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

Monique Castagnat, Yoshimi Takai, Kozo Kaibuchi, Kimihiko Sano, Ushio Kikkawa, and Yasutomi Nishizuka

From the Department of Biochemistry, Kobe University School of Medicine, Kobe 650 and the Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan

Tumor-promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) directly activate in vitro Ca²⁺-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 Ca²⁺ 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 Ca²⁺-activated, phospholipid-dependent protein kinase is activated by unsaturated diacylglycerol which may be transiently formed during the receptor-mediated turnover.

* This investigation has been supported in part by research grants from the Scientific Research Fund of the Ministry of Education, Science and Culture, Japan (1979–1981), the Intractable Diseases Division, Public Health Bureau, the Ministry of Health and Welfare, Japan (1981), a Grant-in-Aid of New Drug Development from the Ministry of Health and Welfare, Japan (1979–1981), the Yamanouchi Foundation for Research on Metabolic Disorders (1977–1981), and the Mitsuhsa Cancer Research Foundation (1981). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.

† Visiting Scientist under the Japan-France exchange program sponsored by the Japan Society for Promotion of Science and the Centre National de la Recherche Scientifique. Present address, Institut de Recherches Scientifique sur le Cancer, Villejuif, France.

§ To whom correspondence should be addressed at Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan.

Materials and Chemicals—Protein kinase C and Ca²⁺-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 Ca²⁺-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 Baenzigier and Majerus (19). TPA and other phorbol derivatives were obtained from P. Borchert, Edin Prairie, MN. Diolein and dimethyl sulfoxide were purchased from Nakarai Chemicals. [³H]Arachidonic acid (78.2 Ci/mm), and [³C]serotonin (58 mCi/mm) were obtained from New England Nuclear and Amersham, respectively. Bovine thrombin was obtained from Mochida Pharmaceutical Co. [γ⁻³P]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 ³²P into H1 histone from [γ⁻³²P]ATP. The standard reaction mixture (0.25 ml) contained 5 nmol of Tris/HCl at pH 7.5, 1.25 µmol of magnesium nitrate, 50 µg of H1 histone, 2.5 µmol of [³²P]ATP (5 to 15 x 10⁶ cpm/nmol), and 0.5 µg of protein kinase C. Phospholipid, diolein, phorbol esters, and Ca²⁺ were added as indicated in each experiment. All reagents were mixed and H1 histone was phosphorylated by a double distillation apparatus followed by passing through a Chelex 100 column to remove as much Ca²⁺ as possible as specified earlier (8). All reactions were carried out in plastic tubes. After incubation for 3 min at 30 ºC, the reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials were collected on filters and counted in a liquid scintillation counter.

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 diolene 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 diolene. Glycogen phosphorylase kinase was assayed by measuring the incorporation of \(^3\)P into phosphorylase from [\(^{32}\)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 \(^3\)H, in 2 ml of Tris/HCl buffer (pH 7.5). The platelet-rich plasma (36 ml) was incubated with 25 μCi of \[^{3}H\]parachidonic 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 × 10^9 cells/ml) and 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% β-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.

**Assay for Diacylglycerol Formation and \(^3\)P Incorporation into Phospholipid**—The platelet-rich plasma (36 ml) was incubated with 25 μCi of \[^{3}H\]parachidonic 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 × 10^9 cells/ml) and stimulated by thrombin or TPA. At various periods of time, the incubation was terminated by the addition 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, the enzyme for Ca\(^{2+}\), 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_v 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 diolene was supplemented to phospholipid, the K_v value for Ca\(^{2+}\) was dramatically decreased to 10^{-4} M range. For instance, in the presence of TPA (10 ng/ml) or diolene (0.8 μg/ml) in addition to phospholipid (20 μg/ml), approximate K_v values of 2 × 10^{-4} and 8 × 10^{-4} M were obtained for this divalent cation, respectively; here diolene was not saturated. With saturating amount of TPA (more than 10 ng/ml) or diolene (more than 1.5 μg/ml), the same Ca\(^{2+}\) titration curves were obtained for these activators. In addition, TPA and diolene 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 sub-maximal concentrations, the effects of TPA and diolene were apparently additive.

**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, like unsaturated diacylglycerol (7, 8), greatly increased the 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_v 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 diolene was supplemented to phospholipid, the K_v value for Ca\(^{2+}\) was dramatically decreased to 10^{-4} M range. For instance, in the presence of TPA (10 ng/ml) or diolene (0.8 μg/ml) in addition to phospholipid (20 μg/ml), approximate K_v values of 2 × 10^{-4} and 8 × 10^{-4} M were obtained for this divalent cation, respectively; here diolene was not saturated. With saturating amount of TPA (more than 10 ng/ml) or diolene (more than 1.5 μg/ml), the same Ca\(^{2+}\) titration curves were obtained for these activators. In addition, TPA and diolene 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 sub-maximal concentrations, the effects of TPA and diolene were apparently additive.
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\(_\text{i}\), 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 \(M_\text{r}\) \(\sim\) 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. 4A) 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. 4A 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. 4B. 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 acid.
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

| 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  |

**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 × 10⁻⁴ M CaCl₂, and 10 μg/ml each of various phorbol derivatives.
promoting phorbol ester once intercalated into membranes remains active for prolonged periods of time, since the diter-
ol is hardly metabolizable (1). In contrast, dioleoylglycerol, 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.

