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# Inhibitory effect of okadaic acid derivatives on protein phosphatases

## A study on structure–affinity relationship

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The effect of structural modifications of okadaic acid (OA), a polyether $C_{38}$ fatty acid, was studied on its inhibitory activity toward type 1 and type 2A protein phosphatases (PP1 and PP2A) by using OA derivatives obtained either by isolation from natural sources or by chemical processes. The dissociation constant ($K_i$) for the interaction of OA with PP2A was estimated to be 30 (26–33) nM [median (95% confidence limits)]. The OA derivatives used and their affinity for PP2A, expressed as $K_i$ (in brackets) were as follows: 35-methyl-OA ($DTX_1$) [19 (12–25) pM], OA-9,10-episulphide (acanthifolicin) [47 (25–60) pM], 7-deoxy-OA [69 (31–138) pM], 14,15-dihydro-OA [315 (275–360) pM], 2-deoxy-OA [899 (763–1044) pM], 7-O-palmitoyl-OA [> 100 nM], 7-O-palmitoyl-$DTX_1$ [> 100 nM], methyl okadaate [≫ 100 nM], 2-oxo-decarboxy-OA [≫ 100 nM] and the C-15–C-38 fragment of OA [≫ 100 nM]. The sequence of the affinity of these derivatives for PP1 was essentially the same as that observed with PP2A, although the absolute values of $K_i$ were very different for the enzymes. The inhibitory effect of OA on PP2A was reversed by applying a murine monoclonal antibody against OA, which recognizes modifications of the 7-hydroxyl group of the OA molecule. It has been shown by n.m.r. spectroscopy and X-ray analysis that one end (C-1–C-24) of the OA molecule assumes a circular conformation. The present results suggest the importance of the conformation for the inhibitory action of OA on the protein phosphatases. The ratios of the $K_i$ values for PP1 to that for PP2A, which were within the range $10^3$–$10^4$, tended to be smaller for the derivatives with lower affinity, indicating that the structural changes in OA impaired the affinity for PP2A more strongly than that for PP1.

## INTRODUCTION

Okadaic acid (OA) is a toxic polyether $C_{38}$ fatty acid (Fig. 1*a*; Tachibana *et al.*, 1981), which is thought to be synthesized together with several derivatives by some kinds of dinoflagellates (plankton) and to accumulate in other marine organisms such as sponges and shellfish which feed on them (Yasumoto *et al.*, 1987; Yasumoto, 1990). Because of its potent inhibitory effect on protein phosphatases, OA is now used as a unique tool for research of various biological systems regulated by reversible protein phosphorylation (for review see Takai, 1988; Cohen *et al.*, 1990).

The specificity of the inhibitory action has been established by studies with purified enzymes (Hescheler *et al.*, 1988; Bialojan & Takai, 1988; Haystead *et al.*, 1989; see also Cohen *et al.*, 1990). Although OA inhibits both type 1 and type 2A protein phosphatases (PP1 and PP2A), two of the major protein phosphatases in eukaryotic cells (Cohen, 1989), its potency towards the enzymes is very different. The values of the dissociation constant $K_i$ for the interaction of OA with PP2A and PP1 have been reported to be in the order of 30 pM and 150 nM respectively (see Takai & Mieskes, 1991).

What molecular characteristics make OA such a strong and specific inhibitor of protein phosphatases? Why is the affinity of OA so different for PP1 and PP2A, which are related enzymes having 50% identity in the amino acid sequence of the catalytic domain (Cohen, 1989)? Although the three-dimensional conformation of OA has been studied in detail by n.m.r. spectroscopy and X-ray analysis (Tachibana *et al.*, 1981; see also Schmitz *et al.*, 1981), little is known about the relation of the chemical structure of OA to its inhibitory effects on the protein phosphatases.

