Res. Bull. Obihiro Univ. I, 15 (1987): 83~90.

Specific binding of phorbol ester tumor promoters in rat liver

Eiki SATOH*, Yoshio SHIMIZU*, Shunji TAMURA*, and Toru KOMAI**

(Received: November 29, 1986)

Abstract

A phorbol ester tumor promoter, [^{3}H] 12-O-tetradecanoylphorbol-13-acetate ([^{3}H] TPA) bound specifically and with high affinity to one class of saturable binding sites in crude mitochondrial fraction from rat liver. The dissociation constant was 1.4 nM. At saturation, 6.9 pmol of [^{3}H] TPA were bound per mg protein. Competition experiments showed displacement of [^{3}H] TPA by ODC inducing phorbol esters (TPA> 4β-phorbol-12, 13-didecanoate> phorbol-12, 13-dibenzoate) in crude mitochondrial fraction. 4α-phorbol, 4β-phorbol, 4α-phorbol-12, 13-didecanoate and phorbol-12, 13-diacetate, phorbol esters devoid of ODC induction had no effect on binding. Specific [^{3}H] TPA binding was sensitive to heat. These data suggest that ODC induction of phorbol esters in rat liver is mediated by this binding activity and that binding activity corresponds to phorbol ester target in mouse skin involved in tumor promotion. Specific [^{3}H] TPA binding was not inhibited by nonphorbol promoters, i. e., phenobarbital (2 mM), estradiol (2 mM) and deoxycholic acid (2 mM). These agents thus appear to act at a target distinct from that of phorbol esters.

Introduction

Tumor promoters are agents which, althouth in themselves neither carcinogenic nor mutagenic, greatly accelerate tumor outgrowth in animals previously exposed to a subthreshold dose of a carcinogen[2, 16, 17, 19]. The phenomenon of tumor promotion has been investigated in most detail in mouse skin system[16, 19]. In that system, the most potent class of tumor promoters is that of 12-O-tetradecanoylphorbol-13-acetate (TPA) and a series of structurally related phorbol esters. Of this class, TPA is the most active.

It has been indicated that phorbol esters can promote tumors in mouse liver[6, 7, 8, 15]. TPA has been shown to induce ornithine decarboxylase (ODC, L-ornithine carboxy-lyase; E. C. 4. 1. 1. 17) activity in mouse and rat liver[1, 3, 9, 14, 20]. Recently, we have observed a close

* Department of Veterinary Pharmacology, School of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan.

** Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140, Japan.

quantitative correlation between the structure-activity requirements for induction of ODC in rat liver and tumor-promoting activity of phorbol esters in mouse skin[14].

The present experiments were carried out to demonstrate and characterize specific binding of phorbol esters to particulate preparations from rat liver.

Materials and Methods

Preparation of particulate fractions: Livers from male Wistar-Imamichi rats (200–250g) were homogenized in 50 mM Tris-HCl (pH 7.4, 4°C) with a Potter-Elvehjem homogenizer. Nuclei, crude mitochondria, and microsome fractions were obtained by centrifugation for 5 min at 600 x g at 4°C, 20 min at 9,000 x g at 4°C, and 60 min at 100,000 x g at 4°C, respectively.

Binding assay: For the saturation studies, 50 μg of protein from the particulate fraction of liver was suspended in 940 μl of BSA-Tris (0.4% bovine serum albumin in 50 mM Tris-HCl, pH 7.4). 5 μl of ethanol and 5 μl of 400 nM ethanolic TPA were added to determine total and nonspecific binding respectively. Then this is followed by the addition of 0.2–40 pmol [^{3}H] TPA in 5 μl of ethanol. For phorbol ester competition studies, 50 μg of crude mitochondrial fraction was added only after dilution of 2 nM of radioactive TPA and various concentrations of nonradioactive competitors in 940 μl of BSA · Tris. Incubation was carried out for 20 min at 37°C in Eppendorf minivials, after which the suspensions were quickly filtered through Whatman GF/F filters and rapidly washed twice with 1 ml of acetone at −20°C. The filter areas were cut away, placed in a draught under a hood to remove residual acetone and, after standing for 24 hr in 1 ml of NCS Solubilizer, radioactivity in each sample was measured by addition of 10 ml of toluene scintillation fluid. The counting efficiency ranged from 37–43%. Specific binding represents the difference between total and nonspecific bindings.

