Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 170-177

TUMOR PROMOTING PHORBOL DIESTERS: SUBSTRATES FOR DIACYLGLYCEROL LIPASE

Myles C. Cabot

Medical and Health Sciences Division, Oak Ridge Associated Universities
Oak Ridge, Tennessee 37831

Received July 2, 1984

SUMMARY--Enzyme activity in rat serum was examined utilizing the potent tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and various glycerolipids as substrates. The serum activity was specific for hydrolysis of the long chain tetradecanoate moiety of TPA, hydrolyzed mono- and diacylglycerols, but was not effective against triacylglycerols, cholesterylesters, or phospholipids. Heating the enzyme preparation at 56°C for 1 min was dually effective in reducing the hydrolysis of both TPA and dioleoylglycerol by 83-86% of control levels. The potent diacylglycerol lipase inhibitor, RHC 80267, inhibited the hydrolysis of TPA in the 0.2-1.0 μM range and was also a potent blocker of monoacyl- and diacylglycerol hydrolysis. In substrate competition studies, exogenous unlabeled TPA was added to the [^{14}C]dioleoylglycerol-containing reaction mixture, however, this produced an approximate 3-fold stimulation of [^{14}]dioleoylglycerol hydrolysis. Although we have not established whether the hydrolysis of TPA and diacylglycerol is the work of one enzyme, the effectiveness of the specific lipase inhibitor, RHC 80267, demonstrates that diacylglycerol lipase can utilize TPA as substrate, a finding never before documented. This point is of interest in light of the theory that phorbol esters act by mimicry of the natural lipid mediator, diacylglycerols.

Current studies provide evidence that the mechanism of phorbol ester-elicited tumor promotion is potentiated by a cascade of myriad biochemical events, the first of which is initiated by phorbol ester -- cell membrane interaction and receptor ligand coupling (1-5). Because these phorboid compounds of plant origin are strong effectors of mammalian cellular response (6-8), it has long been asked whether they act by mimicry of a natural agent. We recently documented the existence of an enzyme in rat serum that destroys the potent tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA)[1], via specific hydrolysis of the 12-O-tetradecanoate moiety (9). The enzyme was also present in mouse, guinea pig, rabbit, and goat serum. With this, and the fact that TPA is a highly lipophilic, membrane-targeted agent (10-11) and a modifier of cellular lipid metabolism (11-21), it seemed that clues to support the idea that TPA parrots diacylglycerol (DG) action (3) could be revealed by examining the serum enzyme and its specificity for other lipid substrates. To this end the results of the present investigation show that of the several lipids as-

[1] Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; DG, diacylglycerol; MG, monoacylglycerol; PA, phorbol acetate; PDB, phorbol dibutyrate; PAF, platelet activating factor

0006-291X/84 $1.50

sayed, including phospholipid, the rat serum preparation only displayed hydrolytic activity towards TPA, DG, and MG (monoacylglycerol). The enzymatic hydrolysis of these substrates was sensitive to heat and blocked by the specific diacylglycerol lipase inhibitor, RHC 80267 (22). Further, and of surprising consequence, exogeneously added TPA stimulated the *in vitro* hydrolysis of [^{14}C]DG by nearly 3-fold, a capacity that weak and nonpromoting phorbol esters lacked. This work shows that DG lipase can hydrolyze the long acyl chain of TPA, an avenue of investigation that is novel and never before explored, and demonstrates that TPA can, in turn, modulate the hydrolysis of DG. These data are timely in light of the current burst of interest in phorbol ester and diacylglycerol research. It is suggested from the results herein that the relationship between TPA and membrane lipids, to the mechanism of phorboid action, is one that is more intimate than previously regarded.

MATERIALS AND METHODS

Detailed methods for the assay of serum TPA lipase have been described (9). Male CDF Charles River rats, 2-5 months old, were used. In the present work a lipid-free acetone powder of rat serum (23) was used as the enzyme source, a preparation which was approximately 8-fold more active than the whole serum used previously. The acetone powder was dissolved by gentle Dounce homogenization (50 mg powder/ml) in phosphate buffered saline. All incubations, initiated by acetone substrate injection, were carried out at 37°C in 1-dram glass vials containing radiolabeled substrate and 25 mol Tris-HCl buffer (pH 8.0), in a final volume of 0.2 ml. Reactions were terminated (^{3}H-phospholipid, cholesteryl[^{14}C]oleate, [^{14}C]acylglycerols) by the lipid extraction method of Bligh and Dyer (24), and enzymatic products were examined by thin-layer chromatography of the lipid extract (Silica Gel G, hexane/diethyl ether/acetic acid, 60:40:1).