The aim of the present paper has been to examine the effect of structural modifications of OA molecule on its affinity for PP1 and PP2A by using OA derivatives which were obtained either by isolation from natural sources or by chemical procedures (see the Experimental section). There are some previous reports on the inhibitory effects of several OA derivatives on protein phosphatases (Sassa *et al.*, 1989; Cohen *et al.*, 1990). However, no attempt appears to have been made in those works at precise determination of the $K_i$ values for the reactions. The present experiments were designed to estimate the $K_i$ values reliably. (i) *p*-Nitrophenyl phosphate (*p*NPP) was used as substrate. Our previous paper (Takai & Mieskes, 1991) has shown that *p*NPP is an especially suitable substrate for quantitative analyses of the inhibitory effect of OA on PP2A, which shows an exceedingly high specific activity with this artificial substrate [for the *p*NPP phosphatase activity of PP2A and its sensitivity to OA, see also Goris *et al.* (1989) and Cayla *et al.* (1990)]. (ii) The concentrations of the OA derivatives, whose availability was limited, were adjusted by a titration procedure (see the Experimental section). (iii) The properties as tightly binding inhibitors (Henderson,

Abbreviations used: OA, okadaic acid; $DTX_1$, dinophysistoxin-1; *p*NPP, *p*-nitrophenyl phosphate; PMLC, phosphorylated myosin light chains; PP1, protein phosphatase-1; PP2A, protein phosphatase-2A; $E_t$, total enzyme concentration; $I_t$, total inhibitor concentration.

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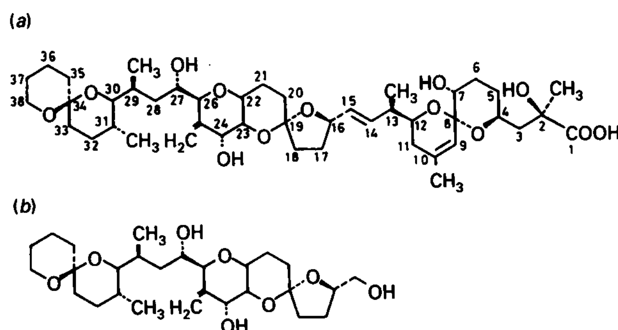


**Fig. 1. Chemical structures of OA and its C-15–C-38 fragment**

(*a*) OA. The backbone carbon atoms (1–38) are numbered. (*b*) The C-15–C-38 fragment of OA.

1972; Williams & Morrison, 1979) were duly considered when OA derivatives with high affinity were used.

We report here that the $K_i$ is markedly increased by modification of some functional groups in a ring-like structure which characterizes the conformation of the OA molecule (Tachibana *et al.*, 1981; Schmitz *et al.*, 1981). We also show that the affinity for PP2A tends to be more strongly impaired by structural changes in the OA molecule than is that for PP1.

A preliminary account of a part of the data has been communicated in an EMBO–FEBS Workshop Course at Leuven, Belgium (Takai *et al.*, 1991).

