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INHIBITION OF 12-*O*-TETRADECANOYL PHORBOL-13-ACETATE PROMOTED TUMORIGENESIS BY CEPHARANTHINE, A BISCOCLAURINE ALKALOID, IN RELATION TO THE INHIBITORY EFFECT ON PROTEIN KINASE C

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Abstract—In two-stage mouse skin carcinogenesis initiated by 7,12-dimethylbenz[α]anthracene (DMBA), cepharanthine inhibited the tumor promoting activity of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Since Ca^{2+}-phospholipid-dependent protein kinase (PKC) was shown to be an intracellular target of TPA, effects of cepharanthine on the activity of this enzyme were investigated. Cepharanthine also inhibited the phosphorylation of H1 histone by PKC in a concentration dependent manner. While cepharanthine inhibited the association of H1 histone with phospholipid vesicles, autophosphorylation of PKC was not inhibited by this drug. Cepharanthine also inhibited TPA-stimulated phosphorylation of some cytoplasmic proteins of mouse skin epidermis. These results indicated the possibility that anti-tumor promoting action of cepharanthine was the result of inhibition of PKC dependent cytoplasmic protein phosphorylation through the reduction of the interaction of these proteins with the plasma membrane.

Cepharanthine, a biscoclaurine alkaloid, is known as one of the potent inhibitors of hemolysis by venom [1], and widely used in therapeutic treatment in Japan as an antiinflammatory agent to allergy [2], an antileukopenia agent after radiation therapy [3], and an antineoplastic agent to multidrug resistant cancer [4]. Cepharanthine suppresses the hemolysis by lisolecithin [5] or hypotonic shock [6], phospholipase A_2 activity [7], lipid peroxidation [8, 9] and several metabolic responses of neutrophils [10, 11] or platelets [12]. The drug is quickly incorporated into the lipid bilayer [13], and thought to stabilize the structure and function of biological membranes, and modulate transmembrane signaling.

In two stage carcinogenesis, several antiinflammatory agents such as steroids [14–16], arachidonic acid metabolism inhibitors [17] and phospholipase A_2 inhibitors [18] inhibited ornithine decarboxylase induction or papillomas formation promoted by tumorigenic phorbol esters. Castagna *et al.* [19] demonstrated that the phorbol esters bind Ca^{2+}-phospholipid-dependent protein kinase (PKC‖) directly and dramatically increase the affinity of this enzyme for Ca^{2+}, resulting the activation of PKC. Therefore, it has been generally accepted that PKC is a receptor of phorbol ester and that the phosphorylation of intracellular proteins by this enzyme is an important event leading to tumor promotion. We previously reported that cepharanthine inhibited the phosphorylation of lysine rich histone by PKC [20]. However, the mechanism of the inhibition was not elucidated. In this study, we demonstrate that cepharanthine inhibits the tumor promoting activity of TPA, reduces TPA-stimulated phosphorylation of some specific proteins in mouse epidermal cytosol fraction, and suggest that the inhibition of PKC activity by this drug appears to be mediated through the inhibition of substrate-phospholipid interaction, rather than direct action on PKC itself.

MATERIALS AND METHODS

Chemicals. Phosphatidylcholin (egg yolk), phenylmethylsulfonyl fluoride (PMSF) and lysine rich histone (type IIIS) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TPA was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Bovine brain phosphatidylserine (bivalent cation free) and DMBA were from Avanti Polar Lipids Inc. and Wako Pure Chemical Industry (Osaka, Japan), respectively. DEAE-cellulose (DE-52) and Glass Filter (GF/C) were obtained from Whatman Ltd. (Maidston, WI, U.S.A.). [γ-^{32}P]ATP was from ICN Radiochemicals (Irvine, CA, U.S.A.). Cepharanthine was from Kaken Showyaku Co. Ltd (Tokyo, Japan). Leupeptin was obtained from Peptide Institute Inc. (Osaka, Japan). Purified H1

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‖ Abbreviations used: CBB, Coomassie brilliant blue; DMBA, 7,12-dimethylbenz[α]anthracene; PC, phosphatidylcholine; PS, phosphatidylserine; PKC, Ca^{2+}-phospholipid-dependent protein kinase; EGTA, ethyleneglycol bis-(aminoethyl ether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin layer chromatography; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

histone was purchased from Boehringer Mannheim (Mannheim, F.R.G.). Other chemicals were from Nakarai Tesque (Kyoto, Japan).

