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Thyrsiferyl 23-acetate is a novel specific inhibitor of protein phosphatase PP2A

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Abstract Thyrsiferyl 23-acetate (TF23A), a cytotoxic compound from marine red alga, has been shown to potently and specifically inhibit serine/threonine protein phosphatase 2A (PP2A) with IC_{50} values of 4–16 μM, depending on the enzyme concentration. TF23A did not affect activity of protein phosphatase 1 (PP1), 2B (PP2B), 2C (PP2C), or protein tyrosine phosphatases (PTP) up to 1 mM. It inhibited PP2A activity in a crude extract of a human T cell line, Jurkat cell, as well as the purified catalytic subunit. Thus, TF23A proved to be a novel useful probe for clearly distinguishing the activity of PP2A from those of the other protein phosphatases in crude cell extracts and identification of cellular processes that are regulated by PP2A.

Key words: Thyrsiferyl 23-acetate; Protein phosphatase 2A

1. Introduction

Okadaic acid (OA), a polyether fatty acid isolated from marine sponges as a cytotoxic compound [1], has been reported to have a potent inhibitory effect on serine/threonine protein phosphatase PP1 and PP2A [2]. Another polyether containing squalene carbon skeleton, thyrsiferyl 23-acetate (TF23A), was isolated from the red alga *L. obtusa* [3]. This compound, like OA, showed strong cytotoxicity against mammalian cells, suggesting that TF23A might also be an inhibitor of protein phosphatases.

A number of substances have an inhibitory effect on serine/threonine protein phosphatase activities, such as calyculin A [4], tautomycin [5,6], microcystin-LR [6,7], and nodularin [9]. However, there had been no known inhibitor which affects only PP2A until now. All these substances show a strong inhibitory effect towards both PP1 and PP2A, and some of them inhibit PP2B to a lesser extent. These facts make it difficult to study the specific function of each molecular species of protein phosphatase.

We report here that TF23A is a potent and specific inhibitor of PP2A but has no effect on PP1, PP2B, PP2C, or PTP.

2. Materials and methods

2.1. Materials

Thyrsiferyl 23-acetate (TF23A) prepared from the red alga *L. obtusa* as previously described [3], was dissolved in dimethyl sulfoxide at 20 mM. Okadaic acid was a generous gift from Dr. Tsukitani (Fujisawa Chemical Co., Japan). Catalytic subunits of PP1 and PP2A [10], phosphorylase *a* [11], phosphorylase kinase [12], and inhibitor-2 [13] were prepared from rabbit skeletal muscle as previously described. PP2C [14] and tyrosine kinases [15] were partially purified from rat liver and spleen, respectively. Myelin basic protein (MBP), histone (type IIA-S), lysozyme, and catalytic subunit of bovine heart cAMP-dependent protein kinase were obtained from Sigma Chemical Co. (St. Louis, MO). Catalytic subunit of bovine brain PP2B was from Upstate Biotechnology (Lake Placid, NY). [γ-^{32}P]ATP was purchased from Dupont/NEN Research Products (Boston, MA).

2.2. Preparation of cell extracts

The human acute T lymphoblastic leukemia cell line, Jurkat cells, was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol. The cells were lysed at a concentration of 2×10^7/ml in cold lysis buffer containing 50 mM Tris-HCl (pH 7.0), 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 0.1 mM PMSF, 0.5 mM TPCK, 1 mM benzamidine, 4 μg/ml leupeptin, and 1.0% (w/v) Nonidet P-40. The lysates were centrifuged at $8,000 \times g$ for 10 min and the resulting supernatants were used as cell extracts.

2.3. Preparation of ^{32}P-labelled proteins and phosphatase assays

^{32}P-Labelled phosphorylase *a* was prepared by using phosphorylase kinase [16]. ^{32}P-Labelled MBP and [^{32}P]histone were prepared by using cAMP-dependent kinase [17]. ^{32}P-Labelled RCM-lysozyme was prepared by using rat spleen tyrosine kinases [18]. Phosphatase assays were performed as previously described [19]. PP1 and PP2A were assayed in the absence of divalent cations by using 10 μM [^{32}P]phosphorylase *a*, 5 μM [^{32}P]MBP or 5 μM [^{32}P]histone as substrate. PP2B was assayed in the presence of 2 mM $CaCl_2$ and 3 μM calmodulin by using 5 μM [^{32}P]MBP [20]. PP2C was assayed in the presence of 10 mM $MgCl_2$ by using 5 μM [^{32}P]histone [19]. Tyrosine phosphatase activity in cell extracts was measured by using 30 μM [^{32}P]RCM-lysozyme [18]. One unit (U) of each activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of phosphate per min.

