Biochemical Pharmacology, Vol. 37, No. 8, pp. 1541–1545, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

COMPARISON OF PROTEIN KINASE C FUNCTIONAL ASSAYS TO CLARIFY MECHANISMS OF INHIBITOR ACTION

TERUO NAKADATE,*† ARCO Y. JENG‡ and PETER M. BLUMBERG†§

† Molecular Mechanisms of Tumor Promotion Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and ‡ Research Department, Pharmaceuticals Division, Ciba-Geigy Corp., Summit, NJ 07901, U.S.A.

(*Received* 15 *June* 1987; *accepted* 14 *September* 1987)

Abstract—The effects of inhibitors of protein kinase C on the activities of the intact enzyme, the proteolytically-generated catalytic domain, and [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding were compared in an effort to evaluate this approach for clarifying mechanisms of inhibitor action. Staurosporine, H-7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], and quercetin inhibited the catalytic fragment with similar potencies as for the intact enzyme while having little or no effect on binding, consistent with reports that they are competitive with ATP. Adriamycin, trifluoperazine, and tamoxifen, suggested to disrupt hydrophobic interactions between the regulatory domain of protein kinase C and phospholipid, were all most effective on the intact enzyme. They appear to possess a mixed mechanism, however, inhibiting activity of the catalytic domain with approximately 3-fold lower potencies. Gossypol inhibited intact enzyme, catalytic fragment, and PDBu binding with similar potencies. In light of multiple apparent sites of action for such protein kinase C inhibitors, comparison of their activities on the individual functional domains of the kinase may provide a useful complement to studies with the intact enzyme.

The intracellular receptor of the phorbol ester tumor promoters is protein kinase C [1, 2], which mediates one arm of the message transduction pathway for a large class of hormones and other cellular effectors which act by inducing phosphatidylinositol 4,5-bisphosphate breakdown [1, 3]. Protein kinase C possesses separate regulatory and catalytic domains [2, 4]. Proteolytic treatment generates the activated Ca^{2+}/phospholipid-independent form, i.e. catalytic fragment of protein kinase C, as well as the regulatory fragment which possesses the phospholipid and phorbol ester binding sites [5–7].

A variety of compounds have been reported to inhibit protein kinase C [8–16], and some of them were shown to work as anti-tumor-promotors [17–19]. Most inhibitors have been simply classified as interacting with the regulatory domain or the catalytic domain based on analysis using intact protein kinase C to determine whether inhibition was competitive or noncompetitive with respect to phospholipid, calcium, and ATP. However, this approach is both involved and, in the case of ligands with multiple sites of action, difficult to interpret. Multiple sites of action may not be unusual for currently described inhibitors. For example, Inagaki *et al.* [20] have shown that naphthalenesulfonamides, which were known as calmodulin inhibitors, inhibited protein phosphorylation by protein kinase not only by inhibition of enzyme activation but also by inhibition of the catalytic activity, using in their analysis the catalytic fragments of myosin light chain kinase and protein kinase C. In the present study, we have broadened this approach to compare the effects of several inhibitors on histone phosphorylation by intact protein kinase C and by its catalytic fragment and on [^{3}H]phorbol 12,13-dibutyrate (PDBu‖) binding to intact protein kinase C. Our results suggest that this approach may provide a useful screening method in the development of inhibitors of protein kinase C as well as for understanding their inhibitory mechanisms.

MATERIALS AND METHODS

Materials. PDBu, phosphatidylserine, histone III-S, fatty acid free bovine serum albumin, bovine gamma globulin, quercetin, gossypol, tamoxifen, trifluoperazine, and adriamycin were purchased from Sigma (St. Louis, MO). H-7 [1-(5-isoquinolinylsulfonyl)2-methylpiperazine] was from Seikagaku America (St. Petersburg, FL). ATP was obtained from Boehringer Mannheim (Indianapolis, IN). Sphingosine was a product of Calbiochem (La Jolla, CA). [20-^{3}H]PDBu (15.8 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from New England Nuclear

* Present address: Department of Pharmacology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

§ Correspondence should be addressed to: Peter M. Blumberg, Ph.D., Molecular Mechanisms of Tumor Promotion Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bldg. 37, Rm. 3B25, National Institutes of Health, Bethesda, MD 20892.