^{3}H-Labeled phospholipids were prepared by incubating human promyelocytic (HL-60) leukemia cells (25), 25 X 10^{6} cells/20 ml serum-free medium, with 10 Ci [9,10-^{3}H(n)]palmitic acid (23.5 Ci/mmol, New England Nuclear) and 10 µCi [9,10-^{3}H(n)]oleic acid (5.04 Ci/mmol, Amersham), introduced in 20 µl absolute ethanol, for 24 hr. Total lipids were extracted from washed cells and the phospholipid, which accounted for 92.5% of the total radioactivity, was isolated by preparative thin-layer chromatography (19). The specific activity (16,590 dpm/nmol) was determined by gravimetric analysis (avg. m.w. phospholipid, 831). ^{14}C-Labeled DG and MG were prepared by incubating 0.5 µmol [carboxyl-^{14}C]trioleoylglycerol, RPI Corp./CEA (51 Ci/mol) with 60 units of *Rhizopus delmar* lipase (Miles) for 45 min at a hexane-buffer (0.13 M sodium acetate, pH 5.6) interface (26). The products were purified by preparative thin-layer chromatography on layers of Silica Gel H using hexane/diethyl ether/acetic acid (60:40:1). The [^{14}C]dioleoylglycerol, 98.7% 1,2 (2,3)-isomer, and [^{14}C]monooleoylglycerol, 100% 1 (3)-isomer were stored dry under N_2 at -80°C and dissolved in acetone for the experiments. All lipase assays were linear within the time frame of each experiment and total hydrolysis of substrates did not exceed 25%.

^{3}H-Phospholipid (5 nmol) was introduced in 10 µl acetone or as a sonicate, 50 mM Tris, pH 8.0, and incubated with enzyme (2.0 mg protein) for 15 and 30 min. The reaction was terminated by lipid extraction (24) and examined for ^{3}H-free fatty acid and ^{3}H-DG by thin-layer chromatography. Cholesterol ester hydrolase activity was assayed using cholesteryl[^{14}C]oleate (56.6 Ci/mol, New England Nuclear), 1.5 nmol, introduced in 5 µl acetone, incubated with 3.4 mg protein for 15, 30, and 60 min. The release of [^{14}C]oleic acid was assessed by thin-layer chromatography. Triacylglycerol lipase activity was assayed using 2

nmol [carboxyl-^{14}C]triolein (51 Ci/mol, RPI Corp./CEA) introduced in 10 µl acetone and incubated for 5, 10, and 20 min with enzyme (1.4, 2.2 mg protein). Reaction extracts were analyzed for free [^{14}C]oleic acid. [^{3}H]TPA (2 nmol/reaction) hydrolysis was followed using [20-^{3}H(n)]prorbol-12-myristate-13-acetate (6.5 Ci/mmol, New England Nuclear). The specific activity was adjusted (100 dpm/pmol) by dilution with unlabeled TPA (Chemicals for Cancer Research). The reaction was terminated and the product, phorbol acetate (PA), was assessed as previously outlined (9). DG lipase was assayed using 4 nmol [^{14}C]dioleoylglycerol (34 Ci/mol), introduced in 5-10 µl acetone, with 2.2 mg protein and incubated for 15-60 min. The lipase product, [^{14}C]oleic acid (17 Ci/mol) was analyzed by thin-layer chromatography. MG lipase was assayed using [^{14}C]monooleoylglycerol (4 nmol, 17 Ci/mol), incubated with 2.2 mg protein, for 5-15 min.