3. Results and discussion

Thyrsiferyl 23-acetate (TF23A) was purified from the marine red alga *Laurencia obtusa* collected from Teuri Island, Hokkaido, Japan (Fig. 1) [3].

The dephosphorylation of [^{32}P]MBP by the catalytic subunit of PP2A from rabbit skeletal muscle was potently inhibited by TF23A. In the standard assay, PP2A was completely inhibited at 100 μM (Fig. 2). On the other hand, TF23A had no effect on the activity of purified PP1, PP2B or PP2C, or PTP activity in a crude cell extract from Jurkat cells. The inhibition of PP2A by TF23A was also observed when other phosphoproteins, [^{32}P]histone or [^{32}P]phosphorylase *a*, were used as substrates (Fig. 3). The concentration required for 50% inhibition (IC_{50})

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Abbreviations: TF23A, thyrsiferyl 23-acetate; OA, okadaic acid; PTP, protein tyrosine phosphatase; MBP, myelin basic protein.

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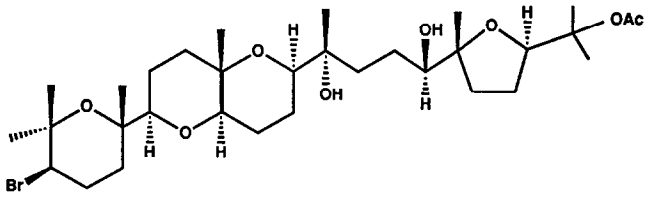

Fig. 1. Structure of thyrsiferyl 23-acetate [3].

of PP2A depended on the enzyme concentration in the assays. As shown in Fig. 3A, a 10-fold dilution of PP2A caused the apparent IC_{50} to change from 16 to 4 μM with [^{32}P]MBP as substrate. Similar decreases in IC_{50} values were observed with [^{32}P]histone and [^{32}P]phosphorylase *a* (Fig. 3B and C). This 'shift' in IC_{50} has been commonly observed with okadaic acid [21], tautomycin [5] microcystin-LR [7,8], and nodularin [9], as previously reported.

To determine whether the potency of TF23A on native forms of PP2A is comparable to that on purified catalytic subunit, we examined the effect of TF23A on protein phosphatase activity in a crude cell extract. Fig. 4 shows the inhibitory effects of OA and TF23A in the crude extract from Jurkat cells. When MBP was used as the substrate, OA potently inhibited the divalent cation-independent phosphatase (PP1 and PP2A) activity in the crude cell extract, causing 90% inhibition at 1 μM (Fig. 4A). The dose–inhibition curve by OA appears to be biphasic, the first inhibition at 1 pM–1 nM and the second at 1 nM–10 μM. The IC_{50} vales for the first and second inhibitions were 35 pM and 24 nM, respectively. The values are almost the same with those obtained by using the purified catalytic subunits of PP2A and PP1, respectively (data not shown), and those reported previously [9,21], suggesting that the extent of the first and second inhibitions represent activities of PP2A and PP1, respectively. On the other hand, TF23A (Fig. 4B) showed monophasic inhibition curves, reaching 60% inhibition of the total activity at above 100 μM. The IC_{50} value for the TF23A inhibition was 8 μM at a 50-fold dilution of extract, and the value changed depending on dilution of the crude cell extract (data not shown) like that for the PP2A catalytic subunit. To determine whether the activity inhibited by TF23A is that of PP2A, additive effects of the PP1-specific inhibitor 2, TF23A, and OA on phosphatases (PP1 and PP2A) in the crude cell extract were

