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SPHINGOMYELIN IS A POSSIBLE CONSTITUENT OF BINDING SITES FOR THE TUMOR PROMOTERS PHORBOL ESTER, INDOLE ALKALOIDS AND POLYACETATES

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Specific binding of 12-O-tetradecanoylphorbol-13-acetate to the epidermal particulate fraction was susceptible to phospholipase A_2 and phospholipase C, but was almost resistant to protease and completely resistant to a mixture of several glycosidases. Of vesicles prepared from five phospholipids associated with the epidermal particulate fraction, sphingomyelin vesicles bound 12-O-tetradecanoylphorbol-13-acetate specifically and most effectively. This binding was inhibited by not only phorbol esters but also two other classes of tumor promoters, indole alkaloids and polyacetates. These results suggest that sphingomyelin is involved in the binding sites of tumor promoters to the cell membrane.

Tumor promoting phorbol esters and two new classes of tumor promoters, indole alkaloids (1) and polyacetates (2), may bind first to receptors on the cell membrane, leading to various effects on cellular functions, including tumor promotion (3,4). Recently, we demonstrated that indole alkaloids and polyacetates, new classes of tumor promoters, inhibited the specific binding of [^{3}H]PDBu[1] to fibroblast cell lines (5), and [^{3}H]PDPr and [^{3}H]TPA to a particulate fraction from mouse epidermis (6, manuscript in preparation) and suggested that phorbol ester receptors might be also used for these new tumor promoters (5,6).

We attempted to characterize the physiological constituents of phorbol ester receptors in a particulate fraction from mouse epidermis using the cold

[1]Abbreviations: PDBu, phorbol-12,13-dibutyrate; PDPr, phorbol-12,13-dipropionate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDD, phorbol-12,13-didecanoate.

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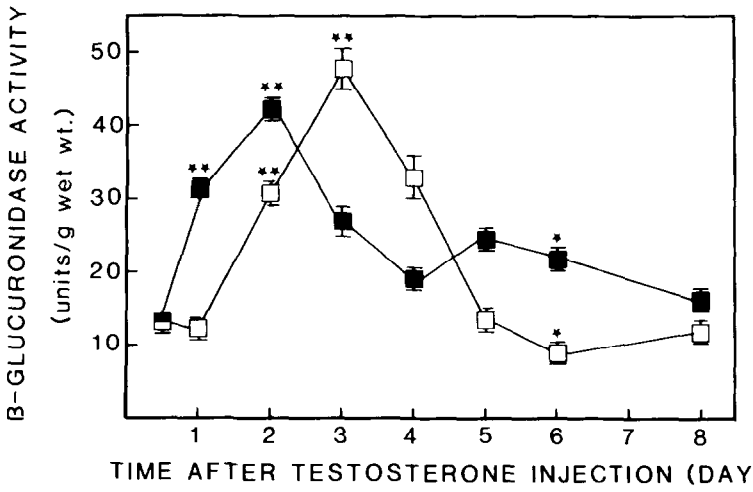

Fig.2. The effect of the combined treatment with DFMO and MGBG on testosterone-induced stimulation of β-glucuronidase activity in mouse kidney. Treatment of animals as described for Fig.1. Testosterone (■); testosterone with inhibitors (□). Each symbol with a vertical bar shows the mean ± S.E. for a given experimental group comprising at least 4 animals. Asterisks indicate significantly different values from the control β-glucuronidase level which was 14.2 ± 0.8 units/g wet wt. * P<0.01; ** P<0.001.

