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EFFECTS OF TANNIC ACID ON 12-*O*-TETRADECANOYLPHORBOL-13-ACETATE-INDUCED PROTEIN KINASE C ACTIVATION IN NIH 3T3 CELLS

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Abstract—Tannic acid (TA) is a naturally occurring phenol, which has been found to display an anti-promotion effect on mouse skin carcinogenesis. In order to explore the molecular mechanism, we have examined the process of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced protein kinase C (PKC) activation, including phorbol ester binding, enzyme translocation, autophosphorylation and substrate phosphorylation, and finally the TPA-stimulated DNA synthesis. In an initial study, we found that TA slightly inhibited [^{3}H]phorbol dibutyrate (PDBu) binding to intact cells, and only 30% reduction of phorbol ester binding was observed at the highest dose of TA (100 μM). Further analysis by Scatchard plot showed that TA reduced the B_{max} of [^{3}H]PDBu from 1.3 pmol/10^6 cells to 1.1 pmol/10^6 cells, but the K_d was increased from 24 to 30 nM. Analysis by western blot indicated that TA did not interfere with the TPA-induced PKC translocation, whereas TA effectively blocked the TPA-evoked phosphorylation of the membrane-bound PKC moiety and its 80 kDa substrate in a dose-dependent manner. We also found that pre- or post-treatment with TA both lead to a similar reduction of 80 kDa protein phosphorylation and that the TPA-stimulated DNA synthesis was also inhibited by TA in a dose-dependent manner, suggesting that the blockage of protein phosphorylation by TA was of biological significance. In conclusion, the work presented here demonstrated that the antitumor promoting effect of TA was not mediated by competing for the binding site with phorbol ester or interrupting the PKC translocation, but rather by effectively blocking phosphorylation by membrane-bound PKC, possibly through altering the biophysical properties of the membrane environment.

Tannic acid (TA§) is a naturally occurring, widespread phenolic compound in wood, bark, fruits, leaves and roots of a large number of plants. It is also found in beverages such as tea, coffee and wine. Numerous studies [1–3] have shown that TA has substantial potential for decreasing the risk of mutagenicity and tumorigenicity induced by some typical carcinogens in different model systems. The inhibition of carcinogenesis by TA may result from decreased metabolic activation and increased conjugation reactions, as indicated by the findings that this phenolic compound inhibits aryl hydrocarbon hydroxylase activity, induces glutathione *S*-transferase or DNA repair enzyme, and decreases the formation and covalent binding of ultimate carcinogens to DNA in the target organ. Recently, two studies [4, 5] have reported that TA inhibited in a dose-dependent manner 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity and skin-tumor promotion in mouse epidermis *in vivo*. Based on these observations, it is believed that this naturally occurring polyphenol may regulate some key enzymes involved in the process of carcinogenesis.

On the other hand, it has been well-documented that protein kinase C (PKC), a serine/threonine-specific kinase, the activity of which is dependent on calcium and phosphatidylserine [6], is the receptor of the tumor promoter TPA [7–9] and may play a key role in triggering the tumor-promotion-signalling pathways [10, 11]. TPA-induced PKC activation process involves translocation of cytosolic PKC to the plasma membrane(s) which, in turn, leads to phosphorylation of target molecules, thereby influencing important cellular processes such as proliferation and/or differentiation [12]. It has been demonstrated that over-expression of PKC-I in NIH 3T3 cells enhances tumorigenicity and produces changes in cellular morphology and other growth properties [13]. In addition, the results [14, 15] from our laboratory also clearly indicated that the characteristics of TPA-mediated PKC activation and tumor promotion can be demonstrated in NIH 3T3 cells. In the present study, we used NIH 3T3 cells as a model to investigate the mechanisms of inhibitory effect of TA on TPA-induced tumor promotion. We showed that TA strongly inhibits phosphorylation of membrane-bound PKC without interfering with phorbol ester binding and PKC translocation.

MATERIALS AND METHODS

Chemicals. Phenylmethylsulfonyl fluoride (PMSF), leupeptin, histone H1 (type III-s), phosphatidylserine, 1,2-diolein (1,2-dioleoly-rac-

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§ Abbreviations: TA, tannic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; PDBu, phorbol dibutyrate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; FCS, fetal calf serum; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

glycerol) and TPA were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.); tannic acid from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) [r-^{32}P]ATP (5000 Ci/mmol) and [^{3}H]phorbol dibutyrate (PDBu, 20 Ci/mmol) from Amersham (Amersham, U.K.).