## EXPERIMENTAL

### Materials

OA, isolated from the black sponge *Halichondria okadia*, was kindly given by Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan). Dinophysistoxin-1 ($DTX_1$; 35-methyl-OA; Murata *et al.*, 1982) and 7-*O*-palmitoyl-OA (Yasumoto *et al.*, 1985) were isolated from the scallop *Patinopecten yessoensis*. Acanthifolicin (OA-9,10-episulphide) was purified from the sponge *Pandaros acanthifolium* as described by Schmitz *et al.* (1981). 2-Deoxy-OA and 7-deoxy-OA were isolated from the dinoflagellate *Prorocentrum lima*, and 14,15-dihydro-OA was isolated from *Halichondria okadai*, by the method of Schmitz & Yasumoto (1991). 7-*O*-Palmitoyl-OA was chemically produced from OA (Yanagi *et al.*, 1989). Methyl okadaate was prepared from OA by a standard method for methylation with the use of diazomethane, which was generated by the reaction of 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) with 6 M-KOH and trapped in ice-cooled diethyl ether (see, e.g., Pizey, 1974). To prepare 2-oxo-decarboxy-OA, OA (8.1 $\mu$mol) was dissolved in 100 $\mu$l of benzene/$CH_2Cl_2$ (1:1, v/v) and mixed with $MnO_2$ (115 $\mu$mol). The mixture was stirred for 12 h and filtered to remove $MnO_2$. The product was chromatographed with a reversed-phase column (Develosil C8, 10 mm × 250 mm; Nomura Kagaku Co., Tokyo, Japan) with $CH_3CN$/water (4:1, v/v) as mobile phase. 2-Oxo-decarboxyl-OA (1.0 $\mu$mol) was eluted at 26.4 ml, while about 80 % of OA was recovered in the fraction of 14–22 ml. The fast-atom-bombardment mass spectrum of the obtained sample revealed an ion at *m/z* 759 corresponding to a protonated form of the molecule. The n.m.r. spectrum (400 MHz; in $C^2HCl_3$) showed a methyl singlet at 2.15 p.p.m. and down-field shifts of $\alpha$-methylene groups at 2.41 and 2.62 p.p.m. (compared with those of OA at 1.66 and 2.09 p.p.m.), whereas it lacked a singlet at 1.38 p.p.m. related to the 2-methyl group of OA. These spectral data unequivocally support the decarboxy structure. The C-15–C-38 fragment of OA was chemically synthesized as described by Ichikawa *et al.* (1987*a–c*).

Murine $IgG_1$ monoclonal antibody against OA, prepared by the method of Usagawa *et al.* (1989), was kindly given by Ube Industries (Ube, Japan).

*p*-Nitrophenyl phosphate (*p*NPP) was a product of Sigma Chemical Co. [$\gamma$-$^{32}$P]ATP was obtained from NEN. All other chemical reagents were of analytical grade.

### Preparation of proteins

The catalytic subunits of PP2A and PP1 were prepared from rabbit skeletal muscle by the method of Tung *et al.* (1984). [$^{32}$P]Phosphorylated chicken gizzard myosin light chains (PMLC) were prepared as described previously (Takai & Mieskes, 1991). The concentrations of proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

### Assay of phosphatase activities

The methods for the assay of protein phosphatases were essentially the same as those used previously (Takai *et al.*, 1989; Takai & Mieskes, 1991), except for the following points. Assays were carried out at 25 °C (rather than at 30 °C). The composition of the buffer for the assay of *p*NPP phosphatase activity was slightly modified; the new buffer contained 40 mM-Tris/HCl, 34 mM-$MgCl_2$, 4 mM-EDTA and 4 mM-DL-dithiothreitol (pH 8.4 at 25 °C). To assay *p*NPP phosphatase activities, PP2A or PP1 was incubated in the buffer for 4 min and then reaction was started by addition of the substrate (*p*NPP).

OA and its derivatives were dissolved in pure dimethyl sulphoxide and added to aqueous buffers. The maximal concentration of dimethyl sulphoxide in reaction mixtures was 0.01 % (v/v). Control activities were not significantly affected by addition of this amount of dimethyl sulphoxide.

### Dose–inhibition relationships

In the following descriptions, $v_0$ and $v_i$ denote the steady-state reaction velocities in the absence and presence of the inhibitor respectively.

OA behaves as a 'tightly binding' inhibitor (Henderson, 1972; Williams & Morrison, 1979) and PP2A. In previous experiments on the inhibition of PP2A by OA (Takai & Mieskes, 1991), we have shown that the dose–inhibition relationship at various enzyme concentrations is well described by the following equation, derived with steady-state assumptions:

$$x = [(E_t - I_t - K_i) + \sqrt{(E_t - I_t - K_i)^2 + 4 \cdot E_t \cdot K_i}]/2E_t \quad (1)$$

where $x$ stands for the fractional activity $v_i/v_0$, $K_i$ is the dissociation constant, and $E_t$ and $I_t$ are the total concentration of the enzyme and that of the inhibitor respectively. In the present experiments, we used this equation to fit the dose–inhibition relationships for the *p*NPP phosphatase activity of PP2A (for the fitting procedure see below).