Protein determination: The protein concentration of the mouse skin extracts was measured by the method of LOWRY et al.[11], with bovine serum albumin as standard.

Reagents: Reagents used and their sources were: 4α-phorbol, 4β-phorbol, 4α-phorbol-12, 13-didecanoate, phorbol-12, 13-diacetate, phorbol-12, 13-dibenzoate, 4β-phorbol-12, 13-didecanoate, 12-O-tetradecanoylphorbol-13-acetate (TPA), and bovine serum albumin (Sigma); phenobarbital sodium, estradiol, and deoxycholic acid (Wako); [20-^{3}H] TPA (specific activity, 6.5 Ci/mmol; New England Nuclear); NCS Solubilizer (Amersham).

Results

To examine the subcellular distribution of specific [^{3}H] TPA binding, rat liver was homogenized and fractionated into 4 parts (nuclei, crude mitochondria, microsomes and cytoplasm) by different centrifugation. As described in "Materials and Methods", specific [^{3}H] TPA binding was determined as the difference in [^{3}H] TPA binding in the absence (total [^{3}H] TPA binding) and presence (nonspecific [^{3}H] TPA binding) of a large excess (400 nM) of nonradioactive TPA. As shown in Table 1, specific [^{3}H] TPA binding of crude mitochondrial fractions were highest in 5 parts (homogenate, nuclei, crude mitochondria, microsomes and cytoplasm).

As shown in Fig. 1, total and nonspecific binding in crude mitochondrial fractions were dependent on the concentrations within the range of 0–20 nM. Nonspecific [^{3}H] TPA binding was linear.

In Fig. 2, specific [^{3}H] TPA binding increased dose-dependently in the range of 0–10 nM, and reached a plateau over 10 nM (7.6 nM of free [^{3}H] TPA). This binding activity was

Table 1. Subcellular distribution of specific [^{3}H] TPA binding in rat liver

	Specific binding (pmol/mg protein)
Homogenate	1.23
Nuclei	1.83
Crude mitochondria	2.36
Microsomes	1.32
Cytoplasm	0.26

Fractionation was carried out as described in "Materials and Methods". The 5 parts were incubated in duplicate with 2 nM [^{3}H] TPA + 400 nM nonradioactive TPA and processed as described in "Materials and Methods." Data is taken from one experiment. A second experiment gave similar results.

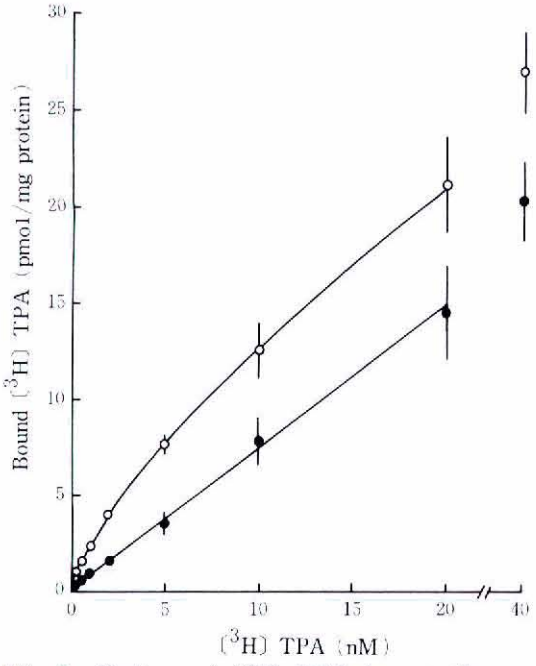

Fig. 1. Binding of [^{3}H] TPA in rat liver. Total binding (○) and nonspecific binding (●) of [^{3}H] TPA to crude mitochondrial fraction were determined as described in "Materials and Methods". Each point is the mean ± S. E. M. of six determinations within a single experiment.