Cell cultures. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), antibiotics (penicillin at 100 units/mL and streptomycin at 50 μg/mL) and 2 mM glutamine. Confluent cells were serum-starved for 36–48 hr before different treatments.

[^{3}H]*PDBu binding to intact cells.* Cells were grown on 35-mm plates, and all studies were conducted on confluent populations of cells (roughly 10^6 cells/plate), approximately 2 days after subculture. All binding assays were conducted at 37° in a total volume of 500 μL and were started by replacing the cell medium with fresh DMEM with 20 mM HEPES. Cells were then exposed either to indicated concentrations of TA for 10 min followed by [^{3}H]-PDBu (20 nM routinely, or as indicated in legends for Scatchard analysis) for another 10 min, or to [^{3}H]PDBu followed by a 10-min post-treatment of TA. All assays were terminated by aspirating the binding mixture, rinsing the plates three times with cold phosphate-buffered saline (PBS), and solubilizing the cells with 0.5 mL of 1 N NaOH. The samples were neutralized with 0.5 mL of 1 N HCl and assessed for tritium content in 3 mL of Hydrofluor. Specific binding represents the difference between total binding and that measured in the presence of 10 μM unlabeled TPA.

Immunoblotting of PKC. Subcellular fractions of NIH 3T3 cells were obtained as described [16]. Protein samples of each cellular fraction [nuclear fraction (NF), cytosolic fraction (CF) and membrane fraction (MF)] were quantitated by the BCA method (Pierce). Immunoblotting of the protein samples was carried out according to Towbin *et al.* [17]. Briefly, each protein sample (30 μg) was denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer and resolved on an 8% SDS–polyacrylamide gel. Following electrophoretic transfer onto Immobilon membrane (Millipore) at 60 V for 3 hr in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine and 20% methanol), the membrane was incubated at 4° for 4 hr in blocking solution (4% skimmed milk and 0.1% sodium azide). The membrane was incubated with anti-PKC primary antibody at room temperature for 3 hr. After that, the membrane was washed with blocking solution three times, incubated with alkaline phosphatase-conjugated secondary antibody (goat antimouse IgG, Promega) for 1 hr, washed with blocking solution three times and TBST (25 mM Tris, pH 8.0, 125 mM NaCl and 0.05% Tween 20) twice, then visualized with dyes NBT and BCIP (Promega) in substrate buffer (100 mM Tris, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$).

80-kDa Protein phosphorylation. NIH 3T3 cells were cultured and labeled essentially as described previously [18]. Briefly, cells were labeled with 0.2 mCi of [^{32}P]Pi in 2 mL of phosphate-free DMEM. Subsequently, cells were treated with TPA (100 ng/mL for 30 min) and/or various doses of TA. After that, cells were washed twice in ice-cold Tris-buffered saline (0.15 M NaCl, 20 mM Tris, pH 7.5), extracted with 5% trichloroacetic acid (TCA) twice, and rinsed with Tris-buffered saline twice, finally lysed in 250 μL of boiling SDS sample buffer. The samples were then boiled for 5 min, centrifuged briefly, and equal amounts of protein (30 mg) in each sample were electrophoresed on 10% SDS–polyacrylamide gels. Gels were fixed and stained with Coomassie Brilliant Blue to detect protein standards, dried and autoradiographed at −80° using Kodak X-ray film.

Autophosphorylation of PKC. NIH 3T3 cells were treated and labeled essentially as described above. Cells were washed with ice cold Tris-buffered saline (0.15 M NaCl, 20 mM Tris, pH 7.5), then lysed with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA) containing 1 mM PMSF, 50 μM leupeptin, 30 mM tetrasodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4. Equal amounts of protein (800 μg) from each lysate were incubated with 20 μg of anti-protein kinase C Ab (MC 5, Amersham) for 1 hr at 4°, then incubated with 75 μL protein A-sepharose for another hour at 4°.

After centrifugation, the protein A bead was pelleted and washed with washing solution (25 mM Tris–Cl, 0.5% Na deoxycholate, 0.2% NP-40) containing 1 M, 0.5 M, or 0.1 M NaCl, respectively, twice each. Proteins were then eluted with 2 × SDS buffer from protein A, and analysed with 8% SDS–PAGE. The gel was autoradiographed at −70° after drying.

