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Direct Activation of Calcium-activated, Phospholipid-dependent Protein Kinase by Tumor-promoting Phorbol Esters*

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Tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) directly activate *in vitro* Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C), which normally requires unsaturated diacylglycerol. Kinetic analysis indicates that TPA can substitute for diacylglycerol and greatly increases the affinity of the enzyme for Ca^{2+} as well as for phospholipid. Under physiological conditions, the activation of this enzyme appears to be linked to the receptor-mediated phosphatidylinositol breakdown which may be provoked by a wide variety of extracellular messengers, eventually leading to the activation of specific cellular functions or proliferation. Using human platelets as a model system, TPA is shown to enhance the protein kinase C-specific phosphorylation associated with the release reaction in the total absence of phosphatidylinositol breakdown. Various phorbol derivatives which have been shown to be active in tumor promotion are also capable of activating this protein kinase in *in vitro* systems.

Although the cellular targets for the action of tumor-promoting phorbol esters have not been definitely identified, studies in cell culture systems strongly suggest that 12-*O*-tetradecanoylphorbol-13-acetate may act directly on cell surface membranes (for review, see Ref. 1). One of the earliest biological effects of phorbol esters is the induction of platelet aggregation associated with release reaction (2-5), and the structural requirements of tigliane-type diterpenes for tumor promotion appear to be similar to those for platelet activation (3, 4). A series of recent reports from this laboratory (6-8) has shown that a Ca^{2+}-activated, phospholipid-dependent protein kinase is activated by unsaturated diacylglycerol which may be transiently formed during the receptor-mediated turnover of phosphatidylinositol. It is suggestive that in human platelets the enzyme activated in this way plays roles in serotonin release, presumably through the phosphorylation of one protein having $M_r \sim 40{,}000$ (9, 10). This protein kinase is present in a wide variety of tissues, and shows apparently neither tissue nor species specificity (11, 12). The enzyme requires absolutely Ca^{2+} and phospholipid, particularly phosphatidylserine for its activation (7, 13). Kinetic analysis indicates that diacylglycerol sharply increases the affinity of enzyme for Ca^{2+} as well as for phospholipid, and thus initiates the selective activation of this protein kinase (7, 8). We wish to describe here that in human platelets tumor-promoting phorbol esters such as TPA[1] can substitute for unsaturated diacylglycerol and, thus activate the protein kinase directly without provoking phosphatidylinositol turnover. Ca^{2+}-activated, phospholipid-dependent protein kinase and cyclic AMP-dependent protein kinase will be referred to as protein kinase C and protein kinase A, respectively.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Protein kinase C and Ca^{2+}-dependent protease were prepared from soluble fraction of rat brain as described previously (14). The catalytic fragment (protein kinase M) of protein kinase C was prepared by limited proteolysis with Ca^{2+}-dependent protease under the conditions specified earlier (14). Rabbit muscle glycogen phosphorylase kinase was prepared by the method of Cohen (15). Rabbit muscle protein kinase A was prepared as described previously (16). These enzyme preparations were free of each other and of endogenous phosphate acceptor proteins. A mixture of phospholipids used for the present studies was extracted from bovine brain by the method of Folch *et al.* (17) and fractionated on a silicic acid column as described by Rouser *et al.* (18). Human platelet-rich plasma and washed platelets were prepared by the method of Baenziger and Majerus (19). TPA and other phorbol derivatives were obtained from P. Borchert, Eden Praire, MN. Diolein and dimethyl sulfoxide were purchased from Nakarai Chemicals. [^{3}H]Arachidonic acid (78.2 Ci/mmol) and [^{14}C]serotonin (58 mCi/mmol) were obtained from New England Nuclear and Amersham, respectively. Bovine thrombin was obtained from Mochida Pharmaceutical Co. [γ-^{32}P]ATP, calf thymus H1 histone, and other materials and chemicals were prepared as described earlier (6, 8).