RESULTS

The data of Table 1 show that of the lipid substrates employed, only DG and MG were hydrolyzed under the reaction conditions established for enzymatic hydrolysis of TPA. The rate of TPA hydrolysis was approximately 2-fold higher than the hydrolysis of DG, and the rate of MG degradation far exceeded that of TPA. The serum preparation did not liberate the acyl chains or the phosphobase group of ^{3}H-phospholipid, the labeled acyl groups of tri[^{14}C]oleate, or hydrolyze cholesteryl[^{14}C]oleate, indicating the absence of triacylglycerol lipase, cholesterol ester hydrolase, and phospholipase activity.

To test the possibility that a diacylglycerol lipase could be acting on TPA, the selective diacylglycerol lipase inhibitor, RHC 80267 (22) was employed in the reaction system (Fig. 1). At a concentration of 0.5 µM, RHC 80267 caused an 83% inhibition of TPA hydrolysis. Likewise, this agent produced an approximate 50% inhibition of DG hydrolysis, albeit at a concentration of 5.0 µM, and a 70% reduction of MG hydrolysis at 1 µM. RHC 80267 has been shown to be a selective inhibitor of dog platelet diacylglycerol lipase in the 1-100 µM range (22), much like the inhibition observed here for MG, DG, and TPA hydrol-

TABLE 1

SUBSTRATE SPECIFICITY OF RAT SERUM LIPID HYDROLASE(S)

Substrate	Activity (pmol fatty acid or PA formed/min/mg protein)
^{3}H-Phospholipid	N.D.
Cholesteryl[^{14}C]oleate	N.D.
[^{14}C]Trioleoylglycerol	N.D.
[^{3}H]TPA	12.0 ± 1.5
[^{14}C]DG	6.1 ± 0.3
[^{14}C]MG	55.5 ± 1.5

[^{3}H]TPA hydrolysis represents the avg. ± S.D. of two or more incubation samples from 10 separate experiments. [^{14}C]DG (dioleoylglycerol) hydrolysis is the avg. ± S.D. of duplicate samples from 9 separate experiments. [^{14}C]MG (monooleoylglycerol) activity is the avg. ± S. D. of 4 samples. N.D. (not detected)

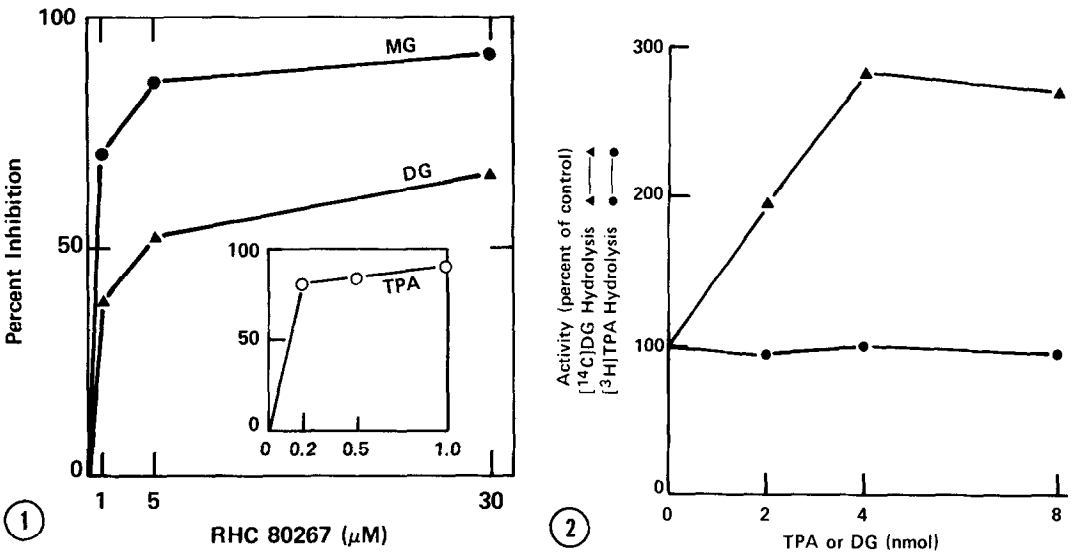

Fig. 1 The effect of diacylglycerol lipase inhibitor, RHC 80267, on serum-catalyzed hydrolysis of TPA, DG, and MG.