Measurements of DNA synthesis. Cells were plated at a density of 10^5 cells/35 mm plastic dish, and made quiescent by incubating for 24 hr with serum starvation (0.5% FCS in DMEM). Quiescent cultures were treated with 100 ng/mL of TPA and various concentrations of TA or 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) for 6 hr at 37°. After that cells were washed with PBS, then [^{3}H]thymidine was added at 2 μCi/mL and incubated for 24 hr. Thymidine incorporation into cellular DNA was determined after washing the cells, followed by three 5% cold-TCA washes, solubilization in 0.25 M NaOH, and counting in a liquid scintillation counter (Beckman).

RESULTS

The binding of phorbol ester derivative, PDBu, to intact cells was used to determine the effect of TA on the ligand binding capacity to cell surface PKC. In initial studies, we examined the kinetics of [^{3}H]PDBu binding to intact NIH 3T3 cells at 37°. A steady-state level of [^{3}H]PDBu binding was reached within 5 min and remained constant for at least 30 min (data not shown). As shown in Fig 1, the K_ds of [^{3}H]PDBu binding were 24 and 30 nM for untreated and 50 μM TA treated cells, respectively. TA also reduced the B_{max} for [^{3}H]PDBu from 1.3 pmol/plate to 1.1 pmol/plate. The inhibition of [^{3}H]PDBu binding to intact cells by TA reached a maximum at 50 μM (Table 1). Nonspecific binding, defined by 10 μM unlabeled TPA, is 10–15% of total binding using 20–100 nM [^{3}H]PDBu.

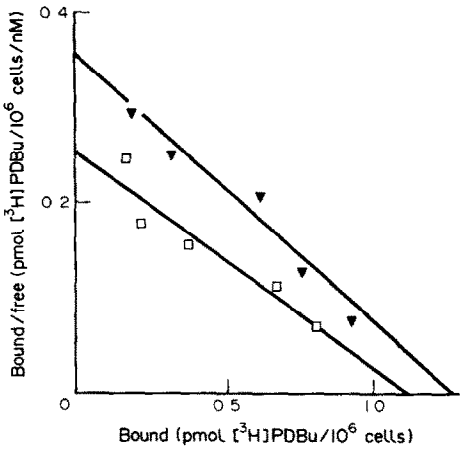

Fig. 1. Scatchard analysis of TA inhibited [^{3}H]PDBu binding to intact NIH 3T3 cells. Cells were cultured as described in Materials and Methods and treated with 50 μM TA for 10 min before addition of various concentrations of [^{3}H]PDBu (20–160 nM). After 10 min of incubation an aliquot of medium was withdrawn for determination of unbound [^{3}H]PDBu. Dishes were rinsed with ice-cold buffer, and cells were obtained as described in Materials and Methods. (▼) Control; (□) TA-treated.

Table 1. Effect of TA on [^{3}H]PDBu binding to intact NIH 3T3 cells

Treatment*	Bound [^{3}H]PDBu cpm/10^6 cells	% of inhibition†
Control	8670	0
TA (12.5 μM)	8336	4
TA (25 μM)	7250	16
TA (50 μM)	6475	25
TA (100 μM)	6126	28

* NIH 3T3 cells were pretreated for 10 min with various doses of TA as indicated above, and then exposed to [^{3}H]-PDBu (40 nM) at 37° for another 10 min. Specific binding was determined as described in Materials and Methods. These values are an average of a single experiment with triplicate samples. Similar results were obtained in two separate experiments.

† % of inhibition

$$= \frac{\text{cpms of (control} - \text{treated) group}}{\text{cpms of control group}} \times 100$$

Translocation of PKC from an inactive, cytosolic form to an active, membrane-bound form is a well-characterized phenomenon during TPA-induced PKC activation [19]. It is of interest to examine whether the TPA-stimulated PKC translocation was interfered with by TA. Immunoblotting shows that treatment with TA did not interfere (or block) the TPA-induced PKC membrane association in intact cells, at the concentration up to 150 μM (Fig. 2). In addition, down regulation of PKC was not accelerated nor delayed by TA (data not shown).