### Fitting procedure: estimation of $E_t$ and $K_i$

In the previous experiments (Takai & Mieskes, 1991), the values of $E_t$ and $K_i$ were determined by fitting dose–inhibition relationships to the theoretical function by an ordinary non-linear least-squares method, which does not provide reliable confidence limits. In this paper, we used the following fitting procedure, in order to estimate the values of $E_t$ and $K_i$ with 95 % confidence limits.

Let $x$ and $x'$ be the values of fractional activity experimentally obtained for two different inhibitor concentrations, $I_t$ and $I_t'$

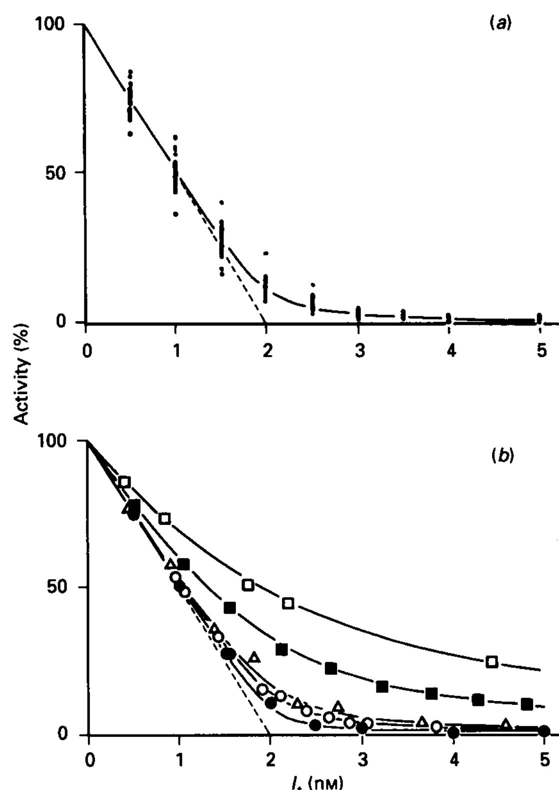


**Fig. 2. Inhibition of PP2A by OA and its derivatives: dose–inhibition relationships**

The inhibitory actions of OA (*a*) and its derivatives (*b*) were examined against 2 nM-PP2A with 5 mM-*p*NPP as substrate. Symbols: ●, 35-methyl-OA ($DTX_1$); ○, 7-deoxy-OA; △, OA-9,10-episulphide (acanthifolicin); ■, 14,15-dihydro-OA; □, 2-deoxy-OA. $I_t$ denotes the total concentration of OA or its derivatives added to the cuvette. The activities are presented as percentages of the values obtained in the absence of inhibitors. In (*b*), each point represents the median of experimental values, and vertical bars indicating experimental errors are omitted for clarity. The curves given by eqn. (2) were drawn by using the values of $E_t$ and $K_i$ obtained by non-parametric fitting, which are listed with 95% confidence limits and total numbers of experiments in Table 1. See the text for further explanations.

respectively. For the set of observations ($I_t$, $x$) and ($I_t'$, $x'$), rearrangement of eqn. (1) gives the following simultaneous equations for $E_t$ and $K_i$

$$x(1-x)E_t+(1-x)K_i = xI_t$$

and

$$x'(1-x')E_t+(1-x')K_i = x'I_t'$$

In the present dose–inhibition analyses for PP2A, the values of $E_t$ and $K_i$ were estimated as the medians of the solutions obtained by solving the simultaneous equations for all possible sets of observations. Ranks corresponding to 95% confidence limits are calculated with correction for replicated observations, as described by Kendall (1970).

This procedure is a modification of the direct-linear plot method for estimation of enzyme-kinetic constants (see Porter & Trager, 1977), which is an extension of the non-parametric statistical method of Sen (1968). Although some theoretical problems have been pointed out for this type of application (Cornish-Bowden *et al.*, 1978), the procedure appears to be adequate because the dose–inhibition relationships were fitted well by using the values of $E_t$ and $K_i$ determined thereby (see the Results section).