Enzyme Assays—Protein kinase C was assayed by measuring the incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP. The standard reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl at pH 7.5, 1.25 μmol of magnesium nitrate, 50 μg of H1 histone, 2.5 nmol of [γ-^{32}P]ATP (5 to 15 $\times$ 10^4 cpm/nmol), and 0.5 μg of protein kinase C. Phospholipid, diolein, phorbol esters, and Ca^{2+} were added as indicated in each experiment. All reagents were taken up in water which was prepared by a double distillation apparatus followed by passing through a Chelex 100 column to remove as much Ca^{2+} as possible as specified earlier (8). All reactions were carried out in plastic tubes. After incubation for 3 min at 30 °C, the reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials

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[1] The abbreviation used is: TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm). The catalytic fragment of protein kinase C was assayed similarly except that Ca^{2+}, phospholipid, and diolein were omitted. Protein kinase A was assayed under similar conditions except that 250 pmol of cyclic AMP was added instead of Ca^{2+}, phospholipid, and diolein. Glycogen phosphorylase kinase was assayed by measuring the incorporation of ^{32}P into phosphorylase from [γ-^{32}P]ATP as specified earlier (20). Ca^{2+}-dependent protease was assayed with ^{125}I-labeled casein as a substrate (21).

Assay for Platelet Protein Phosphorylation—The washed platelets (4×10^9 cells) were labeled with 1 mCi of carrier-free $^{32}P_i$ in 2 ml of Buffer A (0.14 M NaCl, 15 mM Tris/HCl at pH 7.5, and 5.5 mM glucose) as described by Lyons *et al.* (22). The radioactive platelets (6×10^8/ml) were then stimulated by thrombin or TPA as indicated in each experiment. The incubation was terminated by the addition of a half volume of a stop solution which contained 9% sodium dodecyl sulfate, 6% 2-mercaptoethanol, 15% glycerol, 0.186 M Tris/HCl at pH 6.7. The sample was boiled in a water bath for 3 min, and subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis under the conditions described by Laemmli (23). The separating and stacking gels contained 11 and 3% acrylamide, respectively. The gel was stained with Coomassie brilliant blue. After destaining, the gel was dried on a Whatman No. 1 filter paper, and exposed to an x-ray film to prepare the autoradiograph. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograph using a Shimadzu dual wavelength chromatogram scanner, Model CS-910.

Assays for Diacylglycerol Formation and ^{32}P Incorporation into Phospholipid—The platelet-rich plasma (36 ml) was incubated with 25 μCi of [^{3}H]arachidonic acid as described by Rittenhouse-Simmons (24), and platelets were isolated and washed as described (19). The radioactive platelets thus obtained were suspended in Buffer A (6×10^8 cells/ml) and stimulated by thrombin or TPA as indicated in each experiment. The incubation was terminated by the addition of chloroform/methanol (1:2) and the radioactive lipid was extracted by the method of Bligh and Dyer (25). Diacylglycerol was separated from the other lipids by Silica Gel G plate thin layer chromatography with a solvent system of benzene/diethylether/ethanol/ammonia water (50:40:20:0.1). The area corresponding to diacylglycerol was scraped into a vial and the radioactivity was determined.

In another set of experiments to measure phosphatidylinositol turnover, the platelets which were separately labeled with $^{32}P_i$ were suspended in Buffer A (6×10^8 cells/ml), and stimulated by thrombin or TPA. At various periods of time, the incubation was terminated by the addition of chloroform/methanol (1:2). Phospholipids were then extracted and isolated by Silica Gel G plate thin layer chromatography with a solvent system of chloroform/methanol/acetic acid/H_2O (25:15:4:2). The areas corresponding to each phospholipid were scraped into a vial and the radioactivity was determined.

Assay for Serotonin Release—The platelet-rich plasma (20 ml) was incubated with 1 μCi of [^{14}C]serotonin as described by Haslam and Lynham (26), and platelets were isolated and washed as described (19). The radioactive platelets thus obtained were suspended in Buffer A (6×10^8 cells/ml) and stimulated by thrombin or TPA as indicated in each experiment. The incubation was terminated by the addition of formaldehyde followed by centrifugation at 10,000 × *g* for 40 s by the method of Costa and Murphy (27). The radioactive serotonin released was determined.

Determinations—The radioactivity of ^{32}P-, ^3H-, and ^{14}C-labeled samples was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 3320. Protein was determined by the method of Lowry *et al.* (28) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Among various lipids tested so far, including monoacyl-, diacyl-, and triacylglycerols and free fatty acids, only unsaturated diacylglycerol was effective in the activation of protein kinase C (7, 8). However, it was found that, when TPA instead of unsaturated diacylglycerol was directly added to the reaction mixture, the enzymatic activity was greatly enhanced with the concomitant decrease in the Ca^{2+} concentration that was necessary for enzyme activation as shown in Fig. 1. Phospholipid was indispensable, and TPA alone was unable to activate the enzyme. Kinetic analysis indicated that TPA, like unsaturated diacylglycerol (7, 8), greatly increased the