‖ Abbreviations: PDBu, phorbol 12,13-dibutyrate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; and EGTA, ethylene glycol bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

(Boston, MA). Staurosporine was a gift from the Kyowa Hakko Kogyo Co. (Tokyo, Japan).

Protein kinase C and its catalytic fragment. Protein kinase C was purified from mouse brain cytosol according to the method of Jeng *et al.* [21]. The last step using a Phenyl-5-PW column was omitted, but the purity of protein kinase C was high (approximately 95%) at this Mono Q/ATP step [21]. The catalytic fragment of protein kinase C was obtained and purified by Mono Q column chromatography as described previously [22].

Protein kinase assay. Protein kinase C was assayed by measuring ^{32}P transferred from [γ-^{32}P]ATP to histone H1 (Sigma III-S). The reaction mixture contained, in a total volume of 50 μl, 0.01 to 0.05 μg enzyme, 80 mM Tris–Cl, pH 7.4, 7.5 mM magnesium acetate, 750 μg/ml histone H1, 250 μg/ml bovine serum albumin (fatty acid free), 1 mM dithiothreitol, either 100 μM added calcium chloride or ethylene glycol bis(beta-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA), and 25 μg/ml phosphatidylserine and 12 nM PDBu, if used. Since a Ca^{2+}-EGTA buffer system was not used, the actual free Ca^{2+} concentration might differ from the nominal concentration. The drugs were dissolved in water or dimethyl sulfoxide (final 0.4%) and added to the reaction mixture. The reaction mixture was preincubated at 30° for 5 min. After addition of 25 μM [γ-^{32}P]ATP (100–150 cpm/pmol), the reaction mixture was further incubated for 3 min. Reactions were terminated by cooling to 4°, and the incorporated ^{32}P was determined using phosphocellulose paper as described [23].

[^{3}H]*PDBu binding assay.* The conditions for the [^{3}H]PDBu binding assay were similar to those of the kinase assay, except that the reaction volume was 250 μl, 0.15 to 0.25 μg enzyme was used, and the reaction mixture contained 12 nM [^{3}H]PDBu instead of non-radioactive PDBu. The reaction mixture was incubated for 5 min at 30° and then cooled to 4°. The bound and free [^{3}H]PDBu were measured using the polyethylene glycol precipitation method. To promote precipitation, bovine gamma globulin (1000 μg) was added to the tube just before the addition of polyethylene glycol. For estimation of non-specific binding, 30 μM cold PDBu was added to the mixture.

RESULTS

The catalytic fragment of protein kinase C phosphorylates histone H1 at pH 7.4 in a calcium- and phospholipid-independent fashion. Figure 1 shows the effects of various compounds on histone phosphorylation by the catalytic fragment of protein kinase C. Quercetin and H-7 potently inhibited activity of the catalytic fragment. Gossypol showed slight enhancement of phosphorylation at low concentrations (10–30 μM) and complete inhibition at a high concentration (300 μM). Trifluoperazine, tamoxifen, and adriamycin showed partial but significant ($P < 0.05$, Student's *t*-test) inhibition of histone phosphorylation at high concentrations. It has been reported that sphingosine does not interact with the catalytic domain of protein kinase C [16]. In the present study, sphingosine potently inhibited histone phosphorylation by the catalytic fragment of protein kinase C (Fig. 1). However, as shown in Fig. 2, the inhibition by sphingosine was less potent in the presence of 25 μg/ml phosphatidylserine. Moreover, the inhibition was not observed in the presence of 250 μg/ml phosphatidylserine or 0.3% Triton X-100 in the reaction mixture. Staurosporine caused inhi-