Two stage carcinogenesis. Two stage carcinogenesis was carried out as described by Nishino *et al.* [21]. The dorsal skins of 7-week-old female CD-1 mice (Charles River Japan, Atugi, Japan) were clipped with an electroclipper and those mice with resting hair follicles were used in this experiment. Initiation was accomplished by a single application of 100 μg DMBA in 200 μL acetone. One week later, the mice were treated with 2.5 μg TPA in 200 μL acetone twice-weekly for 18 weeks. Cepharanthine dissolved in 200 μL acetone/ethanol (1:1) was applied topically (20 μmol/mouse) 5 min before each promoter application. In the control group, mice were treated with vehicle (acetone/ethanol, 1:1) alone.

Preparation of PKC from rat brain. PKC was partially purified from the brain soluble fractions of Wistar rats weighing 250–300 g as described previously [22].

Preparation of epidermal cytoplasmic proteins. Epidermal cytosol fraction was prepared from the dorsal skin of 8 to 12-week-old female ICR mice as described by Ashendel *et al.* [23]. After killing by cervical dislocation, the dorsal skins were rapidly removed and laid on ice. The epidermis was scraped off with a razor blade and homogenized with a polytron homogenizer at 4° in 20 mM Tris–HCl (pH 7.5) buffer containing 0.15 M KCl, 1 mM PMSF, 0.01% leupeptin and 2 mM EGTA. The homogenate was filtrated through nylon mesh and centrifuged at 100,000 *g* for 1 hr. Floating lipid was discarded and the supernatant was applied to a Sephadex G-25 column equilibrated with 20 mM Tris–HCl (pH 7.5) and 0.15 M KCl. The macromolecular fraction was used as the epidermal cytosolic fraction.

Assay of PKC. PKC activity was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone (200 μg/mL) or lysine rich histone (200 μg/mL) as described previously [22]. Autophosphorylation of PKC was measured by the same method in the absence of substrate. Epidermal cytosolic fraction (1 mg/mL) was phosphorylated by the endogenous protein kinases under the same conditions without purified PKC. The phosphoproteins in the fraction were analysed by SDS–PAGE and autoradiography.

Binding assay of H1 histone to phospholipid vesicles. The interaction of H1 histone with phospholipid vesicles (small unilamella liposomes) was analysed by the floation method [24]. Briefly, 50 μg/mL H1 histone was phosphorylated by PKC at 30° for 10 min in the reaction buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM phospholipid (egg PC, or egg PC/PS = 4:1 in molar ratio and 100 nM TPA), 10 mM magnesium acetate and 1 μM Ca^{2+}. The reaction was stopped by mixing with an equal volume of ice-cold 1.12 density Ficoll 400 solution dissolved in 20 mM Tris–HCl (pH 7.5) buffer containing 1 μM Ca^{2+}. The mixture was layered within a discontinuous Ficoll density gradient of 1.05 and 1.01, and centrifuged at 100,000 *g* at 4° for 1 hr. The liposomes which floated upward through this gradient were collected, and analysed by SDS–PAGE and autoradiography.

