{2+}-activated phospholipid-dependent protein kinase (17–20, 39), an observation that we have confirmed in Swiss 3T3 cells (unpublished results). The enzyme is regulated *in vivo* by ligand-induced phospholipid breakdown and transient generation of diacylglycerol in the plasma membrane (16). Does enhancement of 80k phosphorylation reflect the activation of this protein kinase in the intact cell? As an initial approach to elucidate whether Ca^{2+}-activated phospholipid-dependent protein kinase is responsible for 80k phosphorylation, we determined (*i*) whether other treatments leading to phospholipid breakdown and generation of diacylglycerol in the membrane of intact cells also stimulate 80k phosphorylation and (*ii*) whether prolonged exposure to PBt_2, which down-modulates the PBt_2 binding sites in 3T3 cells (2, 3, 38), not only prevents the enhancement of 80k phosphorylation induced by this ligand but also blocks the effect of the other treatments that enhance 80k phosphorylation. For example, exposure of intact cells to phospholipase C, which causes direct hydrolysis of phosphatidylcholine and generates diacylglycerol in the membrane (32, 33), rapidly enhances 80k phosphorylation, whereas other phospholipases (A_2 or D) were not effective. Further, receptor-mediated phospholipid breakdown and increase of diacylglycerol as potently produced by PDGF in Swiss 3T3 cells (31) also cause a marked enhancement of 80k phosphorylation. In addition, preliminary results indicate that other mitogenic peptides such as vasopressin (12) and bombesin (13), which share with phorbol esters a pathway(s) for eliciting mitogenesis (10, 13), also enhance 80k phosphorylation (unpublished results). In contrast, other mitogenic ligands, including EGF, insulin, and cAMP-increasing agents, which do not induce phospholipid breakdown in 3T3 cells, fail to enhance 80k phosphorylation. That tumor promoters, growth factors that activate endogenous phospholipase C, or exogenous phospholipase C enhance 80k phosphorylation suggests that these chemically diverse agents interact with a common effector system. In line with this conclusion, prolonged treatment of 3T3 cells with PBt_2, which induces down-modulation of PBt_2 receptors, not only prevents the enhancement of 80k phosphorylation brought about by this ligand but also blocks the increase in 80k phosphorylation induced by treatment with phospholipase C (which causes direct breakdown of phospholipids) or by addition of PDGF, which interacts with a receptor (40–42) separate from the phorbol ester receptor (3) and activates an endogenous phospholipase C (31). These findings are consistent

with the possibility that 80k phosphorylation in intact 3T3 cells may result from the activation *in vivo* of Ca^{2+}-activated phospholipid-dependent protein kinase.

Homogeneous Ca^{2+}-activated phospholipid-dependent protein kinase from rat brain migrates in $NaDodSO_4$ gels as if its molecular weight were 82,000 and becomes autophosphorylated when fully activated (43). In view of the molecular weight, the rapidity of the phosphorylation, and the subcellular distribution, it is tempting to speculate that 80k phosphorylation represents the self-phosphorylation of the enzyme when it is activated by phorbol esters or by phospholipid turnover in intact quiescent cells, a proposition that warrants further experimental work.

We suggested that the tumor promoter phorbol esters modulate mitogenesis in fibroblastic cells through pathways that converge with those utilized by other hormones and growth factors (3, 10, 13). Taken together with recent reports on *in vitro* studies (refs. 14–17 and unpublished results), our findings with intact fibroblastic cells suggest that the Ca^{2+}-activated phospholipid-dependent protein kinase could represent a point of convergence in the action of a variety of mitogenic ligands, a proposition of considerable importance for elucidating which physiologically occurring molecules could drive tumor promotion *in vivo*.

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