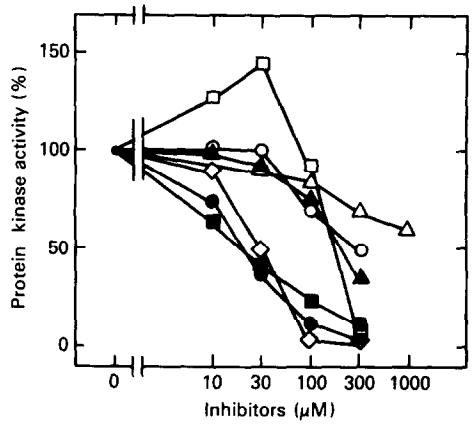

Fig. 1. Effects of various inhibitors on histone phosphorylation by the catalytic fragment of protein kinase C. After preincubation of the reaction mixture containing each inhibitor and the catalytic fragment of protein kinase C for 5 min at 30°, histone phosphorylation was measured by the addition of [γ-^{32}P]ATP and incubation for 3 min in the absence of cofactors, i.e. calcium and phosphatidylserine, and in the presence of EGTA (0.1 mM). Protein kinase activity in the absence of ligand was 273 ± 24 nmol/mg/min (mean ± SD, N = 12) which represents 100%. Each point is the mean of duplicate determinations. Each inhibition curve is a representative result from two to three separate experiments. Symbols: (●) quercetin; (○) tamoxifen; (▲) trifluoperazine; (△) adriamycin; (■) H-7; (□) gossypol; and (◇) sphingosine.

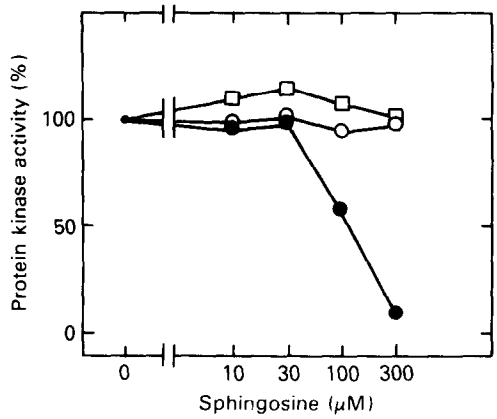

Fig. 2. Effect of sphingosine on histone phosphorylation by the catalytic fragment of protein kinase C in the presence of phosphatidylserine or Triton X-100. Reaction conditions were similar to those in Fig. 1 except that the reaction mixture contained phosphatidylserine (25 and 250 μg/ml) or Triton X-100 (0.3%). Protein kinase activity in the absence of sphingosine represents 100% and corresponds to 320, 380, and 420 nmol/mg/min respectively. Each point is the mean of duplicate determinations. A similar result was obtained in another experiment. Symbols: (●) 25 μg/ml phosphatidylserine; (□) 250 μg/ml phosphatidylserine; and (○) 0.3% Triton X-100.

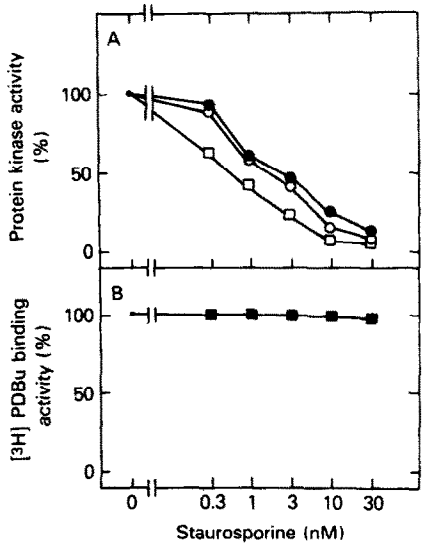

Fig. 3. Effect of staurosporine on histone phosphorylation by the catalytic fragment and intact protein kinase C (A), or on [^{3}H]PDBu binding activity (B). Each result is the mean of duplicate determinations, and similar results were obtained in another experiment. Symbols: in panel A, (□) histone phosphorylation by catalytic fragment; (●) histone phosphorylation by intact protein kinase C in the presence of 0.1 mM calcium, 25 μg/ml phosphatidylserine and 12 nM PDBu; and (○) histone phosphorylation by intact protein kinase C in the presence of 0.1 mM calcium and 25 μg/ml phosphatidylserine; in panel B, (■) [^{3}H]PDBu binding activity.

bition of histone phosphorylation by the catalytic fragment at very low concentrations (Fig. 3A, open squares). The interactions between trypsin-treated protein kinase C and trifluoperazine or H-7 have been reported previously [11, 24] and were found to be similar to the present results.