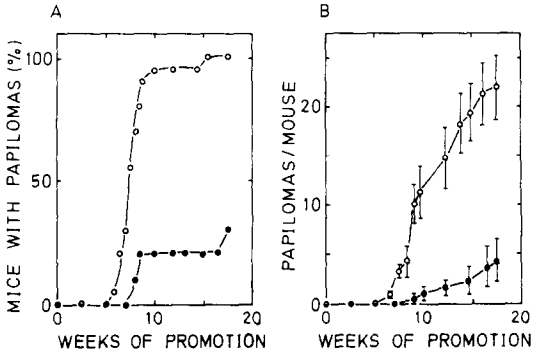

Fig. 1. Inhibitory effect of cepharanthine on skin papilloma formation promoted by TPA. CD-1 mice were initiated by a single topical application of 100 μg DMBA in 200 μL acetone. One week later, the mice were applied with 2.5 μg TPA in 200 μL acetone twice weekly for 18 weeks. In the cepharanthine group (closed circle, consisting of 10 mice), cepharanthine dissolved in 200 μL vehicle (acetone/ethanol = 1:1) was applied topically 5 min before each promoter application. In the control group (open circle, consisting of 20 mice), mice were applied the vehicle alone. (A) Percentage of tumor-bearing mice; (B) average number of papillomas per mouse. Bars indicate ±SE. The cepharanthine applied group was statistically different from the control group after week 8 in both cases, $P < 0.01$ (A: Chi-square test, B: Student's *t*-test).

Preparation of phospholipid vesicles. Phospholipids dissolved in chloroform and methanol (3:1, v/v), and TPA dissolved in ethanol were mixed, evaporated under N_2 gas, and dispersed in 20 mM Tris–HCl (pH 7.5) buffer containing 100 mM NaCl. The suspension was sonicated by Branson sonifier (Cell disruptor 185) at 0° for 1 hr under N_2 gas followed by centrifugation in order to eliminate titanium.

Lipid analysis of mouse skin epidermal cytosol. The lipids in mouse skin epidermal cytosol were extracted by the procedure of Bligh and Dyer [25]. The neutral lipids were separated by TLC developing in petroleum ether/ethyl ether/acetic acid (80:30:1, by vol.) and detected by iodine.

In the case of phospholipid separation, the sample was developed in chloroform/methanol/acetic acid/water (100:60:16:8, by vol.). Phosphate was visualized by the phosphate stain method modified by Vaskovsky and Kostetsky [26] and the density of visualized color at 700 nm was measured by densitometer (Shimadzu type CS-930).

RESULTS

Inhibition of tumor-promoting activity of TPA by cepharanthine

Figure 1 shows the time course of skin tumor formation of the groups treated with DMBA and TPA, with or without cepharanthine. In the cepharanthine applied group, the percentage of tumor bearing mice was decreased to 30% at week 18, although 95% of the control group mice developed tumors by week 10. The average number of tumors per mouse was dramatically decreased by cepharanthine from

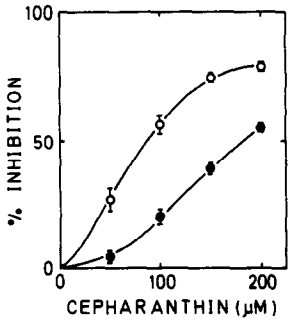

Fig. 2. Inhibitory effect of cepharanthine on the phosphorylation of H1 histone and lysine rich histone by PKC. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone (closed circle) or lysine rich histone (open circle). The reaction was performed at 30° for 10 min in the presence of 10 μM [γ-^{32}P]ATP, 1 μM Ca^{2+}, 10 mM magnesium acetate and 1 mM phospholipids (egg PC, or egg PC/PS = 4:1 and 100 nM TPA). The data are expressed as means ± SE of triplicate determinants. Above 20 μM cepharanthine, the inhibition of H1 histone phosphorylation was significantly different from that of lysine rich histone, $P < 0.01$ (Student's t-test).

23/mouse to 4.8/mouse at week 18. This cepharanthine effect was statistically significant. Control mice bore multiple foci of inflammation, necrosis and scarring, but cepharanthine applied mice had smooth skin. These results indicate that cepharanthine prevents the tumor promoting activity and the inflammatory effect of TPA.

Effect of cepharanthine on the phosphorylation of H1 histone by PKC

Figure 2 shows the inhibitory effect of cepharanthine on the phosphorylation of H1 histone and lysine rich histone by rat brain PKC. The phosphorylations of these substrates were inhibited by this drug in a concentration dependent manner. However, the extent of inhibition was different among substrate proteins. A 50% inhibition dose of cepharanthine on the phosphorylation of lysine rich histone was almost 100 μM. This value was approximately half of that of H1 histone. In the case of protamine sulfate, no cofactor, such as Ca^{2+}, was required to be phosphorylated by PKC [27]. This phosphorylation was slightly enhanced by cepharanthine in either the presence or the absence of Ca^{2+} and PS/TPA (data not shown). These results suggest that the inhibitory effect of cepharanthine on PKC activity is caused by modification of the enzyme–substrate interaction through an indirect mechanism.