The dose–inhibition relationships for PP1 were fitted by the linear least-squares method to the linear form of the Hill function:

$$\ln\{[1-(v_i/v_o)]/(v_i/v_o)\} = h\cdot\ln I_t - h\cdot\ln K_i$$

where $h$ is the Hill coefficient. The values in the range $0.1 < v_i/v_o < 0.9$ were used for the fitting.

### Concentrations of the OA derivatives

The amounts of the OA derivatives (except for $DTX_1$) used for the present experiments were only 10–100 $\mu$g, and it was difficult to determine their weight accurately. The concentrations of their solutions were therefore adjusted by the following procedure.

With an OA derivative, dose–inhibition analysis is done by using the approximate concentration of the derivative $\hat{I}_t$ calculated from the weight, and the apparent enzyme concentration $\hat{E}_t$ and the apparent dissociation constant $\hat{K}_i$ are computed by the above procedure.

Now we define a correction factor $\gamma$ by the equation

$$\hat{I}_t = \gamma I_t \quad (2)$$

It can be shown that

$$\hat{E}_t = \gamma E_t \quad (3)$$

and

$$\hat{K}_i = \gamma K_i \quad (4)$$

The value of $\gamma$ can be estimated experimentally by using eqn. (3) as the ratio of $\hat{E}_t$ to $E_t$ [$E_t$ is determined by dose–inhibition analysis with a standard inhibitor (OA)]. The values of $I_t$ and $K_i$ are calculated by using this $\gamma$ from eqns. (2) and (4) respectively.

### Statistics

The values of $K_i$ obtained from dose–inhibition analyses with PP2A by the above procedure were compared by a non-parametric method (Hollander & Wolfe, 1973) as described by Porter & Trager (1977) for comparison of kinetic constants. The values of $K_i$ for PP1 were compared by a method of co-variance analysis (Snedecor & Cochran, 1980). In every case, differences were evaluated as statistically significant when a two-tailed probability of less than 0.05 was obtained.

## RESULTS

### Inhibitory effect of OA derivatives on PP2A

As shown in Fig. 2, the dose–inhibition relationships for the *p*NPP phosphatase activity of PP2A appear to be well fitted to the theoretical function (eqn. 1) by the procedure described in the Experimental section. Table 1 gives the values of $K_i$ together with the 95% confidence limits obtained. For the OA derivatives whose availability was limited, adjustment of the $K_i$ values has been made by comparing the values of the apparent enzyme concentration $E_t$ with that for OA (= 2.0 nM) as described in the Experimental section.

We have argued about the theoretical advantage of keeping the enzyme concentration low for estimation of $K_i$ (Takai & Mieskes, 1991). During preparation of the OA derivatives, however, we noticed that some of the OA derivatives (especially 7-deoxy-OA) are very liable to be adsorbed to the wall of the container. Our preliminary dose–inhibition analyses also showed that the inhibitory effects of the derivatives were apparently very variable when experiments were done in the concentration range lower than 0.5 nM. In the present dose–inhibition analyses with

**Table 1. Inhibition of PP2A and PP1 by OA and its derivatives**

The dissociation constants $K_i$ estimated by the dose–inhibition analyses are listed. The values for PP2A are presented with 95% confidence limits (in parentheses); *n* denotes the total number of experiments. PP1/PP2A is the ratio of the $K_i$ value for PP1 to that for the *p*NPP phosphatase activity of PP2A. The $K_i$ values for inhibition of PP2A by OA obtained in previous experiments by using *p*NPP (Takai & Mieskes, 1991) and phosphorylase *a* (Bialojan & Takai, 1988) are included for comparison. * PMLC from chicken gizzard (4 μM) were used for the assay of PP1 only. See also the numbered chemical structure of OA shown in Fig. 1.