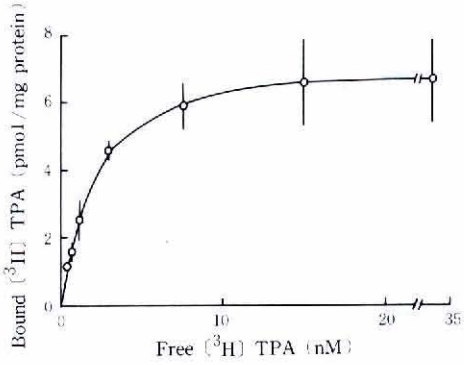

Fig. 2. Specific binding of [^{3}H] TPA to the crude mitochondrial fraction from rat liver. Specific [^{3}H] TPA binding were calculated as described in "Materials and Methods". Each point is the mean ± S. E. M. of six determinations within a single experiment.

destroyed by boiling for 5 min (date not shown).

Scatchard plot of specific [^{3}H] TPA binding was obtained from the experiments in Fig. 2. In Fig. 3, Scatchard plot revealed high affinity binding with a dissociation constant K_D of 1.4 nM and a maximal binding site concentration R_T of 6.9 pmol TPA bound per mg protein.

Specificity of specific [^{3}H] TPA binding was tested by incubation of crude mitochondrial fractions in the presence of a half-saturating amount of [^{3}H] TPA and various concentrations of phorbol ester competitor. As shown in Fig. 4, the 3 tumor promoting phorbol esters, TPA, 4β-phorbol-12, 13-didecanoate (4β-PDD), and phorbol-12, 13-dibenzoate (PDB), all inhibited specific [^{3}H] TPA binding; the slopes of the inhibition curves were

Table 2. Effects of nonphorbol promoters on specific [^{3}H] TPA binding in crude mitochondrial fractions

Additives	Specific binding (pmol/mg protein)
None	2.31 ± 0.36
Phenobarbital (2 mM)	2.45 ± 0.08
Estradiol (2 mM)	2.63 ± 0.16
Deoxycholic acid (2 mM)	2.33 ± 0.23

Binding assay were carried out as described in "Materials and Methods". Each compound was tested in three separate experiments with four determinations per compound per experiment. Values are mean ± S. E. M. for these twelve determinations.

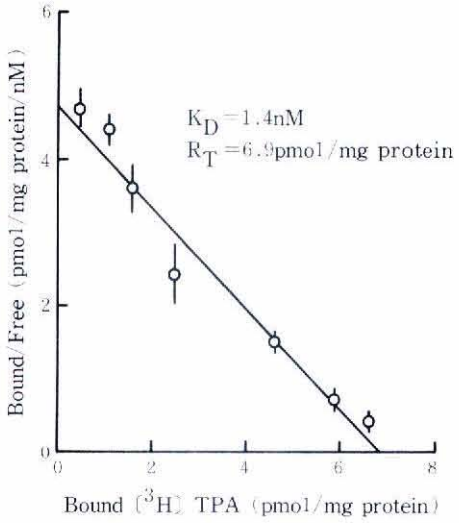

Fig. 3. Scatchard plot of specific binding of [^{3}H] TPA. Data is taken from the experiment shown in Fig. 2.