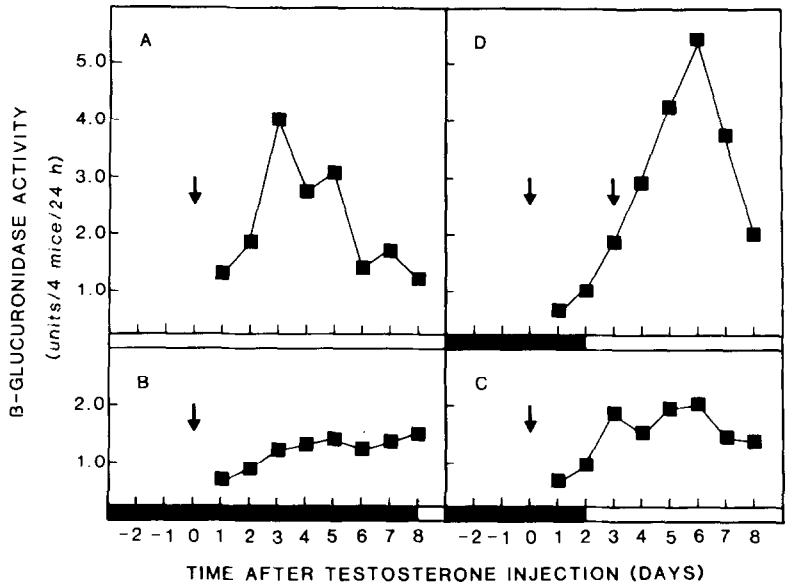

Fig.3. Urinary secretion of β-glucuronidase after testosterone injection. A. Without inhibitors. B. With DFMO and MGBG. C. After withdrawal of inhibitor-treatment. D. After withdrawal of inhibitor-treatment and repeated testosterone injection. Testosterone injection point is indicated by the arrow and inhibitor-treatment period by the black horizontal bar. Doses of testosterone and inhibitors as described for Fig.1 and 2.

Table 1 Effects of enzyme treatment of the epidermal particulate fraction on specific [^{3}H]TPA binding

Enzyme	Specific binding remaining[a] (% of control)
Proteinase K (0.2 mg/ml)	73 ± 11
Phospholipase A_2 (30 units/ml)	50 ± 4
Phospholipase C (2 units/ml)	48 ± 6
Endoglycosidase D (0.05 unit/ml)	130 ± 2
Neuraminidase (0.1 unit/ml)	112 ± 2
Neuraminidase (0.1 unit/ml) +β-galactosidase (0.1 unit/ml) +β-N-acetylglucosaminidase (0.1 unit/ml)	90 ± 2
Endoglycosidase D (0.05 unit/ml) +neuraminidase (0.1 unit/ml) +β-galactosidase (0.1 unit/ml) +β-N-acetylglucosaminidase (0.1 unit/ml)	130 ± 1

[a]After the enzyme treatment, 100 μg protein of samples were assayed for [^{3}H]TPA binding by incubation with 4 nM [^{3}H]TPA at 0°C for 2 h in the presence or absence of 2 μM unlabeled TPA. Specific binding was calculated by subtracting nonspecific binding from total binding.

particulate fraction (data not shown), 73% of the TPA binding activity still remained. Treatments with glycosidases such as endoglycosidase D, neuraminidase, β-galactosidase, β-N-acetylglucosaminidase or mixtures of these enzymes did not affect the binding activity. In addition, monosaccharides, such as glucose, galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid at concentrations of 50 mM did not inhibit the specific binding of [^{3}H]TPA (data not shown).

These results suggest that TPA receptors may be complexes of various membrane components such as phospholipid and protein, phospholipid being the more important for recognition, and that sugars are not involved in TPA receptor recognition.

[^{3}H]TPA binding activity of phospholipid vesicles: The phospholipid content of the particulate fraction of mouse epidermis was determined. The contents

per 100 μg protein were as follows: phosphatidylcholine (14 nmol), phosphatidylethanolamine (9.9 nmol), sphingomyelin (2.2 nmol), phosphatidylinositol (1.9 nmol), phosphatidylserine (1.6 nmol) and lysophosphatidylcholine (1.6 nmol). Vesicles of these five phospholipids were prepared using authentic phospholipids and assayed for direct binding of [^{3}H]TPA. The data were normalized for the amount of phospholipid remaining on the filter. Fig. 1 shows that sphingomyelin vesicles bound [^{3}H]TPA most effectively and dose-dependently and that the other vesicles had little or no binding activity at a dose of 20 nmol.

Characterization of [^{3}H]TPA binding to sphingomyelin vesicles: Fig. 2a shows that [^{3}H]TPA bound to sphingomyelin vesicles specifically and that nonspecific binding in the presence of 500-fold excess of unlabeled TPA was low. Specific binding of TPA gave a sigmoidal curve. Scatchard analysis of the data gave a convex curve (Fig. 2b) and Hill analysis showed that the Hill coefficient was 2 (Fig. 2c). Half maximum binding was observed with more than 13 nM [^{3}H]TPA. In contrast, the specific binding of [^{3}H]TPA to the epidermal particulate fraction gave a hyperbolic curve and a Scatchard plot gave a