| OA and its derivatives | Substrate | PP2A $K_i$ (pM) | PP2A *n* | PP1 $K_i$ (nM) | PP1 *n* | PP1/PP2A |
|---|---|---|---|---|---|---|
| OA | *p*NPP | 30 (26–33) | 164 | 145 | 44 | 4800 |
| | Phosphorylase *a* | – | | 272 | 48 | 9100 |
| $DTX_1$ (= 35-methyl-OA) | *p*NPP | 19 (12–25) | 112 | 165 | 55 | 8700 |
| | PMLC | – | | 180 | 45 | 9500 |
| OA-9,10-episulphide | *p*NPP | 47 (25–60) | 35 | – | | – |
| 7-Deoxy-OA | *p*NPP | 69 (31–138) | 33 | – | | – |
| | PMLC | – | | 215 | 36 | 3100 |
| 14,15-Dihydro-OA | *p*NPP | 315 (275–360) | 40 | 790 | 40 | 2500 |
| 2-Deoxy-OA | *p*NPP | 899 (763–1044) | 40 | 870 | 42 | 970 |
| | PMLC | – | | 990 | 36 | 1100 |
| 7-*O*-Palmitoyl-OA | *p*NPP, PMLC* | > 100 nM | 12 | > 1 μM | 10 | – |
| 7-*O*-Palmitoyl-$DTX_1$ | *p*NPP, PMLC* | > 100 nM | 12 | > 1 μM | 10 | – |
| Methyl okadaate | *p*NPP, PMLC* | ≫ 100 nM | 12 | > 10 μM | 10 | – |
| 2-Oxo-decarboxyl OA | *p*NPP, PMLC* | ≫ 100 nM | 12 | ≫ 10 μM | 10 | – |
| C-15–C-38 fragment | *p*NPP, PMLC* | ≫ 100 nM | 12 | ≫ 10 μM | 10 | – |

PP2A, we therefore decided to keep the enzyme concentration at 2.0 nM so that the main part of the dose–inhibition curves might appear in the range of inhibitor concentration higher than 0.5 nM. For the OA derivatives with dissociation constants lower than 1.0 nM, this experimental condition is relatively severe to estimate $K_i$. However, the value (30 pM) of the $K_i$ for OA obtained with 2.0 nM-PP2A (Table 1) agreed well with that (32 pM) obtained in the previous experiments with 50 pM-PP2A (Takai & Mieskes, 1991). Thus the value of $K_i$ appeared to be estimated well with 2.0 nM-PP2A, as long as the number of experiments was sufficiently large.

Most of the OA derivatives examined in the present experiments exhibited lower affinity for PP2A than did OA (Table 1). The extent of the decrease in the affinity differed widely, depending on the position and the type of structural modifications of the OA molecule. Replacement of the 7-hydroxyl group with hydrogen (7-deoxy-OA) caused only a slight (2.3-fold) decrease in the affinity, whereas esterification of the same hydroxyl group with palmitic acid (7-*O*-palmitoyl-OA) resulted in a more than 3000-fold increase in the $K_i$ value. A significant increase in the $K_i$ value was also observed with 14,15-dihydro-OA (11-fold) as well as with 2-deoxy-OA (30-fold). The affinity to PP2A was essentially abolished by esterifying (methyl okadaate) or removing (2-oxo-decarboxy-OA) the carboxyl group ($C_1$). [The preparation of methyl okadaate did inhibit PP2A (2 nM) in the apparent concentration range higher than 0.5 μM. However, it is likely to be due to contamination by a small amount (about 0.6%) of okadaic acid, from which it was chemically induced, as judged from the linear appearance of the dose–inhibition relationship (results not shown).] The C-15–C-38 fragment had no effect on PP2A.

The $K_i$ value was thus larger for most of the OA derivatives, whereas it was slightly but significantly smaller for $DTX_1$ than for OA (Table 1). There was only a small difference between the $K_i$ values for OA-9,10-episulphide (acanthifolicin) and OA; unfortunately, its limited availability did not allow us to obtain for this derivative a sufficient number of results to examine whether the difference is significant.