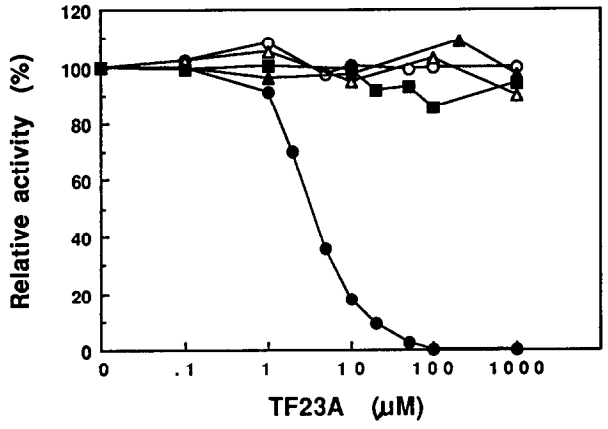

Fig. 2. Effect of TF23A on various protein phosphatases. The catalytic subunit of PP1 (○) was assayed at 0.015 mU/ml by using [^{32}P]phosphorylase *a* as the substrate. Assay for PP2A (●) and PP2B (▲) catalytic subunits were at 0.015 mU/ml and 0.09 mU/ml, respectively, with [^{32}P]MBP. Rat liver PP2C (△) was at 0.05 mU/ml with [^{32}P]histone, and PTP (■) in the Jurkat cell extract was at 0.15 mU/ml with [^{32}P]RCM-lysozyme.

examined. The activity resistant to 1 nM OA (Fig. 5; OA) was equivalent to that resistant to 100 μM TF23A (Fig. 5; T), and both could be suppressed to 10% activity by addition of 0.1 μM inhibitor 2 (Fig. 5; OA + I-2 and T + I-2). However, the activity resistant to 1 nM OA was not affected by addition of 100 μM TF23A (Fig. 5; OA+T). Thus 1 nM OA and 100 μM TF23A showed the same effect on the phosphatase activity, suggesting that TF23A specifically inhibits PP2A activity in the crude cell extract. Therefore, we reached the conclusion that the activity inhibited by 1 nM OA and that by 100 μM TF23A are identical and represent PP2A activity.

Thus, we have shown here that TF23A is a specific inhibitor of PP2A activity. The IC_{50} value of TF23A was 10^5–10^6 fold higher than those of OA, microcystin-LR, and tautomycin. However, unlike those inhibitors, inhibitory effect of TF23A was specific for PP2A. These results indicate that TF23A is a novel useful probe not only to distinguish PP2A activity in a crude cell extracts, but also to identify physiological substrates of PP2A and analyse cellular processes that are regulated by PP2A.