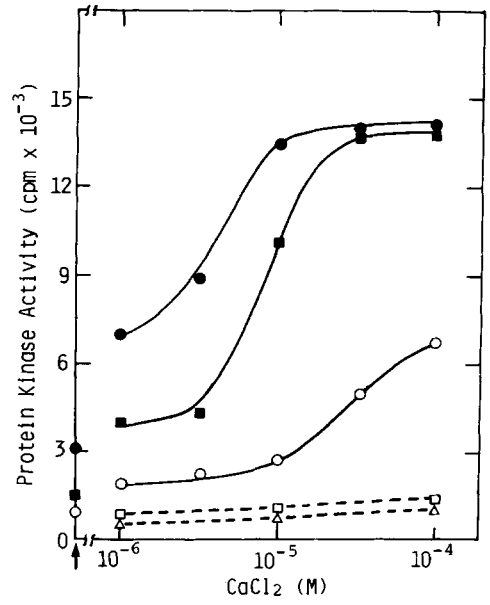

FIG. 1. **Activation of protein kinase C by TPA and unsaturated diacylglycerol at various concentrations of $CaCl_2$.** Protein kinase C was assayed under the standard conditions in the presence of $CaCl_2$ as indicated. TPA, diolein, and phospholipid were added as specified. Diolein, phospholipid, or both were dissolved in a small volume of chloroform. After chloroform was removed *in vacuo*, the residue was suspended in 20 mM Tris/HCl at pH 7.5 by sonication as described (7). TPA, which was dissolved in dimethyl sulfoxide, was directly mixed with phospholipid suspended in the buffer before being added to the reaction mixture. The final concentration of dimethyl sulfoxide in the reaction mixture was 0.01%. Where indicated with an *arrow*, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (0.5 mM at a final concentration) was added instead of $CaCl_2$. ○——○, in the presence of 20 μg/ml of phospholipid alone; □- - -□, in the presence of 10 ng/ml of TPA alone; △- - -△, in the presence of 0.8 μg/ml of diolein alone; ●——●, in the presence of 20 μg/ml of phospholipid plus 10 ng/ml of TPA; ■——■, in the presence of 20 μg/ml of phospholipid plus 0.8 μg/ml of diolein.

apparent affinity of enzyme for Ca^{2+} as well as for the phospholipid, and thus enhanced the enzyme activation. Namely, in the presence of phospholipid alone, relatively higher concentrations of Ca^{2+} were needed irrespective of the amount of phospholipid present, and the reaction velocity was accelerated by increasing amounts of phospholipid employed. When a saturating amount of phospholipid (about 100 μg/ml) was added, full enzymatic activity was obtained even though the K_a value for Ca^{2+}, the concentration needed for half-maximum activation, remained higher (about 7×10^{-5} M). If, however, a small amount of either TPA or diolein was supplemented to phospholipid, the K_a value for Ca^{2+} was dramatically decreased to be 10^{-6} M range. For instance, in the presence of TPA (10 ng/ml) or diolein (0.8 μg/ml) in addition to phospholipid (20 μg/ml), approximate K_a values of 2×10^{-6} and 8×10^{-6} M were obtained for this divalent cation, respectively; here diolein was not saturated. With saturating amount of TPA (more than 10 ng/ml) or diolein (more than 1.5 μg/ml), the same Ca^{2+} titration curves were obtained for these activators. In addition, TPA and diolein did not act as synergistic allies, and in the presence of a saturating amount of one of these two activators, no further enhancement of the reaction was observed by the addition of the other. However, at submaximal concentrations, the effects of TPA and diolein were apparently additive.

It has been described previously (14) that protein kinase C may alternatively be activated through limited proteolysis by Ca^{2+}-dependent neutral protease. The enzyme activated in

this way (protein kinase M) was catalytically fully active in the absence of Ca^{2+}, phospholipid, and diacylglycerol and was not susceptible to TPA. The result seems to indicate that the tumor promoter does not interact with the catalytically active site of the enzyme. Rather, it is suggestive that TPA may associate with lipid lamellae or micelles and modify the phospholipid-enzyme interaction to express full catalytic activity at physiologically lower concentrations of Ca^{2+}. Ca^{2+}-dependent neutral protease was not affected by TPA as assayed with casein as a substrate. Experiments shown in Fig. 2 indicated that low concentrations of TPA in the order of nanograms/ml showed significant effects; this tumor promoter at an amount of roughly one- to five-thousandths of that of diacylglycerol fully activated the protein kinase *in vitro*. Dimethyl sulfoxide itself showed practically no effect at the concentrations employed in these experiments. In similar *in vitro* systems, neither protein kinase A nor calmodulin-dependent protein kinase such as glycogen phosphorylase kinase was affected by TPA.

