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Specific binding of okadaic acid, a new tumor promoter in mouse skin

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The tumor promoter okadaic acid binds specifically to a particulate as well as a cytosolic fraction of various mouse tissues, e.g., skin, brain, lung and colon. The K_D value was 21.7 nM for receptors in the particulate fraction and 1.0 nM for those in the cytosolic fraction of mouse skin. The specific binding of [^{3}H]okadaic acid to the particulate fraction of mouse skin was inhibited dose-dependently by okadaic acid, but not okaidaic acid tetramethyl ether, an inactive compound, or by other tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate and teleocidin. The results suggest a new pathway of tumor promotion mediated through the okadaic acid receptor(s).

Okadaic acid; Tumor promoter; Specific binding

1. INTRODUCTION

Okadaic acid, which is a polyether compound of a C_{38} fatty acid isolated from a black sponge *Halichondria okadai* [1], showed potent tumor-promoting activity in a two-stage carcinogenesis experiment on mouse skin [2]. The tumor-promoting activity of okadaic acid is as strong as that of teleocidin, but these two tumor promoters act on the cells in different ways. Namely, okadaic acid does not inhibit the specific binding of [^{3}H]12-*O*-tetradecanoylphorbol-13-acetate (TPA) to a particulate fraction of mouse skin, or activate protein kinase C isolated from mouse brain in vitro, whereas teleocidin, like TPA and aplysiatoxin, does both [2,3]. We are studying this new pathway for tumor promotion with okadaic acid. Recently, we obtained [27-^{3}H]okadaic acid by tritium-labeling of methyl 27-ketookadaate with sodium boro[^{3}H]hydride [4]. In this paper, we report the specific binding of [^{3}H]okadaic acid to both a particulate fraction and a cytosolic fraction of mouse skin. DEAE-cellulose column chromatography of the cytosolic fraction of mouse skin gave a protein peak, eluted with 0.2 M NaCl, that also showed significant specific binding of [^{3}H]okadaic acid. The binding fraction of mouse skin contained protein phosphatases and protein kinases (data not shown). Similar results have also been found in mouse brain [5]. The specific binding of [^{3}H]okadaic acid is intimately associated with protein phosphatase activity. The results indicate a new mechanism of action of the tumor promoter, okadaic acid.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; K_D, dissociation constant; B_{max}, maximum binding capacity

2. MATERIALS AND METHODS

2.1. *Chemicals*

Okadaic acid and dinophysistoxin-1 (35-methylokadaic acid) were isolated from a black sponge, *Halichondria okadai* as described previously [1,2,6]. Okadaic acid tetramethyl ether was synthesized chemically from okadaic acid [7]. [27-^{3}H]Okadaic

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acid (14 Ci/mmol) was prepared at Amersham (Buckinghamshire, England) as reported previously [4].

2.2. *Specific binding of [^{3}H]okadaic acid*

Particulate and cytosolic fractions from the epidermis of the back of CD-1 mice were prepared as described previously [8]. Specific binding of [^{3}H]okadaic acid to a particulate fraction was measured by the filtration method that follows. [^{3}H]-Okadaic acid was incubated with the particulate fraction in a total volume of 1 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM 2-mercaptoethanol for 20 min at 37°C. Then the mixture was filtered on a GF/F glass fiber filter with 50 mM Tris-HCl buffer, pH 7.4, containing bovine serum albumin at a concentration of 1 mg/ml. Specific binding of [^{3}H]okadaic acid to the cytosolic fraction was assayed by the filtration method using cold acetone [8,9]. [^{3}H]Okadaic acid was incubated with the cytosolic fraction for 2 h at 0°C. The incubation mixture was filtered on a GF/C glass fiber filter with acetone cooled to −78°C as described previously [8]. Non-specific binding was measured in the presence of 500-fold excess of unlabeled okadaic acid. Specific binding was calculated by subtracting non-specific binding from total binding.

2.3. *Isolation of a specific binding protein(s) of okadaic acid from the cytosolic fraction of mouse skin by DEAE-cellulose column chromatography*

The cytosolic fraction was prepared from the epidermis of the backs of 60 mice. The total supernatant, containing 140 mg protein, was applied to a DEAE-cellulose column (2.6 × 25.0 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 mM leupeptin and 10% glycerol. The fractions of eluate were tested for ability to bind [^{3}H]okadaic acid specifically.