Effect of cepharanthine on autophosphorylation of PKC

The autophosphorylation of PKC is known to occur following the activation of PKC [22], even though the rate of autophosphorylation is not entirely consistent with that of substrate phosphorylation [28]. Figure 3 shows the effect of cepharanthine on the autophosphorylation in the absence of the substrate. The autophosphorylation required Ca^{2+} and PS/TPA, and cepharanthine did not exhibit any inhibitory effect on the autophosphorylation at 100 μM. However, the autophosphorylation was increased by a high concentration of cepharanthine (200 μM). In this case, cepharanthine could not increase the autophosphorylation in the absence of cofactors. These results suggest that cepharanthine does not inhibit the activation of PKC itself. Thus, it is unlikely that cepharanthine inhibits PKC activation through direct interaction with PKC.

Effect of cepharanthine on H1 histone–phospholipid vesicle interaction

To obtain further insight, the effect of cepharanthine on phospholipid–H1 histone interaction was investigated. After phosphorylation of H1 histone by PKC in the presence of 1 μM Ca^{2+} and 1 mM phospholipid vesicles (egg PC, or egg PC/PS = 4:1 and 100 nM TPA), liposome-associated H1 histone was isolated from free H1 histone by Ficoll density gradient centrifugation. As shown in Fig. 4, H1 histone associated with the phospholipid vesicles consisting of egg PC, PS and TPA. The amount of associating H1 histone was decreased by cepharanthine. In the absence of PS and TPA, 200 μM cepharanthine caused neither the association of H1 histone with the vesicles nor the phosphorylation of H1 histone by PKC (data not shown). Namely, cepharanthine inhibited the H1 histone–phospholipid interaction and the phosphorylation by PKC. The inhibition of total histone phosphorylation was consistent with that of liposome–H1 histone association (Figs 2 and 4), although the phosphoprotein distributed in both associated and non-associated fractions. In this case, the distribution of phosphorylated protein was almost parallel with that of H1 histone. These results lead us to speculate that the inhibitory effect of cepharanthine on PKC activity is a consequence of the inhibition of the interaction of H1 histone with the phospholipid vesicles.

Effect of cepharanthine on the phosphorylation of mouse skin epidermal cytoplasmic proteins by endogenous protein kinases

Figure 5 shows the inhibitory effect of cepharanthine on the phosphorylation of skin epidermis cytoplasmic proteins by endogenous PKC. Many of the cytoplasmic proteins, such as 90, 85, 60, 50, 45 and 31 kDa, were phosphorylated in a Ca^{2+} and PS/TPA dependent fashion. Cepharanthine inhibited the phosphorylation of these proteins in a dose dependent manner, although the phosphorylation of some proteins, such as 64 and 54 kDa, was not inhibited. On the contrary, the phosphorylation of 80 kDa protein, corresponding to PKC, was increased by this drug as observed on the autophosphorylation of PKC (Fig. 3).

The phosphorylation of mouse skin epidermal cytosol proteins was also observed in the absence of PS, and was inhibited by cepharanthine. The following possibility was considered to explain these similarities: PKC activating factors or other protein kinases existed in the epidermal cytosolic fraction. Thus, we examined whether the cytosolic fractions contained activating factors for PKC or not.