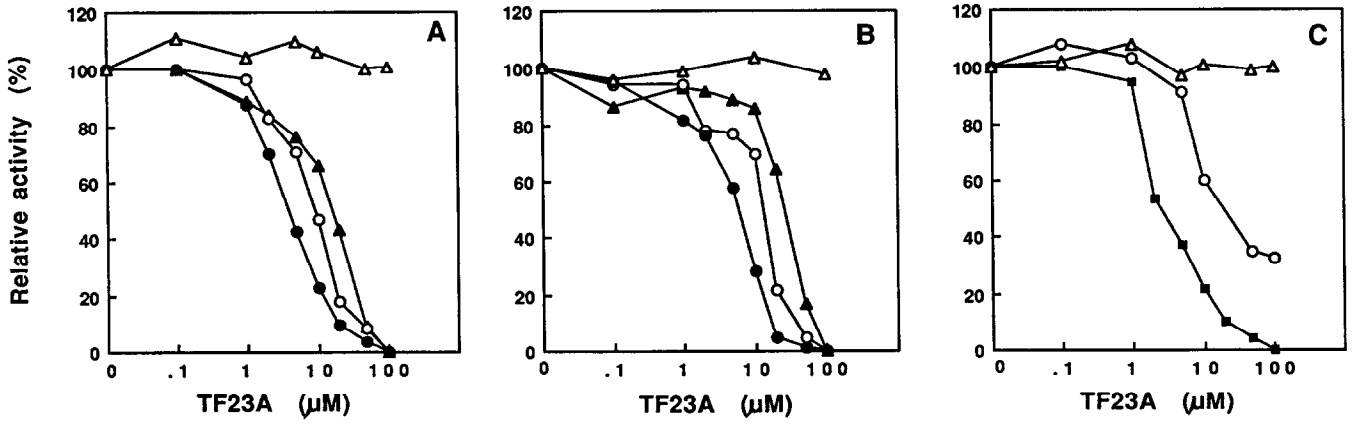

Fig. 3. Effect of enzyme concentration on the inhibition of purified catalytic subunit of PP2A by TF23A. The catalytic subunit of PP2A was assayed at 0.005 mU/ml (■), 0.015 mU/ml (●), 0.05 mU/ml (○), and 0.15 mU/ml (▲). The catalytic subunit of PP1 (△) was assayed at 0.015 mU/ml. ^{32}P-Labelled MBP (A), histone (B), and phosphorylase *a* (C) were used as substrates.

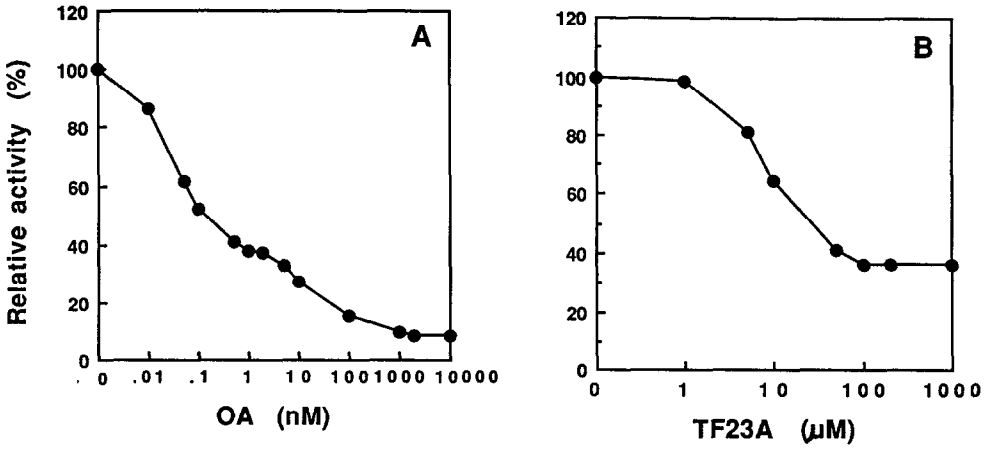

Fig. 4. Effect of OA (A) and TF23A (B) on protein phosphatase activity in Jurkat cell extract. The activity was measured by using [^{32}P]MBP as the substrate in the presence of indicated concentrations of the inhibitors at 50-fold dilution (15 μg/ml of protein) of the extract.

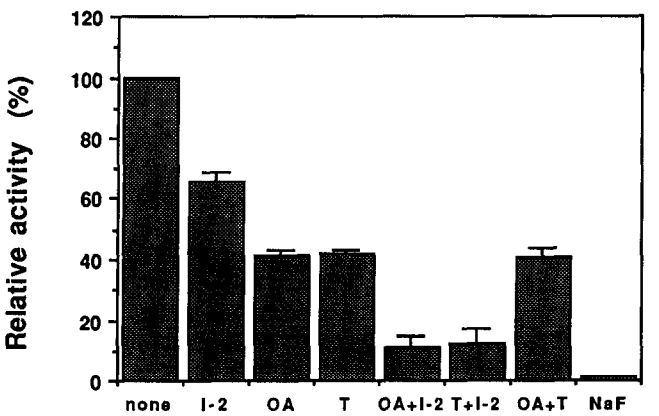

Fig. 5. The differential assay conditions for PP1 and PP2A in Jurkat cell extract. Assay was carried out by using [^{32}P]MBP at 50-fold dilution of the extract in the presence of 0.1 μM inhibitor-2 (I-2), 1 nM OA (OA), 100 μM TF23A (T), 1 nM OA plus 0.1 μM inhibitor-2 (OA + I-2), 100 μM TF23A plus 0.1 μM inhibitor-2 (T + I-2), 1 nM OA plus 100 μM TF23A (OA + T), 50 mM NaF (NaF), or in their absence (none).

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