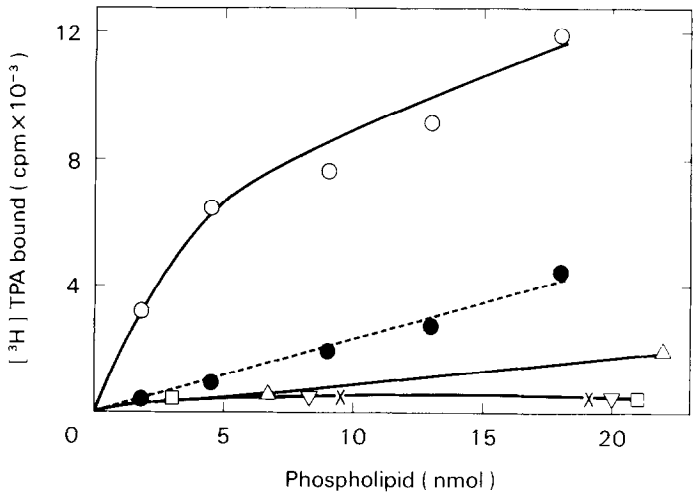

Fig. 1 [^{3}H]TPA binding to various phospholipid vesicles. Vesicles were incubated with 20 nM [^{3}H]TPA at 0°C for 2 h in the presence (●, △, □, ▽, X) or absence (○, △, □, ▽, X) of 10 μM unlabeled TPA. After incubation, binding activity was determined by the filter method and expressed as the amount of phospholipid retained on the filter; ○ ●, sphingomyelin; △, phosphatidylcholine; □, phosphatidylethanolamine; ▽, phosphatidylinositol; X, phosphatidylserine.

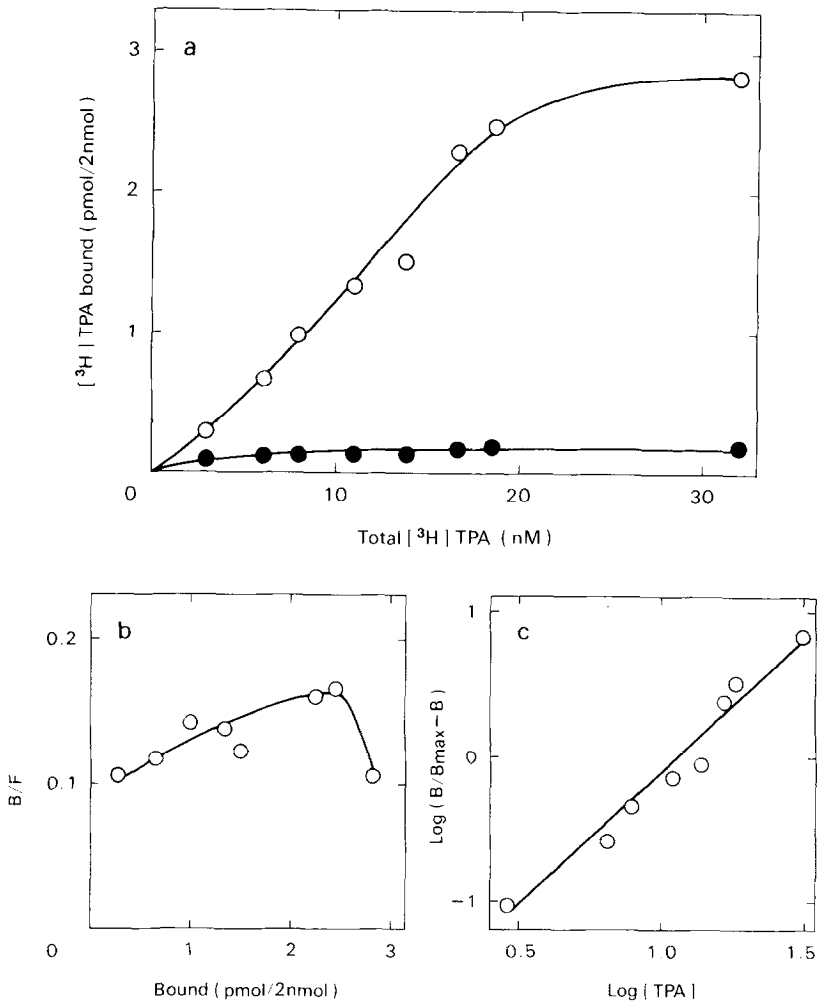

Fig. 2 [^{3}H]TPA binding to sphingomyelin vesicles. Two nmol of sphingomyelin vesicles were incubated with various concentrations of [^{3}H]TPA in the presence or absence of a 500-fold excess of unlabeled TPA at 0°C for 2 h. (a) Dose-response of specific binding (○) and nonspecific binding (●) of [^{3}H]TPA. (b) Scatchard plot and (c) Hill plot of data (a).

straight line with a K_D of 1.8 nM. Thus specific binding of TPA to sphingomyelin was different from physiological binding: the former showed positive cooperation of binding whereas the latter did not, and its binding affinity was lower than that of physiological binding.