Figure 6 shows the phosphorylation of lysine rich

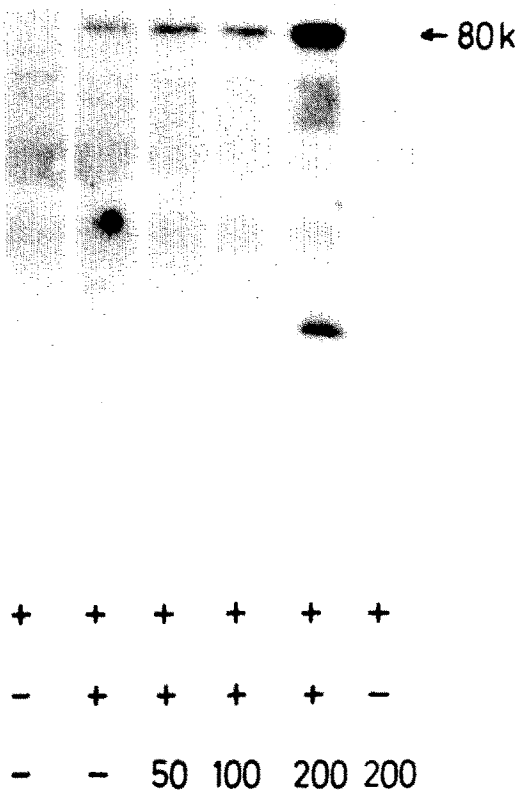

Fig. 3. Effect of cepharanthine on autophosphorylation of PKC. PKC was autophosphorylated in the presence of 1 μM Ca^{2+}, 10 μM [γ-^{32}P]ATP, 10 mM magnesium acetate, 1 mM phospholipids (egg PC, or egg PC/PS = 4:1 and 100 nM TPA) without addition of substrate proteins. The phosphorylation was analysed by SDS–PAGE (10% gel) and autoradiography. The arrow indicates the position of PKC.

histone or the endogeneous proteins by PKC in the presence of heat denatured skin epidermal cytosolic fraction. PKC was activated by addition of Ca^{2+} in the absence of PS and TPA, and the phosphorylation was further increased by addition of PS and TPA. The rate of histone phosphorylation was much higher than that of the endogeneous proteins. This phenomenon was due to the low concentration of PKC substrate proteins in the heat denatured epidermal cytosol fraction. In order to examine the type of PKC activating factors, we analysed the lipid composition of the epidermal cytosol fractions by TLC. As shown in Fig. 7, PS and diacylglycerol could be detected in the cytosol fraction. The factors might activate the endogeneous PKC in the presence of Ca^{2+} (Fig. 5). These results suggest that most of the phosphorylations induced by Ca^{2+} and/or PS/TPA are caused by PKC, and cepharanthine selectively inhibits the PKC dependent phosphorylations.

DISCUSSION

Like diacylglycerol, tumorigenic phorbol esters interacted with PKC directly and activated PKC both *in vitro* and *in vivo* [19]. It has been generally accepted that PKC is the receptor of phorbol ester [29] and that protein phosphorylation by this enzyme is an important event in tumor promotion. Some types of PKC inhibitor bearing both positively charged moiety and hydrophobic region, could inhibit tumor promotion activity of TPA [30]. Cepharanthine is also a cationic amphipath. As shown in Fig. 8, cepharanthine is a diester composed of two phenolic bases, *d*-1-(4′-hydroxybenzyl)-6-hydroxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline and 1-1-(4′-methoxybenzyl)-6-methoxy-7-hydroxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline [31]. Cationic amphipathic drugs were reported to interact with polar lipids [32], so that cepharanthine may also interact with negatively charged phospholipids, such as PS.

Sphingosine is a lipid with positively charged moiety and thought to be an endogeneous PKC inhibitor [33]. Hannun *et al.* [33] showed that sphingosine competitively intereacted with Ca^{2+}, PS and phorbol ester, resulting in the inhibition of PKC