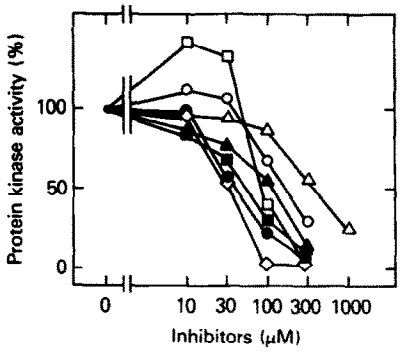

Fig. 4. Effects of various compounds on histone phosphorylation by intact protein kinase C. After preincubation of the reaction mixture containing each inhibitor and intact protein kinase C for 5 min at 30°, histone phosphorylation was measured by the addition of [γ-^{32}P]ATP and incubation for 3 min in the presence of cofactors, i.e. 0.1 mM calcium, 25 μg/ml phosphatidylserine and 12 nM PDBu. Protein kinase C activity in the absence of test compound was 240 ± 19 nmol/mg/min (mean ± SD, N = 8) and represents 100%. Each point is the mean of duplicate determinations. Each inhibition curve is a representative result from two to three separate experiments. Symbols: same as in Fig. 1.

Figure 4 shows the effects of various compounds on histone phosphorylation by intact protein kinase C in the presence of calcium, phosphatidylserine, and PDBu. Quercetin, H-7, gossypol, trifluoperazine, and tamoxifen inhibited histone phosphorylation by intact protein kinase C at 100–300 μM. Sphingosine inhibited activity at less than 100 μM. Adriamycin inhibited activity at 300–1000 μM. Low concentrations (10–30 μM) of gossypol had a tendency to enhance histone phosphorylation by protein kinase C. Inhibition of protein kinase C by staurosporine was observed in the nanomolar range (Fig. 3A, open and closed circles) as reported previously [15].

The highly purified protein kinase C from mouse brain used in the present study was fully activated by calcium (0.1 mM) and phosphatidylserine (25 μg/ml) under our assay conditions, and further stimulation of phosphorylation upon the addition of PDBu at 12 nM (Fig. 3) or higher concentrations (120 and 1200 nM) (data not shown) was not evident in this system. Similar results have been reported by others [25–27]. The effects of all inhibitors used in the present study were similar if assayed in the absence of PDBu (data not shown) rather than in its presence.

In many reports, the effects of inhibitors on protein kinase C enzymatic activity and [^{3}H]PDBu binding activity have been studied using different incubation conditions, complicating comparison. Inhibition of [^{3}H]PDBu binding activity was therefore determined using the same incubation conditions as for the kinase assay. Gossypol and sphingosine inhibited [^{3}H]-PDBu binding (Fig. 5) at concentrations very similar to those inhibiting activity of the intact kinase. Trifluoperazine and quercetin showed slight inhibition of [^{3}H]PDBu binding at high concentrations