The reaction mixtures, incubation conditions, and activity assays are as detailed in the Methods section. A stock solution of 10 mM RHC 80267 in acetone was diluted in 10 mM Tris-HCl buffer, pH 8.0, and introduced to achieve the concentrations designated in the Figure. Proper aliquots of acetone-buffer were added to controls.

Fig. 2 Effect of exogenous addition of TPA on ^{14}C-DG hydrolysis by rat serum. DG hydrolysis was assayed by incubating 4 nmol [^{14}C]dioleoylglycerol, introduced in 5 μl acetone, with unlabeled TPA (acetone addition), and 2.2 mg protein for 30 min. The amount of acetone was adjusted to 9 1 in all incubation vessels. The effect of exogenous DG on [^{3}H]TPA hydrolysis was assayed as described above except that [^{3}H]TPA degradation was measured. Values represent the avg. of duplicate samples which differed <10% from the mean. Control values (measured in the absence of exogenous TPA or DG) represent 100%. (▲): [^{14}C] DG hydrolysis in the presence of unlabeled TPA; (●): [^{3}H] TPA hyrolysis in the presence of unlabeled DG.

ysis using the serum preparation. Additionally, the hydrolysis of TPA and DG was severely inhibited by heat treatment. Preincubation of the enzyme preparation at 56°C for 1 min produced an 83 and 86% inhibition of DG and TPA hydrolysis, respectively.

From the data accrued it seemed possible that TPA, serving as competitor, could exhibit an *in vitro* effector relationship on the hydrolysis of DG. However, Fig. 2 shows that when [^{14}C]dioleoylglycerol is incubated with unlabeled TPA a stimulation of labeled DG hydrolysis ensues. This stimulation is maximal (approximately 3-fold) at a molar ratio of 1:1 (DG to TPA). On the other hand exogeneous addition of DG, over the same concentration range, was completely ineffective in modulating the hydrolysis of [^{3}H]TPA. Because TPA is the most potent phorboid tumor promoter of croton oil, weaker and nonpromoting phorbol compounds were tested for their ability to modify labeled DG hydrolysis. As shown in Fig. 3, the weaker promoter, phorbol dibutyrate (PDB), and the non-

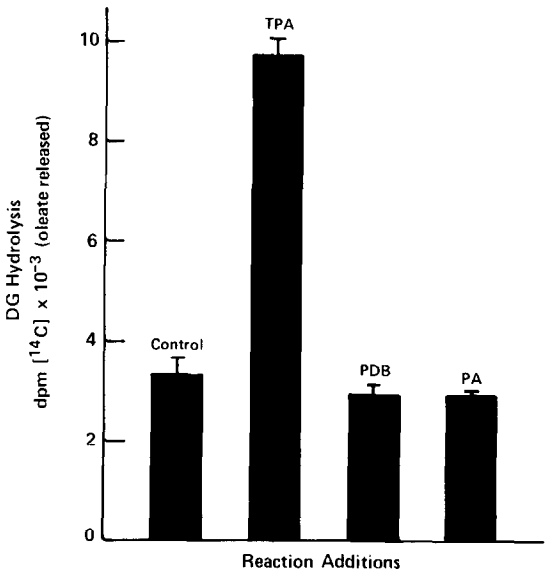

Fig. 3 The effect of TPA and less potent phorbol analogs on [^{14}C]dioleoylglycerol hydrolysis by rat serum enzyme.

[^{14}C]Dioleoylglycerol and unlabeled phorbol esters were present in a 1:1 molar ratio. The reaction mixtures contained 4 nmol [^{14}C]dioleoylglycerol (control) or [^{14}C]dioleoylglycerol plus phorbol ester as shown. All agents were introduced in acetone, and the incubation (2.2 mg protein) was for 30 min.

promoter, PA, were essentially without influence, compared to the near 3-fold stimulation of DG hydrolysis evoked by TPA. The nonpromoter and weak irritant, 4-O-methyl-TPA, was also investigated because this analog is structurally only slightly different from TPA. This 4-methylether of TPA caused a 1.7-fold stimulation of [^{14}C] dioleoylglycerol hydrolysis at a molar ratio of 1:1, DG to 4-O-methyl-TPA (data not depicted in Fig. 3).