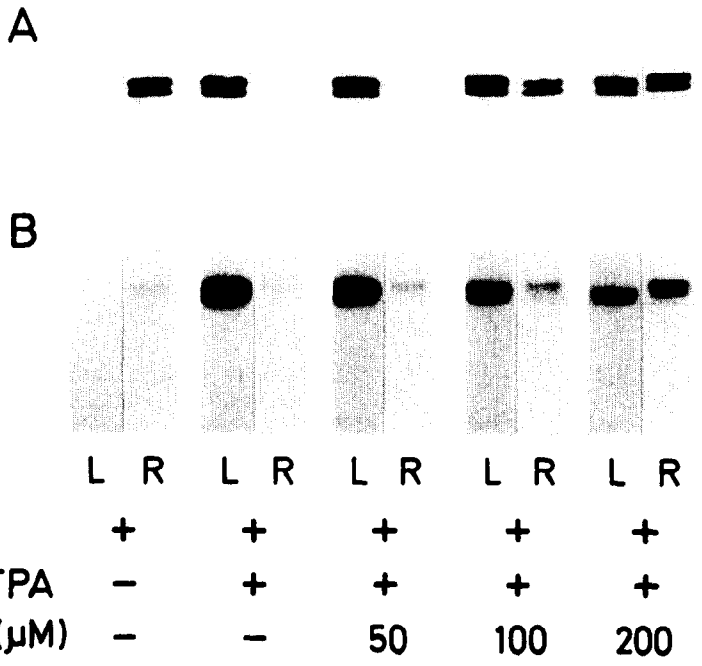

Fig. 4. Inhibitory effect of cepharanthine on the interaction of H1 histone and phospholipid vesicles. H1 histone was phosphorylated by PKC under the same conditions as Fig. 2. The reaction buffer was then mixed with an equal volume of ice-cold Ficoll 400 (1.12 density). The phospholipid vesicles were separated by the flotation method as described in Materials and Methods, and analysed by SDS–PAGE (10% gel) and autoradiography. (A) Silver stain; (B) autoradiography; L, liposome associated protein fraction; R, residual protein fraction.

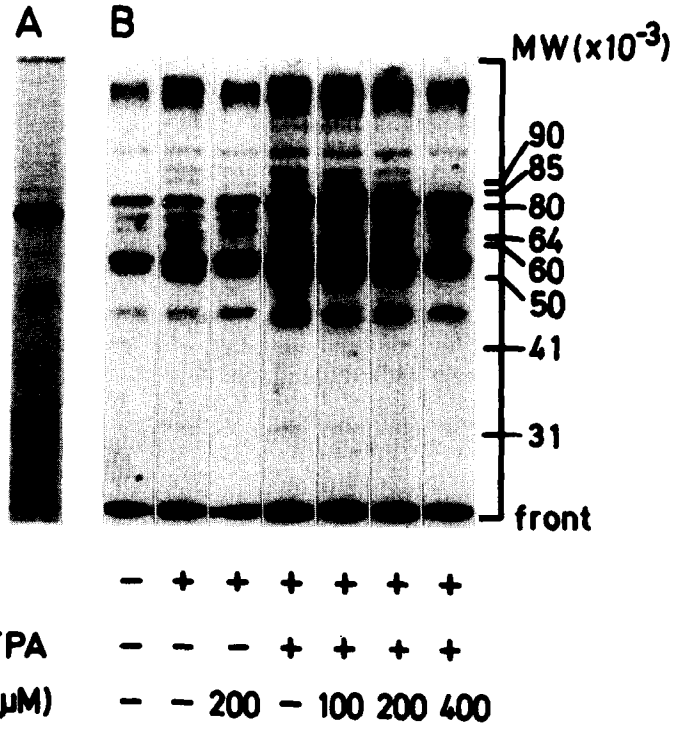

Fig. 5. Effect of cepharanthine on the phosphorylation of mouse skin epidermal cytoplasmic proteins by endogeneous protein kinases. CBB staining (A) and autoradiography (B) show the inhibitory effect of cepharanthine on the phosphorylation of specific proteins in the presence of Ca^{2+} and PS/TPA. The arrows indicate the position of major phosphoproteins. The macromolecular fraction of mouse skin epidermis cytosolic proteins was incubated with 1 mM EGTA or 1 μM Ca^{2+}, 10 μM [γ-^{32}P]ATP, 10 mM magnesium acetate, 100 μM phospholipids (egg PC, or egg PC/PS = 4:1 and 100 nM TPA) at 30° for 3 min. The reaction was stopped by addition of SDS–PAGE sample buffer, and SDS–PAGE (10% gel) was performed.