Autophosphorylation of protein kinase C has an important role in regulation of the enzymic activity subsequent to signal transduction [20]. The autophosphorylated pattern of PKC was examined in TA-treated cells. As shown in Fig. 3, 50 μM TA significantly reduced the TPA-induced autophosphorylation of PKC, and 100 μM of TA completely prevented autophosphorylation of PKC in the cells. It has been reported that PKC exhibited multiple sites which are capable of being phosphorylated [20]. Hence, the different mobility of the PKC on polyacrylamide gel (Fig. 3) between TPA alone and TPA plus TA-treated cells may be due to the degree of phosphorylation. It is important to investigate whether the inhibition of PKC autophosphorylation by TA also affects its substrate (80 kDa protein) phosphorylation. As shown in Fig. 4, TPA-induced 80 kDa protein phosphorylation was significantly inhibited by TA. This inhibition pattern was clearly dose dependent. By densitometry, 50 μM TA resulted in 50% inhibition of the substrate protein phosphorylation, and 100 μM resulted in complete inhibition. Additionally an approximately 45 kDa phosphorylated protein specifically induced by TPA was also remarkably suppressed by TA. In addition, we further investigated whether the sequence of addition would result in a different mode or extent of inhibition. Table 2 summarizes the phosphorylation of the 80 kDa protein obtained from the cells which were pre- or post-treated with 50 and 100 μM of TA for 10 min. The bands corresponding to the 80 kDa protein were measured and quantitated by a densitometer. The results shown here suggest that both treatments made a substantial reduction of the phosphorylated 80 kDa substrate.

Finally, we examined the effect of TA on DNA synthesis triggered by TPA in NIH 3T3 cells, to determine if the perturbations of PKC activity are of functional significance. Figure 5 shows [^{3}H]-thymidine incorporation sharply decreased to 45% when cells were concomitantly treated with TA (50 μM) and TPA (100 ng/mL) as compared with the TPA-treated group. TA cannot completely prevent the TPA-mediated [^{3}H]thymidine incorporation (DNA synthesis) in NIH 3T3 cells even at 100 μM concentration. We did not increase the concentration of TA since TA was cytotoxic to cells when the concentration was higher than 100 μM. H-7, a well known PKC inhibitor [21, 22] was used to examine its effect on TPA-caused [^{3}H]thymidine incorporation. The result (Fig. 5) showed that TPA-elicited [^{3}H]thymidine incorporation was almost completely inhibited by 30 μM H-7. In addition, we found that treatment with TA alone did not alter [^{3}H]thymidine incorporation and protein phosphorylation (data not shown) as compared to a control without any treatment.

DISCUSSION

In [^{3}H]PDBu binding study, we found that the decrease in [^{3}H]PDBu binding corresponds to a decrease in binding sites (B_{max}) and affinities (K_d)

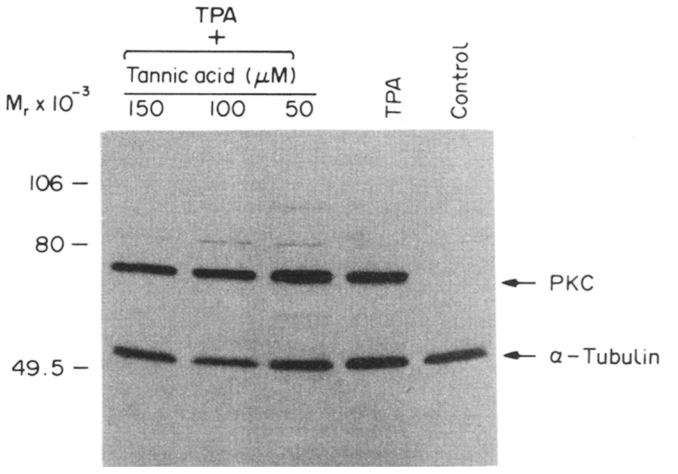

Fig. 2. Immunoblot analysis of the membrane-bound PKC in NIH3T3 cells after treatment with TPA plus TA or TPA alone. Confluent NIH 3T3 cells were treated with TPA (100 ng/mL) plus various concentrations of TA (25, 50, 75 and 100 μM) or TPA (100 mg/mL) for 30 min. Membrane fraction of each sample (30 μg protein/lane) was analysed by SDS–PAGE. After electrotransfer of the gel, PKC was immunoblotted with anti-PKC antibody (MC5) as described in Materials and Methods. α-Tubulin was blotted with monoclonal anti-α-tubulin as an internal control