The next set of experiments was conducted to examine whether in intact cells TPA activates directly protein kinase C and causes some cellular response in an analogous manner to receptor-linked natural extracellular messengers. For this purpose, human platelets were employed. Preceding reports from this laboratory (9, 10) have proposed that in thrombin-stimulated platelets protein kinase C is activated by diacylglycerol which is derived from the receptor-linked breakdown of phosphatidylinositol and that the enzyme thus activated is probably responsible for the release of serotonin. In the experiment given in Fig. 3, washed human platelets were preincubated with $^{32}P_i$, and then stimulated by either thrombin or TPA. Consistent with the recent observations made by Chiang *et al.* (5), when platelets were activated by TPA, some endogenous platelet proteins were rapidly phosphorylated; in the present experiment, most predominantly 40-kilodalton protein and to some extent another protein having $M_r \sim 20{,}000$ were labeled. It has been described earlier (9) that 40-kilodalton protein serves as a preferred substrate for protein kinase C *in vitro* and that the phosphorylation of this particular protein is most likely related to release reaction.[2] In fact, in all experiments thus far done with intact platelets, the diacylglycerol formation that was induced either by thrombin (Fig. 4*A*) or by exogenously added phospholipase C (9) was always associated with 40-kilodalton protein phosphorylation as well as with serotonin release. On the other hand, 20-kilodalton protein has been identified as myosin light chain, and another species of protein kinase, that is Ca^{2+}-calmodulin-regulated myosin light chain kinase, has been proposed to be responsible for the phosphorylation of this protein (30, 31). It is evident from the autoradiograph that 20-kilodalton protein was phosphorylated only slightly, when platelets were stimulated by TPA. It is likely that Ca^{2+} influx or movement may be limited at least in the early phase of the TPA-induced platelet activation. The rapid disappearance of diacylglycerol shown in Fig. 4*A* was probably due to the conversion to phosphatidic acid and also to further degradation to arachidonic acid and its metabolites.

In a marked contrast to thrombin, TPA induced serotonin release in parallel with 40-kilodalton protein phosphorylation, but did not produce diacylglycerol under similar conditions as shown in Fig. 4*B*. In the experiments shown in Fig. 4, *A* and *B*, however, the rates and extents of the release of serotonin were different, although the extents of 40-kilodalton protein phosphorylation in both systems were roughly the same. The reason for this difference is not known, but it is possible that Ca^{2+} plays some roles in the secretory process of serotonin. Another possibility which may not be ruled out is that diacylglycerol has additional roles during the platelet activation. For instance, diacylglycerol is known as a membrane fusigen (32, 33) and also serves as a precursor to ionophoric phosphatidic acid (for review, see Ref. 34) as well as to arachidonic

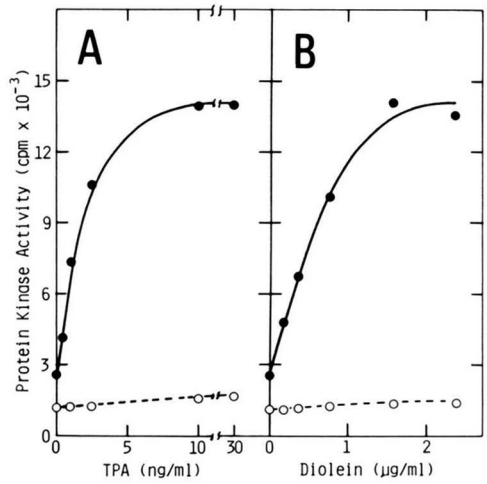

FIG. 2. **Dose-dependent activation of protein kinase C by TPA and diolein.** Protein kinase C was assayed under the standard conditions in the presence of 20 μg/ml of phospholipid, 1×10^{-5} M $CaCl_2$, and various amounts of either TPA or diolein as indicated. *A*, with TPA; *B*, with diolein. ●——●, in the presence of phospholipid plus $CaCl_2$; ○- - -○, in the presence of $CaCl_2$ and without phospholipid. The enzymatic activity obtained in the presence of phospholipid and without $CaCl_2$ was nearly the same as that obtained in the presence of $CaCl_2$ and without phospholipid (data not shown).