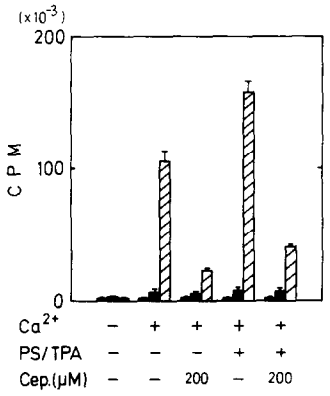

Fig. 6. Activation of PKC in heat denatured mouse skin epidermal cytoplasmic proteins in the absence of phospholipid. The macromolecular fraction of mouse epidermal cytosol was denatured at 100° for 3 min. PKC was purified from rat cerebral tissues. Lysine rich histone (200 μg/mL) was phosphorylated by PKC in the presence of denatured cytosol fraction (1 mg/mL), 10 μM [γ-^{32}P]ATP, 1 mM EGTA or 1 μM Ca^{2+}, 10 mM magnesium acetate, 100 μM phospholipids (egg PC, or egg PC/PS = 4:1 and 100 nM TPA) at 30° for 3 min. ^{32}P incorporation was measured by liquid scintillation counting. Open column, denatured cytosol + lysing rich histone; closed column, PKC + denatured cytosol; hatched column, PKC + denatured cytosol + lysine rich histone. The data are expressed as means + SE of triplicate determinants.

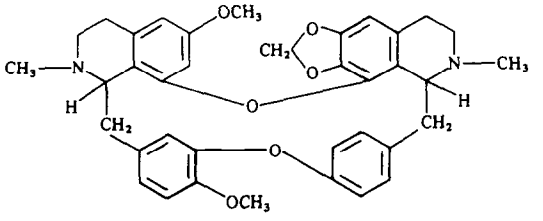

Fig. 8. Structure of cepharanthine.

activity while maintaining the association of the enzyme to the surface in Triton–PS micelles. On the other hand, Bazzi and Nelsestuen [34] demonstrated that histone could interact with PS and aggregate phospholipid vesicles consisting of egg PC and PS as well as Triton–PS mixed micelles. Furthermore, they suggested that the inhibition of PKC activity by sphingosine should be related to simple neutralization of the lipid, thereby preventing interaction with PKC and/or its substrate protein.

In our experiment, autophosphorylation of PKC was not inhibited by cepharanthine, suggesting that cepharanthine has no inhibitory action on PKC itself. On the contrary, cepharanthine inhibited the association of H1 histone with the liposomes consisting of egg PC and PS. The extent of inhibition for the association was almost paralleled with that for the phosphorylation of total proteins (Figs 2 and 4).