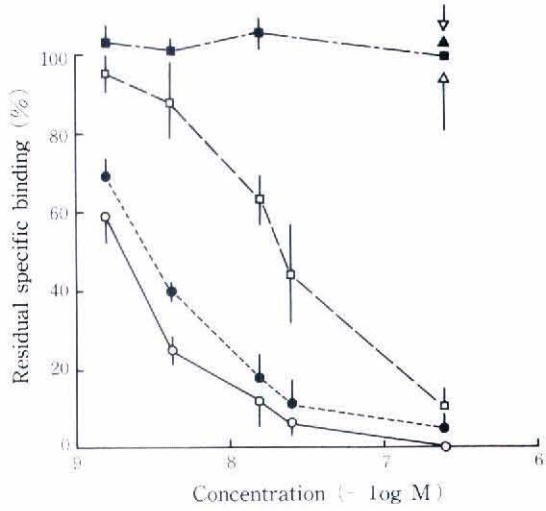

Fig. 4. Dose-response curves for the inhibition of specific [^{3}H] TPA binding by nonradioactive phorbol esters. Binding experiments were carried out as described in "Materials and Methods" in the presence of 2 nM [^{3}H] TPA and the indicated concentrations of nonradioactive phorbol esters. Specific binding in the presence of the nonradioactive phorbol esters was expressed as a fraction of the total specific binding determined for each experiment. Each point represents the mean ± S. E. M. of six separate experiments with triplicate determinations at each concentration in each experiment. 4α-phorbol (▲); 4β-phorbol (■); 4α-PDD (▽); PDA (△); PDB (□); 4β-PDD (●); TPA (○).

similar. No inhibition was observed for the non-tumor promoting phorbol esters, 4α-phorbol, 4β-phorbol, 4α-phorbol-12, 13-didecanoate (4α-PDD), and phorbol-12, 13-diacetate (PDA).

Effects of nonphorbol promoters on specific [^{3}H] TPA binding were examined. In Table 2, specific [^{3}H] TPA binding was not inhibited by phenobarbital (2 mM), estradiol (2 mM), and deoxycholic acid (2 mM), the three compounds known as liver tumor promoters.

The ID_{50} values of various phorbol esters

Table 3. Comparison of specific [^{3}H] TPA binding inhibition and ODC induction in rat liver by phorbol esters

Treatment	50% binding inhibition[a] (nM)	ODC activity[b] (nmol CO_2/30 min/g liver)
Vehicle	N. I.	0.35±0.05
4α-phorbol	N. I.	0.39±0.06
4β-phorbol	N. I.	0.43±0.16
4α-PDD	N. I.	0.48±0.25
PDA	N. I.	0.91±0.57
PDB	13.2	4.61±1.13*
4β-PDD	4.5	10.75±1.03*
TPA	3.0	14.67±0.42*

a) 50% binding inhibition was obtained from the results of Fig. 4.
b) Induction of ODC was the activity tested (from Ref. 14)
*P<0.001 versus Vehicle; N. I., No inhibition

for specific [^{3}H] TPA binding were compared with rat liver ODC activity that we determined previously. In Table 3, the ID_{50} values of various phorbol esters correlated well with their ODC inductive ability in rat liver.

Discussion

We determined the subcellular distribution of specific [^{3}H] TPA binding to examine the cellular sites of action of phorbol esters in rat liver. Specific [^{3}H] TPA binding of crude mitochondrial fractions were highest in 5 parts (homogenate, nuclei, crude mitochondria, microsomes and cytoplasm). It has been demonstrated that specific binding sites for [^{3}H] TPA exist in the rat liver.

Specific [^{3}H] TPA binding of crude mitochondrial fractions increased dose-dependently in the range of 0-10 nM, and reached a plateau over 10 nM. This binding was sensitive to heat. As Scatchard plot on this binding revealed linear regression, it has been suggested that this specific [^{3}H] TPA binding has a single binding site.