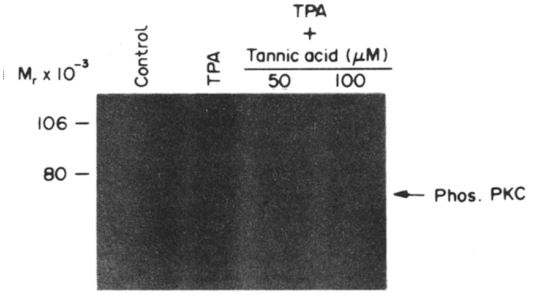

Fig. 3. Effect of TA on autophosphorylation of PKC induced by TPA in NIH 3T3 cells. Cells were treated with TPA (100 ng/mL) and 50 or 100 μM TA for 30 min, then washed with ice-cold Tris-buffered saline. After that, cells were lysed with RIPA buffer and immunoprecipitated with anti-protein kinase C Ab (MC-5) for 1 hr at 4°. See Materials and Methods for detailed procedure. The phosphorylated form of PKC (Phos. PKC) is indicated by an arrow.

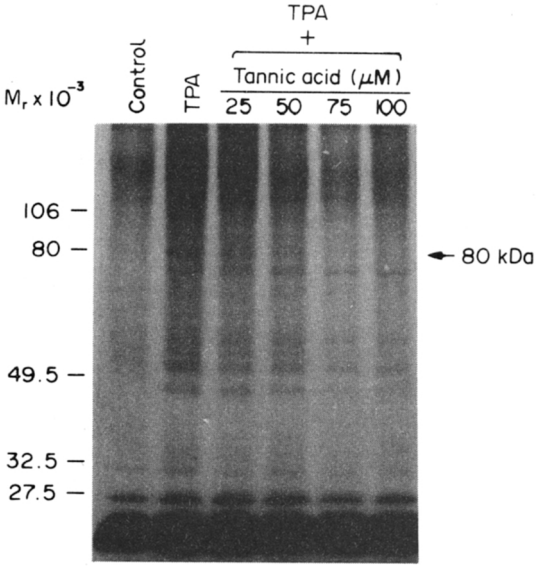

Fig. 4. Effect of TA on phosphorylation of 80 kDa protein induced by TPA in NIH 3T3 cells. The detailed procedure is as described in Materials and Methods The TPA-stimulated phosphorylation of 80 kDa is indicated by an arrow, and the positions of molecular weight standards are indicated to the left of the gel. The concentrations of TA and TPA are indicated.

for the phorbol ester. However, the decrease of K_d and B_{max} was not obvious, implying that TA could not specifically compete for the phorbol ester-binding domain of PKC, but interfered with the phorbol ester binding through an indirect way. On the other hand, it could be argued that this inhibition of [^{3}H]PDBu binding to intact cells might result from the TA moiety directly binding to the phorbol ester but this can be excluded because the concentration of TA used in the binding study is 1000-fold higher than [^{3}H]PDBu. Hence, if TA could chelate with phorbol esters, we should obtain 100% inhibition of [^{3}H]PDBu binding by treatment with high doses (50 or 100 μM) of TA, but in fact, the maximum inhibition observed was 30%.

Western blot analysis (Fig. 2) showed that TA failed to block the TPA-induced translocation of PKC from cytosol to membrane, suggesting that in

Table 2. Inhibition of phosphorylation of 80 kDa protein by treatment with TA

Treatment	Dose (μM)	Level of phosphorylation‡
Control	none	<2
TPA (100 ng/mL)		100
Pretreatment*	100	5
with TA	50	24
Post-treatment†	100	12
with TA	50	30

* NIH 3T3 cells were cultured and labeled with [^{32}P]Pi as described in Materials and Methods. Before addition of TPA, cells were pretreated with various doses (as indicated in the Table) of TA for 10 min, and then 100 ng/mL of TPA were added to the cultures for a further 30 min. Finally, cells were lysed and electrophoresed on 5–15% gradient gels, then gels were dried and autoradiographed.

† [^{32}P]Pi-labeled cells (as described above) were firstly treated with TPA (100 ng/mL) for 10 min, after that, various doses of TA were added to the cells and incubated for a further 25 min.

‡ Autoradiographs of 5–15% SDS–PAGE gradients gels were quantitated using Kodak XAR preflashed film and a Biometra densitometer. All the data were normalized to the phosphorylation observed in the parental line in the presence of TPA (100%). A value of 2% was determined to be the limit for specific detection of the 80 kDa protein above background phosphorylation in the 80 kDa region of the densitometer scan. Values are the means of three separate determinations.