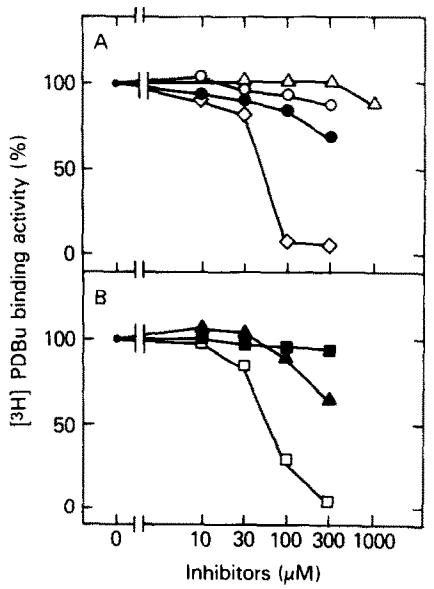

Fig. 5. Effects of various inhibitors on [^{3}H]PDBu binding activity to protein kinase C. [^{3}H]PDBu binding activity in the absence of test compound was 4089 ± 576 pmol/mg (mean ± SD, N = 7) and represents 100%. Each result is the mean of duplicate determinations, and each inhibition curve is a representative result from two to three separate experiments. Symbols: same as in Fig. 1.

(Fig. 5). Adriamycin and H-7 (Fig. 5) and staurosporine (Fig. 3B) did not show inhibition. Similar results were obtained under incubation conditions of 37° and 30 min.

DISCUSSION

Quercetin, H-7, and staurosporine were shown to be potent inhibitors for the catalytic fragment of protein kinase C and to have little or no activity on the binding domain. Inhibition of the intact kinase was similar to that for the catalytic fragment. These results are consistent with earlier evidence that these compounds interact at the active site of the intact enzyme as shown by competition with ATP [11, 28] and lack of competition with phospholipid [15].

Tamoxifen, gossypol, trifluoperazine, and adriamycin contain lipophilic or non-polar regions, and these compounds have been thought to inhibit protein kinase C by their ability to interfere with a hydrophobic interaction between phospholipid and the enzyme without an interaction with the catalytic site [8, 10, 12, 13]. The present results show that these compounds in fact caused inhibition of histone phosphorylation by the catalytic fragment. Although the IC_{50} value for the catalytic fragment was bigger than that for intact protein kinase C, the difference was only 2- to 3-fold in each case. Interaction at the catalytic domain should therefore be partially responsible for the overall inhibition observed with the intact enzyme. A similar result was reported for *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7); W-7 inhibited the trypsin-treated myosin light chain kinase and protein kinase C in a competitive fashion with respect to ATP, although it also inhibited the native enzymes in a competitive fashion with respect to calmodulin and phospholipid [20].

Sphingosine has been reported recently to inhibit protein kinase C activity and [^{3}H]PDBu binding by interfering with the function of its regulatory domain [16], and the possible physiological role of sphingosine as a negative effector of protein kinase C has been suggested [19]. However, the interaction of the catalytic fragment with sphingosine was determined using the mixed micellar assay which includes 0.03% Triton X-100. Under such conditions or in the presence of high concentrations of phosphatidylserine, we likewise observed no inhibition of histone phosphorylation by the catalytic fragment. However, in the absence or at low concentrations of phosphatidylserine, sphingosine inhibited the catalytic fragment with a potency comparable to H-7. The difference presumably reflects sequestration of the sphingosine in the hydrophobic phase at high lipid and detergent concentrations. Unless assay conditions are carefully evaluated, sphingosine therefore ought not to be regarded as a simple negative effector of protein kinase C with a single site of action.

Inhibition of [^{3}H]PDBu binding to the phorbol ester binding site on protein kinase C is one index of the interaction between a compound and the regulatory domain of the kinase. On the one hand, tamoxifen and adriamycin inhibited protein kinase C activity without inhibiting [^{3}H]PDBu binding activity, suggesting that these compounds interfere with the interaction between phospholipid and the regulatory domain at a site independent of the phorbol ester binding site. On the other hand, inhibition by gossypol correlated well with inhibition of [^{3}H]PDBu binding, and trifluoperazine was intermediate. These latter compounds may either interfere directly with the phorbol ester binding site or else with the lipid requirement for binding.

The present results showed that we could detect direct interaction of inhibitors with the active site of the enzyme by using the catalytic fragment to eliminate actions on the regulatory domain. Comparison of inhibitory activity on intact protein kinase C, on its catalytic fragment, and on [^{3}H]PDBu binding may be a useful screening method for assessing the mechanism of protein kinase C inhibitors.