DISCUSSION

TPA hydrolytic enzymes have been described (27-29); however, slight regard has been given to the question of substrate specificity of these enzymes. Owing to the lipid-like nature of TPA and the presence and proximity of two hydrolizable ester groups, it was reasoned from our previous study (9) that a neutral lipid hydrolase may display enzymatic activity towards phorbol diesters. This hypothesis is in concert with recent findings showing that TPA directly activates phospholipid-dependent protein kinase C (3), the putative phorbol ester receptor (4). This activation is normally regulated by unsaturated diacylglycerols (30-32). Whether the TPA and DG hydrolytic activity of serum is the work of one enzyme cannot presently be verified; although the potent DG lipase inhibitor, RHC 80267, was an extremely effective blocker of TPA hydrolysis.

The mechanism by which exogenously added TPA stimulated the *in vitro* enzymatic hydrolysis of [^{14}C]dioleoylglycerol is not known. The exact nature of this stimulation is under investigation. The weak promoter, PDB, failed to elicit a response over the nonpromoter, PA; however, the nonpromoting compound, 4-*O*-methyl-TPA, did stimulate [^{14}C]DG hydrolysis, and this analog contains the exact tetradecanoate-acetate functional groupings as the protent promoter, TPA. Therefore the acyl chain moieties may be a necessary requisite for stimulation. This would imply that the mechanism of stimulation is not linked to tumor promotability but to an enzyme-diacylglycerol-phorbol diester interaction, a phenomenon never before documented. Unorthodoxy encountered in lipid enzyme kinetics (33) or allostearic activation, as in the case of kinetic cooperativity (34), may be involved in the stimulatory reaction.

From several lines of investigation it is evident that membrane lipids serve a regulatory role in phorbol ester binding (11,35). High specific phorbol diester binding occurs in the 100,000x*g* particulate fraction of mouse brain (36). Acylglycerol lipases, with both acidic and basic pH optima, similar to the pH profile for TPA hydrolysis (9), have been described in rat (26,37-39) and mouse brain subcellular fractions (40). TPA is a membrane-targeted, highly lipophilic agent, and although the relevance of a serum enzyme to a cellular membrane system must be addressed, the present data establish a basis for such investigation. Utilization of a lipase-containing, *in vitro* membrane system, in which membrane-resident diacylglycerol can be generated *in situ* (38), would be useful to assess the relationship between phorbol esters, diacylglycerols, and lipase.

Full activation of protein kinase C, *in vitro*, occurs at a very low concentration of TPA, compared to the amount of dioleoylglycerol required to achieve like stimulation (3). Activation of protein kinase C and calcium mobilization can be induced using synthetic analogs of diacylglycerol (41-42). Thus, the possibility exists that more potent diradylglycerols, other than the long chain diacyl type, take part in the *in vivo* activation of protein kinase C. A case in point would be the neutral backbone of alkylacetyl-GPC [PAF, platelet activating factor (43)]. This diradyl backbone, 1-alkyl-2-acetyl-*sn*-glycerol, is physiological (44), and can be produced via phospholipase C hydrolysis of PAF. Further, the O-alkyl linkage is resistant to lipase attack, and the *sn*-2 acetate moiety is only slightly labile (serum lipase, unpublished results).

ACKNOWLEDGEMENT

This report is based on work performed under Contract No. DE-AC05-760R00033 between the Department of Energy, Office of Health and Energy Research, and Oak Ridge Associated Universities.