Specific [^{3}H] TPA binding of crude mitochondrial fractions in the presence of a half-saturating amount of [^{3}H] TPA was inhibited by tumor-promoting phorbol esters (TPA <4β-PDD <PDB). The non-tumor promoting phorbol esters (4α-phorbol, 4β-phorbol, 4α-PDD, PDA) had no effect on this binding. We have already demonstrated that there is a close quantitative correlation between tumor-promoting ability in skin by phorbol esters and their ability of ODC induction in rat liver[14]. This experiment has also demonstrated that there is a clear correlation between competition for TPA binding sites by various phorbol esters and their ability of ODC induction in liver. From this result, it has been suggested that at least the induction of ODC by phorbol esters is mediated through binding to the receptor detected with [^{3}H] TPA.

It has been shown that phenobarbital[10, 13], estradiol[4, 18] and deoxycholic acid[5] have the promoting ability in hepatocarcinogenesis. Since specific [^{3}H] TPA binding was not inhibited by these liver tumor promoters, these agents appear to act at a target distinct from that of phorbol esters.

Microtubule-containing structures may constitute a general mechanism for the transmission of signals generated at the plasma membrane into the interior of the cell where these signals effect on gene expression[12]. As colchicine, microtubule-disrupting agents,

inhibited significantly the induction of rat liver ODC by TPA, it is suggested that the first acting site of TPA is the membrane of rat liver[14]. Further studies concerning the constituents in the crude mitochondrial fraction of rat liver are currently being carried out.

References

1) BISSCHOP, A., VAN ROOIJEN, L. A. A., DERKS, H. J. G. M., and VAN WIJK, R. (1981): Induction of rat hepatic ornithine decarboxylase by the tumor promotors 12-O-tetradecanoylphorbol-13-acetate and phenobarbital in vivo; effect of retinyl-acetate. Carcinogenesis, **2**, 1283–1287.

2) BOUTWELL, R. K. (1964): Some biological aspects of skin carcinogenesis. Prog. Exp. Tumor Res., **4**, 207–250.

3) BYUS, C. V., and WEINER, R. A. (1982): Tumor promoting phorbolester derivatives increase ornithine decarboxylase activity and polyamine biosynthesis in the liver of the rat and mouse. Carcinogenesis, **3**, 751–755.

4) CAMERON, R., IMAIDA, K., and ITO, N. (1981): Promotive effects of ethinyl estradiol in hepatocarcinogenesis initiated by diethylnitrosamine in male rats. Gann, **72**, 339–340.

5) CAMERON, R., IMAIDA, K., and ITO, N. (1981): Promotive effects of deoxycholic acid on hepatocarcinogenesis initiated by diethylnitrosamine in male rats. Gann, **72**, 635–636.

6) GOERTTLER, K., and LOEHRKE, H. (1976): Transmaternal variation of the BERENBLUM experiment with NMRI-mice. Tumor initiation with DMBA via mothers milk followed by promotion with the phorbol ester TPA. Virchow Arch. A Path. Anat. and Histol., **370**, 97–102.

7) GOERTTLER, K., and LOEHRKE, H. (1977): Diaplacental carcinogenesis: Tumor localization and tumor incidence in NMRI mice after diaplacental initiation with DMBA and urethane and postnatal promotion with the phorbol ester TPA in a modified 2-stage BERENBLUM/MOTTRAN experiment. Virchows Arch. A Path. Anat. and Histol., **376**, 117–132.

8) GOERTTLER, K., LOEHRKE, H., HESSE, B., MILZ, A., and SCHWEIZER, J. (1981): Diaplacental initiation of NMRI mice with 7, 12-dimethyl-benz[a]anthracene during gestation days 6–20 and postnatal treatment of the F1-generation with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate: tumor incidence in organs other than the skin. Carcinogenesis, **2**, 1087–1094.

9) KISHORE, G. S., and BOUTWELL, R. K. (1981): Induction of mouse hepatic ornithine decarboxylase by skin application of 12-O-tetradecanoylphorbol-13-acetate. Experientia, **37**, 179–180.

10) KITAGAWA, T., PITOT, H. C., MILLER, J.A. (1979): Promotion by dietary phenobarbital of hepatocarcinogenesis by 2-methyl-N^1, N-dimethyl-4-aminoazobenzene in the rat. Cancer Res., **39**, 112–115.

11) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**, 265–275.

12) O'BRIEN, T. G., SIMSIMAN, R. C., and BOUTWELL, R. K. (1976): The effect of colchicine on the induction of ornithine decarboxylase by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res., **36**, 3766–3770.

13) PERAINO, C., STAFFELDT, E., HAUGEN, D. A., LOMBARD, L. S., STEVENS, F. J., and FREY, R. J. M. (1980): Effect of varying the dietary concentration of phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumorigenesis. Cancer Res., **40**, 3268–3273.

14) Satoh, E., Shimizu, Y., Inoue, K. Tamura, S., and Komai, T. (1986): Induction of rat hepatic ornithine decarboxylase by tumor-promoting phorbol esters. Res. Bull. Obihiro Univ., **14**, 329–338.
15) Schweizer, J., Loehrke, H., and Goerttler, K. (1978): Transmaternal modification of the BERENBLUM/MOTTRAN experiment in mice. Bull. Cancer, **65**, 265–270.
16) Scribner, J. D., and Süss, R. (1978): Tumor initiation and promotion. Int. Rev. Pathol., **18**, 137–198.
17) Sivak, A. (1979): Cocarcinogenesis. Biochim. Biophys. Acta., **560**, 67–89.
18) Taper, H. S. (1978): Effect of estradiol-17-phenylpropionate and estradiol benzoate on N-nitrosomorpholine-induced liver carcinogenesis in ovariectomized female rats. Cancer, **42**, 462–467.
19) Van Duuren, B. L. (1969): Tumor promoting agents in two-stage carcinogenesis. Prog. Exp. Tumor Res., **11**, 31–68.
20) Weiner, R. A., and Byus, C. V. (1980): Induction of ornithine decarboxylase by 12-O-tetradecanoylphorbol-13-acetate in rat tissues. Biochem. Biophys. Res. Commun., **97**, 1575–1581.

ラット肝臓における発癌プロモーターである Phorbol Esters の Specific Binding

佐藤栄輝・清水祥夫・田村俊二
帯広畜産大学獣医学科家畜薬理学教室
（北海道帯広市稲田町）

駒井　亨
三共株式会社分析代謝研究所
（東京都品川区広町1－2－58）

摘　要

ラット肝臓の細胞下画分（Nuclei, Crude Mitochondria, Microsomes および Cytoplasm）において，発癌プロモーターである［^{3}H］12-O-Tetradecanoylphorbol-13-Acetate（［^{3}H］TPA）の Specific Binding について検討した。

［^{3}H］TPA の Specific Binding は Crude Mitochondria 画分に多かった。以下，この画分について実験を行った。Dissociation Constant (K_D) は 1.4nM で，Maximal Binding Site Concentration (R_T) は 6.9 pmol/mg protein であった。

［^{3}H］TPA の Specific Binding は肝臓のオルニチン脱炭酸酵素（ODC）を誘導する非標識の Phorbol Esters: TPA, 4β-Phorbol-12, 13-Didecanoate (4β-PDD) および Phorbol-12, 13-Dibenzoate (PDB) により抑制された（TPA>4β-PDD>PDB）。それらの Binding 抑制率と ODC 誘導能とは良く対応した。この Binding は ODC を誘導しない Phorbol Esters: 4α-Phorbol, 4β-Phorbol, 4α-Phorbol-12, 13-Didecanoate (4α-PDD) および Phorbol-12, 13-Diacetate (PDA) により抑制されなかった。

以上の成績から，Phorbol Esters には肝臓に Specific Binding が存在すること，さらにこの Site への Binding を介して ODC が誘導されることが示唆される。

［^{3}H］TPA の Specific Binding は肝発癌プロモーター：Phenobarbital, Estradiol および Deoxycholic Acid によって抑制されなかった。これらの発癌プロモーターは Phorbol Esters の Binding

Site と異なった Site に作用すると推察される。