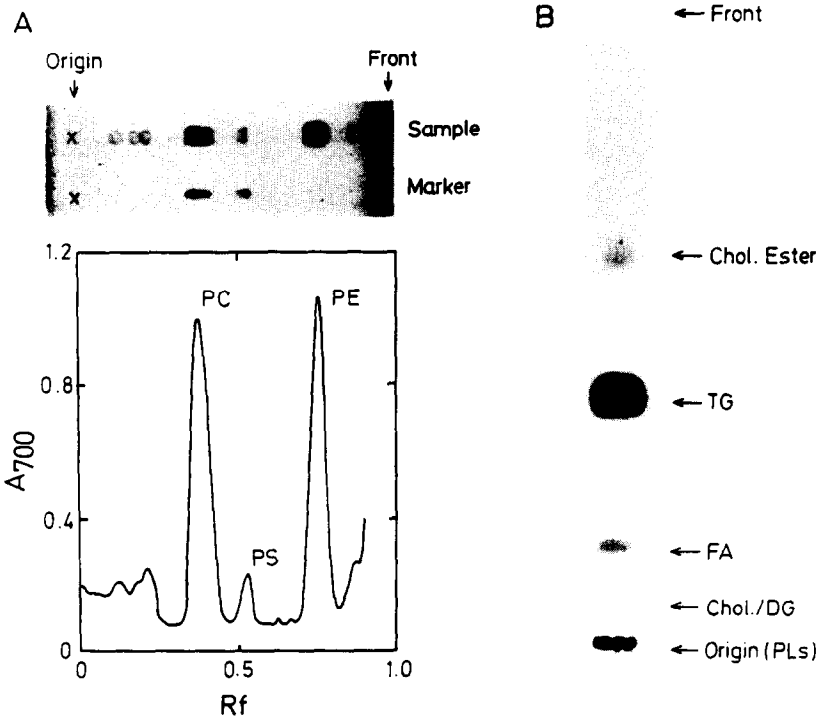

Fig. 7. Distribution of the lipids in skin epidermal cytosolic fraction. The distribution of the lipids in mouse epidermal cytosol was analysed by TLC. The extract of the cytosol fraction was developed in petroleum ether/ethyl ether/acetic acid (80:30:1, by vol.) or in chloroform/methanol/acetic acid/water (100:60:16:8, by vol.) for neutral lipids (B) or polar lipids (A), respectively. The lipids were visualized by molybdate (A) or iodine (B).

Thus, we presume that the mechanism of the inhibitory effect of cepharanthine on PKC activity is the consequence of the reduction of substrate association with the phospholipid vesicles.

The distribution of phosphorylated H1 histone in the liposome associated or non-associated fraction was almost parallel with that of H1 histone (Fig. 4). A possible explanation for this phenomenon is that there is little difference between phosphorylated and non-phosphorylated H1 histone in its affinity for PS, so that H1 histone associating with the liposomes was phosphorylated by PKC and easily released from the liposomes, resulting in the increase of phosphorylated H1 histone in the non-associated fraction.

We demonstrated that the phosphorylation of skin epidermal cytosolic proteins, such as 90, 85, 80, 64, 60, 54, 50 and 31 kDa, was increased by Ca^{2+} and PS/TPA. The phosphorylation of many proteins, such as 90, 85 and 60 kDa was inhibited by cepharanthine, although the phosphorylation of 64 and 54 kDa was not inhibited. The reason for the lack of the inhibitory effect on the phosphorylation of 64 and 54 kDa is not clear yet. However, several studies suggested that the PKC substrates required the ability of membrane association, Buss *et al.* [35] indicated that the absence of myristic acid decreased the membrane binding of $p60^{src}$ and that $p60^{src}$ lost the ability to be phosphorylated by PKC. Furthermore, we previously reported that the acylated lysozyme associated with liposomes and was phosphorylated by PKC [36, 37]. Thus, we speculate that the association of substrate protein to membrane is prerequisite for the phosphorylation by PKC.

As shown in Fig. 5, Ca^{2+} induced the same phosphorylation in the absence of PS/TPA and this phosphorylation also was inhibited by cepharanthine. This phosphorylation depended on the factors for PKC activation in the epidermal cytosol fraction, because purified PKC could be activated by Ca^{2+} in heat denatured cytosol without addition of PS and TPA (Fig. 6). In fact, we found PS and diacylglycerol in the cytosolic fraction (Fig. 7), so that these materials may have activated endogeneous PKC by addition of Ca^{2+} in the absence of PS.

Chida *et al.* [38] reported that TPA increased phosphorylation of 40 and 34 kDa cytosolic proteins of mouse skin *in vivo*, and purified PKC could phosphorylate these proteins *in vitro*. In our results, however, 41 kDa protein, which is similar in molecular weight to 40 kDa, was phosphorylated slightly and no phosphorylation was observed in 34 kDa protein. The reason for this discrepancy is not clear. Several annexin family proteins, which are distributed in skin epidermal cytosol, have similar molecular weights to these proteins. Phosphorylation of annexin 1 by PKC is known to be dependent on Ca^{2+} concentration [39, 40]. Therefore, it is conceivable that the 40 and 34 kDa proteins in skin epidermal cells were Ca^{2+} dependent phospholipid binding proteins. Therefore, the following two possibilities are considered as explanations for the failure of these protein phosphorylations. One is low concentration of Ca^{2+} in the PKC assay system, and the other is dephosphorylation by endogeneous phosphatase activity.

In the present study, we have shown the possible PKC substrate proteins in mouse skin epidermal cytosol fraction and preferential inhibition of these protein phosphorylations by cepharanthine. More direct evidence of participation of these proteins in tumor promotion by TPA will be the subject of further investigation.

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