3. RESULTS AND DISCUSSION

3.1. *Specific binding of [^{3}H]okadaic acid*

The specific binding of [^{3}H]okadaic acid to the particulate fraction increased linearly with increase in protein concentration (fig.1A). With 200 μg of protein of the particulate fraction, the specific binding of [^{3}H]okadaic acid became saturated at about 80 nM [^{3}H]okadaic acid (fig.1B). These results demonstrate the specific binding of [^{3}H]-okadaic acid to the particulate fraction of mouse epidermis. Scatchard analysis of the equilibrium binding data (fig.1B, inset) indicated that the dissociation constant, K_D, was 21.7 nM and the maximum binding capacity, B_{max}, was 2.5 pmol/mg protein. Unlabeled okadaic acid caused dose-dependent inhibition of the specific binding of [^{3}H]okadaic acid to the particulate fraction (fig.2). The concentration of okadaic acid for 50% inhibition, ED_{50}, was 30 nM. Okadaic acid tetramethyl ether, which is an inactive compound

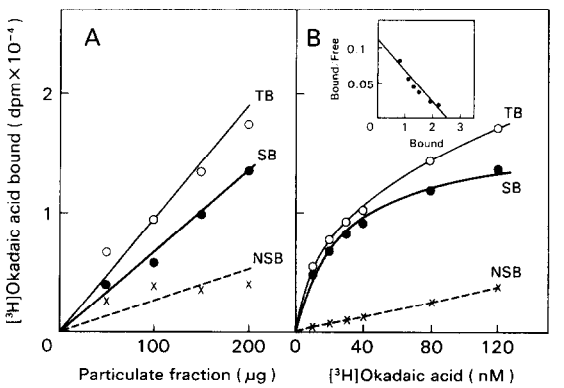

Fig.1. Specific binding of [^{3}H]okadaic acid to the particulate fraction. Various concentrations of the particulate fraction were incubated with 80 nM [^{3}H]okadaic acid (A) and 200 μg of the particulate fraction was incubated with various concentrations of [^{3}H]okadaic acid (B). Total bound [^{3}H]okadaic acid (TB, ○), specifically bound [^{3}H]okadaic acid (SB, ●) and non-specifically bound [^{3}H]okadaic acid (NSB, ×). (Inset) Scatchard analysis [14].

[7], did not inhibit the specific binding of [^{3}H]-okadaic acid at concentrations of up to 100 μM (fig.2). The specific binding of [^{3}H]okadaic acid was also not inhibited by TPA-type tumor promoters, TPA or teleocidin (fig.2), or by the non-TPA type tumor promoters palytoxin and thapsigargin (data not shown), which do not bind to the phorbol ester receptor [3,11], at concentrations of

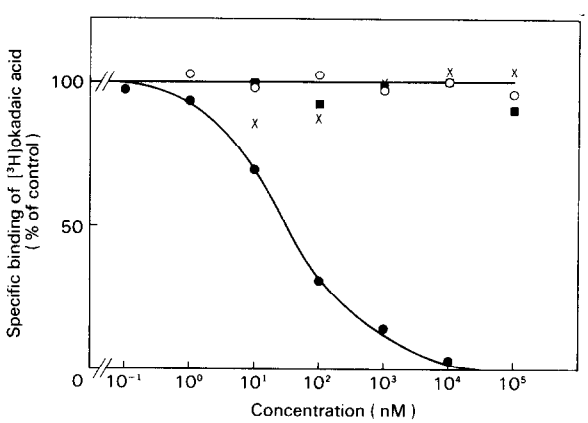

Fig.2. Inhibition of specific binding of [^{3}H]okadaic acid to the particulate fraction by various compounds. A mixture of 150 μg of the particulate fraction and 30 nM [^{3}H]okadaic acid was incubated with various concentrations of unlabeled okadaic acid (●), okadaic acid tetramethyl ether (○), TPA (×) or teleocidin (■).

up to 100 μM. These results clearly indicated that the binding of [^{3}H]okadaic acid is highly specific and that the specificity correlates well with the biological activities of okadaic acid class tumor promoters.

The specific binding of [^{3}H]okadaic acid to a cytosolic fraction of mouse epidermis was also demonstrated (fig.3). The K_D for binding was 1.0 nM and the B_{max} was 6.8 pmol/mg protein. Scatchard analysis (fig.3B, inset) indicated that receptors in the cytosolic fraction appeared to have higher affinity for binding than those in the particulate fraction. It is unknown how the binding of okadaic acid to the cytosolic fraction is related to its binding to the particulate fraction. We determined the specific binding of [^{3}H]okadaic acid per mg of these fractions of various mouse tissues (table 1). Both the particulate and cytosolic fractions of various mouse tissues, such as brain, lung, and colon showed specific binding, and the total amount of the binding molecule(s) seemed to be greater in the cytosolic fractions than in the particulate fractions. If there are several binding molecules with different binding affinities of [^{3}H]-okadaic acid in particulate and cytosolic fractions, the amounts of the binding molecule(s) of various tissues, as shown in table 1, might not indicate the exact total amount of binding molecule(s). But we thought that the cytosolic fraction contained a larger amount of binding molecule(s) for [^{3}H]-okadaic acid than did the particulate fraction. Therefore we have isolated the binding molecule(s) from a cytosolic fraction of mouse skin.