Acknowledgements—We thank Stuart H. Yuspa for critical reading of the manuscript and Charlotte Crumley for typing.

REFERENCES

1. Y. Nishizuka, *Nature, Lond.* **308**, 693 (1984).
2. C. L. Ashendel, *Biochim. biophys. Acta* **822**, 219 (1985).
3. Y. Nishizuka, *Science* **233**, 305 (1986).
4. A. Kishimoto, N. Kajikawa, M. Shiota and Y. Nishizuka, *J. biol. Chem.* **258**, 1156 (1983).
5. M. Hoshijima, A. Kikuchi, T. Tanimoto, K. Kaibuchi and Y. Takai, *Cancer Res.* **46**, 3000 (1986).
6. K-P. Huang and F. L. Huang, *Biochem. biophys. Res. Commun.* **139**, 320 (1986).
7. M-H. Lee and R. M. Bell, *J. biol. Chem.* **261**, 14867 (1986).
8. B. C. Wise and J. F. Kuo, *Biochem. Pharmac.* **32**, 1259 (1983).
9. N. Katoh, B. C. Wise, R. W. Wrenn and J. F. Kuo, *Biochem. J.* **198**, 199 (1981).
10. K. Kimura, K. Sakurada and N. Katoh, *Biochim. biophys. Acta* **839**, 276 (1985).
11. H. Hidaka, M. Inagaki, S. Kawamoto and Y. Sasaki, *Biochemistry* **23**, 5036 (1984).
12. C. A. O'Brien, R. M. Liskamp, D. H. Solomon and I. B. Weinstein, *Cancer Res.* **45**, 2462 (1985).
13. H-D. Su, G. J. Mazzei, W. R. Vogler and J. F. Kuo, *Biochem. Pharmac.* **34**, 3649 (1985).
14. M. Gschwendt, F. Horn, W. Kittstein and F. Marks, *Biochem. biophys. Res. Commun.* **117**, 444 (1983).
15. T. Tamaoki, H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto and F. Tomita, *Biochem. biophys. Res. Commun.* **135**, 397 (1986).
16. Y. A. Hannun, C. R. Loomis, A. H. Merrill, Jr. and R. M. Bell, *J. biol. Chem.* **261**, 12604 (1986).
17. T. Matsui, Y. Nakao, T. Koizumi, Y. Katakami and T. Fujita, *Cancer Res.* **46**, 583 (1986).
18. R. Kato, T. Nakadate, S. Yamamoto and T. Sugimura, *Carcinogenesis* **4**, 1301 (1983).
19. A. H. Merrill, A. M. Sereni, V. L. Stevens, Y. A. Hannun, R. M. Bell and J. M. Kinkade, Jr., *J. biol. Chem.* **261**, 12610 (1986).
20. M. Inagaki, S. Kawamoto, H. Itoh, M. Satoh, M. Hagiwara, J. Takahashi and H. Hidaka, *Molec. Pharmac.* **29**, 577 (1986).
21. A. Y. Jeng, N. A. Sharkey and P. M. Blumberg, *Cancer Res.* **46**, 1966 (1986).
22. T. Nakadate, A. Y. Jeng and P. M. Blumberg, *J. biol. Chem.* **262**, 11507 (1987).
23. J. J. Witt and R. Roskoski, Jr., *Analyt. Biochem.* **66**, 253 (1975).

24. S. Pontremoli, E. Melloni, M. Michetti, O. Sacco, F. Salamino, B. Sparatore and B. L. Horecker, *J. biol. Chem.* **261**, 8309 (1986).
25. J. E. Niedel, L. J. Kuhn and G. R. Vandenbark, *Proc. natn. Acad. Sci. U.S.A.* **80**, 36 (1983).
26. N. O. Christiansen and H. Juhl, *Biochim. biophys. Acta* **882**, 170 (1986).
27. R. Kumar and O. Holian, *J. invest. Derm.* **86**, 316 (1986).
28. F. Horn, M. Gschwendt and F. Marks, *Eur. J. Biochem.* **148**, 533 (1985).