To examine the specificity of TPA binding sites on sphingomyelin vesicles, we tested the inhibitions by TPA, PDD, PDBu, phorbol, teleocidin and aplysiatoxin of [^{3}H]TPA binding to sphingomyelin vesicles (Fig. 3). Teleocidin and aplysiatoxin are members of new classes of tumor promoters, indole alkaloids

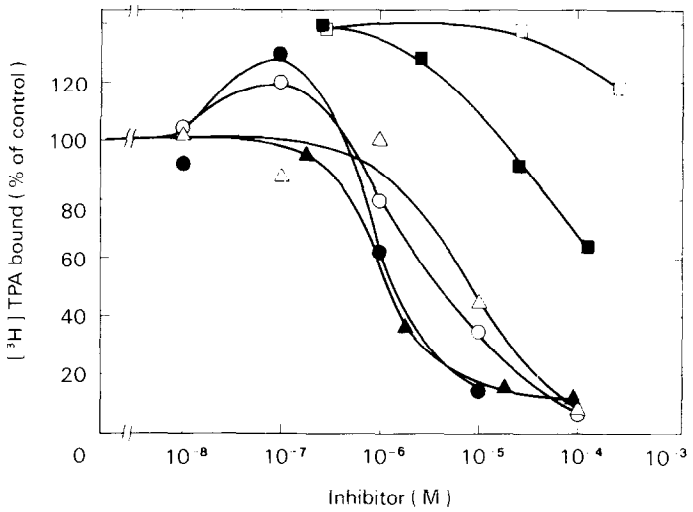

Fig. 3 Inhibition of [^{3}H]TPA binding to sphingomyelin vesicles by TPA (●), PDD (▲), PDBu (■), teleocidin (○), aplysiatoxin (△), and phorbol (□). Two nmol of sphingomyelin vesicles were incubated with 10 nM [^{3}H]TPA and test compounds at 0°C for 2 h.

and polyacetates, respectively, which are structurally quite different from phorbol ester (1,2). Not only TPA and PDD, but also teleocidin and aplysiatoxin inhibited [^{3}H]TPA binding to sphingomyelin vesicles. PDBu was weakly inhibitory, but phorbol did not inhibit the binding at a concentration of 10^{-4} M. Thus TPA binding sites on sphingomyelin vesicles were specific for phorbol esters and the new classes of tumor promoters, indole alkaloids and polyacetates. These results were compatible with the inhibition of specific binding of phorbol ester to cells or the epidermal particulate fraction by these tumor promoters (5,6).

DISCUSSION

A very interesting finding in this work was that TPA binding to the epidermal particulate fraction was almost resistant to proteinase K. Moreover, the binding activity remaining after proteinase K-treatment had the same high affinity as that before treatment (data not shown). Thus some molecule(s) other than protein may be involved in TPA binding in our system.

We found that of vesicles of the five phospholipids in cell membranes only sphingomyelin vesicles could bind TPA specifically and that this binding was specific for tumor promoters. The specific binding of TPA to sphingomyelin

vesicles differed from the TPA binding to the epidermal particulate fraction in showing positive cooperation, and in binding affinity and susceptibility to enzymes. The reason for these differences is still unknown. We suppose that TPA receptors on the epidermal particulate fraction may be constructed by a complex of phospholipid and protein, and that addition of some other components of the cell membrane to sphingomyelin vesicles may restore the binding characteristics of TPA receptors on cell membrane.

Recently, Perrella et al. (13) reported that binding of phorbol ester to nuclear extracts of liver gave a sigmoidal curve, like the specific binding to sphingomyelin vesicles in our study. A sigmoidal pattern of binding may even occur physiologically and sphingomyelin may be involved in receptor molecules for phorbol esters.

Several investigators have reported the characteristics of phorbol ester receptors; specific [^{3}H]PDBu binding to the brain particulate fraction was sensitive to papain and phospholipase A_2 (14), so phorbol ester receptors may be protein with some lipid environment (15). PDBu receptor activity was associated with protein kinase C from rat brain in the presence of phospholipid (16). TPA binding sites in a nuclear extract of liver were found to be receptor protein (13), and the PDBu binding sites on isolated epidermal cells not to be glycoconjugates or phospholipids (17). However, a glycoprotein was involved in [^{3}H]PDBu binding to the surface of cultured epidermal cells (18). TPA binding sites on red blood cell were shown to be gangliosides (19). Here we obtained a new finding about constituents of phorbol ester receptors in an epidermal particulate fraction. These conflicting results may be due to differences in experimental conditions such as differences in the cells or tissues or binding assay systems used, or may indicate heterogeneity in the phorbol ester receptors.

TPA is highly lipophilic, and phorbol ester receptors are widely distributed on mammalian cells (4,8,20) and are present in especially large amounts in brain (8,20). The receptors also interacted with other tumor promoters (5,6). Taken together with other results, our findings are consist-

ent with the idea that sphingomyelin as a membrane component may be involved in binding sites of tumor promoters, as ganglioside GM1 is a receptor of cholera toxin (21).

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