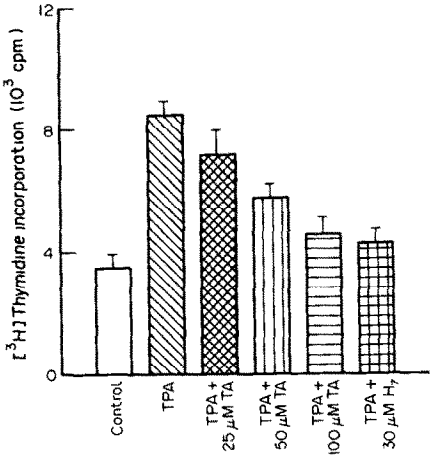

Fig. 5. Effect of TA on DNA synthesis by TPA. Cells were plated onto 24-well plates at a cell density of 10^5 cells per well in DMEM–10% FCS. After attaching, cells were serum-starved (0.5% FCS) for 24 hr. On the following day, 100 ng/mL of TPA and various concentrations of TA or H-7 were added to the cultures for 6 hr at 37°. At the end of the incubation, medium was replaced and incubation of the cells continued in serum-free medium containing 2 μCi/mL [^{3}H]thymidine. Thymidine incorporation into cellular DNA was determined as described in Materials and Methods. Columns, mean of values from two independent experiments; bars, SE.

the case of TA inhibition of [^{3}H]PDBu binding to cells was not associated with PKC translocation. Although PKC translocation is thought to be a prerequisite of enzyme activation, it is not sufficient [23]. Phosphorylation of PKC and its 80 kDa protein substrate can occur or be sustained in the presence of significant increases in membrane-associated PKC. Moreover, it has been suggested that only these two phosphorylated markers actually reflect the activity of PKC in intact cells [24].

In the present study, we clearly demonstrated that TA inhibits TPA-mediated phosphorylation of PKC moiety and 80 kDa protein in a dose-dependent manner. In addition, it is of interest to note that the inhibitory potency of TA toward these two phosphorylated markers was quite similar, for example, 50 μM of TA caused approximately a 50 and 60% reduction for 80 kDa protein and PKC phosphorylation, respectively. Therefore, it is apparent that TA may prevent these two phosphorylation processes through the same mechanism.

Taken together, it is proposed that treatment of cells with TA cannot interrupt TPA-induced cytosolic PKC translocation to membrane, but it can modulate the membrane-bound PKC phosphorylation. Owing to the hydrophilic and bulky nature of TA, it would be difficult for TA to pass through the membrane, but TA might intercalate into the membrane bilayer and perturb the surroundings of membrane PKC, thus leading to inactivation of its kinase activity. In accordance with this hypothesis, it has recently been proposed that some specific membrane-active agents alter the biophysical properties of the lipid environment and then modulate PKC activity [25]. As described above, it is well-documented that PKC is activated by TPA *in vivo* and *in vitro* in the presence of Ca^{2+} and phospholipid [6, 26]. It has also been reported that TA exhibited a high reducing power and formed complexes with various metal ions and cofactors [27]. To exclude the possibility that this inhibition is due to chelation by TA, we have examined the affinity between TA and Ca^{2+} by UV spectrophotometry. The results showed TA did not complex with Ca^{2+} in the physiological condition (data not shown).

TA was found to have an inhibitory effect on the phosphorylation of PKC itself and of 80 kDa protein, implying that the tumor promoting signal induced by TPA might also be blocked by TA. To address this, we have shown that TA can significantly inhibit DNA synthesis stimulated by TPA in a dose-dependent manner (Fig. 5). In the same experiment, H-7, a well known PKC inhibitor, was found to be more potent than TA in inhibiting TPA-stimulated DNA synthesis. Although H-7 could significantly inhibit PKC activity *in vivo* and *in vitro*, it did not interfere with the TPA-induced PKC translocation [22]. Thus, the mode of inhibition of PKC activity by TA was similar to H-7, and may influence greatly the TPA-induced biological response. The results of this study suggest that the antitumor effect of TA was not mediated by competing for the binding site with phorbol ester or blocking the translocation of PKC, but rather by effectively interrupting the phosphorylation of membrane-bound PKC through biophysical modulation.

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