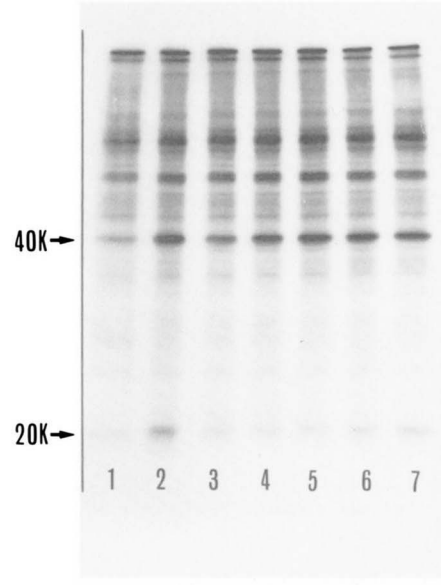

FIG. 3. **Autoradiograph of TPA-induced platelet protein phosphorylation.** The platelets, which were labeled with $^{32}P_i$, were stimulated at 37 °C by 0.25 unit/ml of thrombin or by 100 ng/ml TPA for various periods of time as indicated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were done as described under "Experimental Procedures." *Lane 1*, control; *Lane 2*, with thrombin for 60 s; *Lane 3*, with TPA for 10 s; *Lane 4*, with TPA for 30 s; *Lane 5*, with TPA for 60 s; *Lane 6*, with TPA for 90 s; *Lane 7*, with TPA for 120 s.

[2] This 40-kilodalton protein corresponds to the 47-kilodalton protein designated by other workers (for review, see Ref. 29). It is noted that the sites of phosphorylation in the purified *in vitro* 40-kilodalton protein-protein kinase C system are shown to be identical with those in the thrombin-stimulated platelet system *in vivo* as judged by fingerprint analysis (manuscript in preparation).

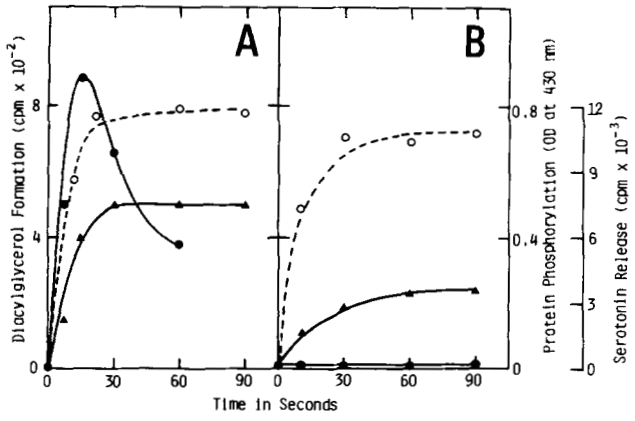

FIG. 4. **Time courses of diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release in platelets.** The platelets, which were labeled with either [^{3}H]arachidonic acid, $^{32}P_i$, or [^{14}C]serotonin, were stimulated at 37 °C by 0.25 unit/ml of thrombin or by 100 ng/ml of TPA for various periods of time as indicated. Diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release were assayed as described under "Experimental Procedures." *A*, with thrombin; *B*, with TPA. ●——●, diacylglycerol formation; ○- - -○, 40-kilodalton protein phosphorylation; ▲——▲, serotonin release.

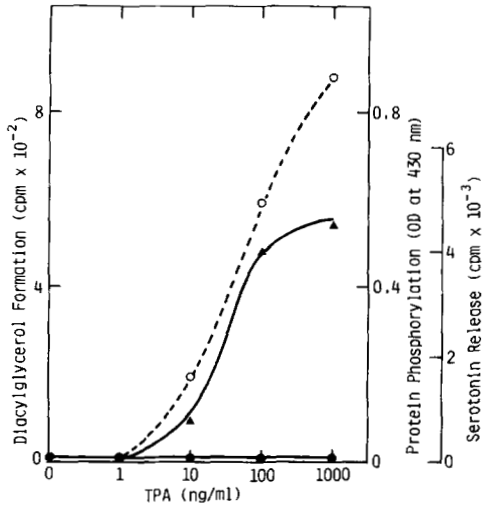

FIG. 5. **Effect of TPA concentration on diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release in platelets.** The platelets, which were labeled with either [^{3}H]arachidonic acid, $^{32}P_i$, or [^{14}C]serotonin, were stimulated for 1 min at 37 °C by various amounts of TPA as indicated. Diacylglycerol formation, 40-kilodalton protein phosphorylation, and serotonin release were assayed as described under "Experimental Procedures." The *symbols* are the same as those given in Fig. 4.