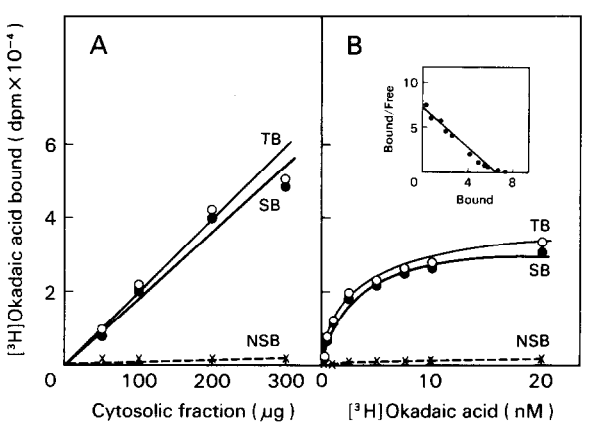

Fig.3. Specific binding of [^{3}H]okadaic acid to the cytosolic fraction. Various concentrations of the cytosolic fraction were incubated with 20 nM [^{3}H]okadaic acid (A) and 150 μg of the cytosolic fraction was incubated with various concentrations of [^{3}H]okadaic acid (B). Total bound [^{3}H]okadaic acid (TB, ○), specifically bound [^{3}H]okadaic acid (SB, ●) and nonspecifically bound [^{3}H]okadaic acid (NSB, ×). (Inset) Scatchard analysis.

Table 1

Specific [^{3}H]okadaic acid binding to fractions of various mouse tissues

	Specific [^{3}H]okadaic acid binding to	
	Particulate fraction[a] (pmol/mg protein)	Cytosolic fraction[b] (pmol/mg protein)
Brain	2.6	16.6
Lung	2.3	7.8
Colon	1.9	12.7
Kidney	1.7	5.6
Stomach	1.3	10.5
Thymus	1.2	9.9
Skin	1.1	9.2
Spleen	1.1	8.1
Ovaries	0.8	13.3
Small intestine	0.7	18.5
Liver	0.6	6.8

[a] The particulate fraction (150 μg) was incubated with 80 nM [^{3}H]okadaic acid. Specific binding of [^{3}H]okadaic acid was measured by the filtration method with the buffer as described in the text

[b] The cytosolic fraction (150 μg) was incubated with 40 nM [^{3}H]okadaic acid. Specific binding of [^{3}H]okadaic acid was measured by the filtration method with cold acetone as described in the text

Tissue was homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA, 2 mM EGTA and 0.2 mM PMSF by Polytron homogenizer for 1 min. The homogenate was centrifuged at 1000 × g for 30 min at 4°C. The supernatant was separated into cytosolic and particulate fractions by centrifugation at 100 000 × g for 60 min at 4°C

3.2. *Isolation of specific binding protein(s) of okadaic acid from the cytosolic fraction of mouse skin*

It was applied to DEAE-cellulose column chromatography to isolate the binding molecule(s). As seen in fig.4, one peak of material eluted with 0.2 M NaCl showed high specific binding of [^{3}H]-okadaic acid. We also found phosphatase activity and protein kinase activity in the binding fraction of mouse skin, and that the phosphatase activity was inhibited by okadaic acid (data not shown). Similar results have been found in mouse brain [5]. Based on our evidence that [^{3}H]okadaic acid does not bind to the protein kinases contained in the binding fraction, the protein phosphatases seem to

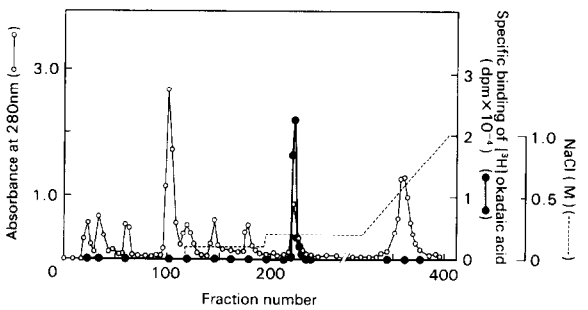

Fig.4. DEAE-cellulose column chromatography of a cytosolic fraction of mouse epidermis. Material was eluted with 450 ml equilibrating buffer at 60 ml/h and then with the same buffer containing different concentrations of NaCl as follows, 320 ml of 0.1 M NaCl, 480 ml of 0.2 M NaCl, gradient (200 ml of 0.2 M NaCl–200 ml of 1.0 M NaCl). 4 ml fractions were collected. Specific binding of each fraction (50 μl) was measured with 10 nM [^{3}H]okadaic acid.

be binding molecules (receptors) for the okadaic acid class of tumor promoters (manuscript in preparation). Recently, it has been reported that okadaic acid inhibits protein phosphatase 1 and 2A, but not protein phosphatase 2B [12,13]. We are now isolating protein phosphatase 1 and 2A and trying to study the direct binding of [^{3}H]-okadaic acid to protein phosphatase 1 and 2A. Protein phosphatases are contained in larger amounts in brain than in other tissues. This fact is compatible with the results that brain contained a larger amount of the binding molecule(s) for [^{3}H]okadaic acid than other tissues.

Recently, we found calyculin A to be an additional tumor promoter of the okadaic acid class, which was screened by the inhibition of the specific binding of [^{3}H]okadaic acid to a particulate fraction (manuscript in preparation). This binding assay is useful to find further compounds of the okadaic acid class. These findings suggest the existence of a new pathway of tumor promotion other than by activation of protein kinase C, the so-called phorbol ester receptor pathway.

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