Acknowledgments—We are grateful to S. Nishiyama and K. Yamashita for their skillful secretarial assistance.

REFERENCES
1. Blumberg, P. M. (1980) CRC Crit Rev. Toxicol. 8, 153–234
2. Zucker, M., Troll, W., and Belman, S. (1974) J. Cell Biol. 60, 325–336
3. White, J. G., Rao, G. H. R., and Estensen, R. D. (1974) Am. J. Pathol. 75, 301–314
4. Munson, R. A., Kulkarni, P., Eakins, K. E., and Weinstein, I. B. (1979) Cancer Res. 39, 3692–3696
5. Chiang, T. M., Cagen, L. M., and Kang, A. M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1981) Thromb. Res. 21, 561–562
6. Takai, Y., Kishimoto, A., Iwasa, K., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695
7. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218–1224
8. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276
9. Kawahara, Y., Takai, Y., Minakuchi, S., Sano, K., and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 97, 369–371
10. Takai, Y., Kaibuchi, K., Matsubara, T., and Nishizuka, Y. (1981) Biochem. Biophys. Res. Commun. 101, 61–67
11. Minakuchi, R., Takai, Y., Yu, B., and Nishizuka, Y. (1981) J. Biochem. (Tokyo) 89, 1651–1654
12. Kuo, F. J., Andresson, R. G. G., Wine, B. C., Macklerova, L., Salomonsson, I., Brackett, N. L., Kato, N., Shoji, M., and Wrenn, R. W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7039–7043
13. Kaibuchi, K., Takai, Y., and Nishizuka, Y. (1981) J. Biol. Chem. 256, 7146–7149
14. Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610–7616
15. Cohen, P. (1973) Eur. J. Biochem. 34, 1–14
16. Yamamura, H., Nishiyama, K., Shimomura, R., and Nishizuka, Y. (1979) Biochemistry 18, 856–862
17. Folch, J., Lees, M., and Stanley, G. H. S. (1957) J. Biol. Chem. 226, 497–509
18. Rouyer, G., Kritchevsky, G., and Yamamoto, A. (1987) in Lipid Chromatographic Analysis, Vol. 1, pp. 99–162, Marcel Dekker, Inc., New York
19. Baenziger, N. L., and Majerus, P. W. (1974) Methods Enzymol. 31, 149–155
20. Sakai, K., Matsumura, S., Okinura, Y., Yamamura, H., and Nishizuka, Y. (1979) J. Biol. Chem. 254, 6631–6637
21. Kishimoto, A., Kajikawa, N., Tabuchi, H., Shiota, M., and Nishizuka, Y. (1981) J. Biochem. (Tokyo) 90, 889–892
22. Lyons, R. M., Stanford, N., and Majerus, P. W. (1975) J. Clin. Invest. 56, 924–936
23. Laemmli, U. K. (1970) Nature (Lond.) 227, 680–686
24. Rutenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580–587
25. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
26. Haslam, R. J., and Lynham, J. A. (1977) Biochem. Biophys. Res. Commun. 77, 714–722
27. Costa, J. L., and Murphy, D. L. (1975) Nature 255, 407–408
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
29. Haslam, R. J., Salam, S. E., Fox, J. E. B., Lynham, J. A., and Davidson, M. M. L. (1980) in Cellular Response Mechanisms and their Biological Significance (Rotman, A., Meyer, F. A., Gilger, C., and Silberberg, A., eds) pp. 213–231, John Wiley & Sons Ltd, New York
30. Daniel, J. L., Holmsen, H., and Adelstein, R. S. (1977) Thromb. Haemostas. 38, 984–989
31. Hathaway, D. R., and Adelstein, R. S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1653–1657
32. Allan, D., and Michell, R. A. (1975) Nature 258, 349–349
33. Allan, D., Bilah, M. M., Finean, J. B., and Michell, R. H. (1978) Nature 261, 58–60
34. Michell, R. H. (1975) Biochim. Biophys. Acta 415, 81–147
35. Bell, R. L., Kennerly, D. A., Stanford, N., and Majerus, P. W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3238–3241
36. Bohlenschieter, L. R., O’Brien, D. H., and Boutwell, R. K. (1972) Biochim. Biophys. Acta 280, 57–70
37. Wertz, P. W., and Mueller, G. C. (1978) Cancer Res. 38, 2900–2904
38. Walsh, C. E., Waite, B. M., Thomas, M. J., and DeChatelet, L. R. (1981) J. Biol. Chem. 256, 7228–7234
39. Hecker, E. (1976) in Carcinogenesis (Slaga, T. J., Sivak, A., and Boutwell, R. K., eds) Vol. 2, pp. 11–48, Raven Press, New York

Activation of Protein Kinase by Phorbol Esters

7851