acid which is rapidly converted to thromboxane (35). Fig. 5 shows dose responses to TPA for such 40-kilodalton protein phosphorylation and release reaction. It is shown that the concentration of TPA necessary for platelet activation was roughly 10 times higher than that required for protein kinase C activation *in vitro*. Presumably, in intact platelets, more TPA is necessary to intercalate into the lipid bilayer structure leading to the protein kinase activation. Again, essentially no diacylglycerol was produced over a wide range of TPA concentrations. The results seem to indicate that this tumor promoter directly activates protein kinase C without provoking phosphatidylinositol breakdown. Further evidence supporting this assumption was provided by the fact that the incorporation of radioactive inorganic phosphate into phosphatidylinositol and phosphatidic acid (phosphatidylinositol turnover) was markedly accelerated by thrombin but not by TPA, although 40-kilodalton protein was actively phosphorylated and serotonin was released under the same conditions (data not shown). It has been described that TPA enhances the incorporation of radioactive choline into phosphatidylcholine in mouse epidermis (36) and bovine lymphocytes (37) several minutes or hours after stimulation, presumably as a result of increased membrane perturbation. However, in human platelets which were stimulated by TPA, the degradation of phosphatidylcholine as well as the incorporation of ^{32}P into this phospholipid was negligible at least at early phase of the platelet activation. The activation of platelets by TPA was usually completed within 1 min, and the appearance of lysophospholipids, if any, was negligible under the present conditions. Using human neutrophils (38), it has been recently reported that TPA shows no effect on arachidonate release at doses which maximally stimulate both degranulation and oxidative metabolism. Probably, the effect of TPA on phosphatidylcholine metabolism may largely depend on cell types employed and/or may be observed at a later phase of TPA actions. The detailed metabolic cascade of various phospholipids that might occur after the addition of TPA remains to be explored.

Table I shows relative activities of various phorbol derivatives to activate protein kinase C in *in vitro* systems. It was noted that phorbol derivatives showing tumor-promoting activity could activate protein kinase C as well. The structural requirements of phorbol-related diterpenes for tumor promotion on mouse skin (for review, see Ref. 39) appear to be roughly similar to those for protein kinase C activation.

A number of kinetic studies using a variety of cell systems appear to suggest that the biochemical target of phorbol esters leading to activation of specific cellular functions or proliferation may be located on membranes. The pleiotropic actions as well as the structure-activity relations of these tumor promoters seem to be compatible with the supposition that there is a specific cell surface receptor that is widespread on various tissues and organs. The results presented above seem to indicate that one of the possible targets of TPA actions is protein kinase C, although it is not known at present whether the activation of this enzyme is directly related to the mechanism involved in the tumor promotion. Under normal conditions, the activation of this enzyme appears to be induced by a large number of hormones, neurotransmitters, and many other biologically active substances including epidermal growth factor and lymphocyte mitogens of plant origin, which are all able to provoke phosphatidylinositol turnover (34). However, this may not necessarily rule out a role of protein kinase C in the tumor promotion. It is possible that the tumor-

TABLE I

Effects of various phorbol derivatives on activation of protein kinase C in vitro

Protein kinase C was assayed under the standard conditions in the presence of 20 μg/ml of phospholipid, 1×10^{-5} M $CaCl_2$, and 10 ng/ml each of various phorbol derivatives.

Phorbol derivative	Protein kinase C activity
	%
TPA	100
Phorbol-12,13-didecanoate	81
Phorbol-12,13-dibutyrate	88
Phorbol-12,13-dibenzoate	100
Phorbol-12-tetradecanoate	0
Phorbol-13-acetate	0
4α-Phorbol-12,13-didecanoate	0
Phorbol	0

promoting phorbol ester once intercalated into membranes remains active for prolonged periods of time, since the diterpene is hardly metabolizable (1). In contrast, diacylglycerol, the natural activator of this enzyme, occurs transiently during the phosphatidylinositol turnover and disappears very quickly. Nevertheless, possible roles of this protein kinase in the regulation which may be essential to the activation of specific functions or proliferation of mammalian cells remain largely unexplored.

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