REFERENCES

1. Blumberg, P. M., Delclos, K. B., Dunn, J. A., Jaken, S., Leach, K. L. and Yeh, E. (1983) Ann. N.Y. Acad. Sci. 407, 303-315.
2. Weinstein, I. B. (1983) Nature 302, 750.
3. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
4. Niedel, J. E., Kuhn, L. J. and Vandenbark, G. R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40.
5. Kraft, A. S., Anderson, W. B., Cooper, H. L. and Sando, J. J. (1982) J. Biol. Chem. 257, 13193-13196.
6. Diamond, L., O'Brien, T. G. and Baird, W. M. (1980) Adv. Cancer Res. 32, 1-74.
7. Weinstein, I. B., Wigler, M. and Pietropaolo, C. (1977) Cold Spring Harbor Conf. Cell Proliferation 4, 751-783.
8. Diamond, L., O'Brien, T. G. and Rovera, G. (1978) Life Sci. 23, 1979-1988.
9. Lackey, R. J. and Cabot, M. C. (1983) Cancer Lett. 19, 165-172.
10. Weinstein, I. B., Lee, L-S. Fisher, P. B., Mufson, A. and Yamasaki, H. (1979) J. Supramol. Struct. 12, 195-208.
11. Cabot, M. C. (1983) Cancer Res. 43, 4233-4238.
12. Rohrschneider, L. R. and Boutwell, R. K. (1973) Cancer Res. 33, 1945-1952.
13. Levine, L. and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 79, 477-484.
14. Dobbs, L. G. and Mason, R. (1978) J. Am. Rev. Respir. Dis. 118, 705-713.
15. Wertz, P. W. and Mueller, G. C. (1978) Cancer Res. 38, 2900-2904.
16. Kinzel, V., Kreibich, G. Hecker, E. and Suss, R. (1979) Cancer Res. 39, 2743-2750.
17. Hoffman, D. R. and Huberman, E. (1982) Carcinogenesis 3, 875-880.
18. Cassileth, P. A., Suholet, D. and Cooper, R. A. (1981) Blood 58, 237-243.
19. Cabot, M. C., Welsh, C. J., Callaham, M. F. and Huberman, E. (1980) Cancer Res. 40, 3674-3679.
20. Cabot, M.C. and Welsh, C. J. (1981) Cancer Res. 41, 4910-4915.
21. Lackey, R. J. and Cabot, M. C. (1983) Cancer Lett. 20, 291-297.
22. Sutherland, C. A. and Amin, D. (1982) J. Biol. Chem. 257, 14006-14010.
23. Rothblat, E. H., Arbogast, L. Y., Ouellette, L. and Howard, B. V. (1976) In Vitro 12, 554-557.
24. Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol., 37, 911-917.
25. Collins, S. J., Gallo, R. C., and Gallagher, R. E. (1977) Nature (Lond.) 270, 347-349.
26. Cabot, M. C. and Gatt, S. (1978) Adv. Exp. Med. Biol. 101, 101-111.
27. Barrett, J. C., Brown, M. T. and Sisskin, E. E. (1982) Cancer Res. 42, 3098-3101.
28. O'Brien, T. G. and Diamond, L. (1978) Cancer Res. 38, 2562-2566.
29. Shoyab, M., Warren, T. C. and Todaro, G. J. (1981) J. Biol. Chem. 256, 12529-12534.
30. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695.
31. Takai, Y., Kishimoto, A., Kikkawa, Y,., Mori, T. and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218-1224.
32. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273-2276.
33. Gatt, S., and Barenholz, Y. (1973) Ann. Rev. Biochem. 42, 61-90.
34. Sandermann, H. Jr. (1983) Trends Biochem. Sci. 8, 408-411.
35. Dunphy, W. G., Delclos, K. B. and Blumberg, P. M. (1980) Cancer Res. 40, 3635-3641.

36. Dunphy, W. G., Kochenburger, R. J., Castagna, M., and Blumberg, P. M. (1981) Cancer Res. 41, 2640-2647.
37. Cabot, M. C. and Gatt, S. (1976) Biochim. Biophys. Acta 431, 105-115.
38. Cabot, M. C. and Gatt, S. (1977) Biochemistry 16, 2330-2334.
39. Cabot, M. C. and Gatt, S. (1978) Biochim. Biophys. Acta 530, 508-512.
40. Rousseau, A., Dubois, G. and Gatt, S. (1983) Neurochem. Res. 8, 417-422.
41. Kaibuchi, K., Takai, J., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701-6704.
42. Mori, T., Takai, Y., Yu, B., Takahashi, J., Nishizuka, Y, and Fujikura, T. (1982) J. Biochem. 91, 427-431.
43. Snyder, F. (1982) Annu. Rep. Med. Chem. 17, 243-252.
44. Blank, M.L., Cress, E. A., and Snyder, F. (1984) Biochem. Biophys. Res. Commun. 118, 344-350.