**Inhibitory effect of OA derivatives on PP1**

The effect of the OA derivatives was also examined against PP1. The dose–inhibition relationships (results not shown) were well fitted to the Hill function ($h$ = 0.85–1.0). Table 1 gives the values of $K_i$.

When the assays of PP1 were done with PMLC from chicken gizzard as substrate (4 μM), the $K_i$ values obtained for OA and its derivatives against PP1 were in the sequence $DTX_1 \approx$ OA $\leqslant$ 7-deoxy-OA < 14,15-dihydro-OA < 2-deoxy-OA. Methyl okadaate, 2-oxo-decarboxyl-OA and the C-15–C-38 fragment of OA had essentially no effect on PP1. [A small but significant decrease in PP1 activity was observed with 10 μM-methyl okadaate. However, this is likely due to contamination with OA, rather than to a weak inhibitory action of methyl okadaate itself (see above).] Thus the affinity sequence with PP1 was essentially the same as that with PP2A (Table 1).

In our previous paper we reported that the PP1 also has a weak *p*NPP phosphatase activity with sensitivity to OA (Takai & Mieskes, 1991). For some derivatives, the inhibitory effect was examined also on the *p*NPP phosphatase activity. The $K_i$ values were within the same range as those obtained with PMLC (Table 1).

The $K_i$ values for PP1 were much higher than those for PP2A (cf. Table 1). The ratios of the $K_i$ values for PP1 to those for PP2A (the PP1/PP2A ratio) were within the range $10^3$–$10^4$ (Table 1). There was a tendency for the PP1/PP2A ratio to be smaller for derivatives with lower affinity for the enzymes (Table 1).

**Reversal of the inhibitory effect of OA and PP2A by the monoclonal antibody against OA**

The monoclonal antibody against OA reversed the inhibitory effect of OA on the *p*NPP phosphatase activity of PP2A in a dose-dependent manner. In order to measure the steady-state velocity, PP2A (1 nM) was first incubated with various concentrations (5–500 nM) of the antibody and then treated with OA (5 nM) for 5 min before reaction was started by addition of the substrate (5 mM-*p*NPP). [Reflecting the very high affinity of OA

for PP2A, the recovery of the activity was markedly time-dependent when the antibody was added after the reaction was suppressed by OA (A. Takai, unpublished work).] Under this condition, the concentrations of the antibody required for 50% and 95% recovery were about 30 nM and 200 nM respectively. The velocity of reaction was not significantly changed when the antibody was added in the absence of OA.

## DISCUSSION

It seems pertinent to depict briefly the characteristics of the three-dimensional structure of OA, which has been studied in detail by n.m.r. spectroscopy and X-ray analysis (Tachibana *et al.*, 1981; see also the paper on the chemical structure of OA-9,10-episulphide by Schmitz *et al.*, 1981). One end (C-1–C-24) of the OA molecule assumes a circular conformation, forming a cavity 0.5–0.7 nm in diameter which is held together by an intramolecular hydrogen bond between the carboxyl group (C-1) and the 24-hydroxyl group. The remaining part of the OA molecule (C-25–C-38), which consists of an extended alkyl chain and two tetrahydropyran rings, is thought to reside outside the cavity. The circular conformation is probably stabilized by hydrogen bondings between the four hydroxyl groups and the other oxygen atoms in the molecule. For example, the 2-hydroxyl group forms a hydrogen bond with the oxygen between C-4 and C-8. The double bond between C-14 and C-15 also probably contributes to stabilization of the conformation by keeping the *trans*-configuration.

In the present experiments we have shown that the affinity of OA for PP2A is decreased by modifications of the OA molecule which appear to disturb or destabilize the circular conformation. OA loses its inhibitory effect on PP2A when the carboxyl group (C-1) is either esterified (methyl okadaate) or removed (2-oxo-decarboxy-OA). The dissociation constant $K_i$ is increased 31-fold by removal of the 2-hydroxyl group (2-deoxy-OA) and 11-fold by conversion of the double bond between C-14 and C-15 into a single bond (14,15-dihydro-OA). Sassa *et al.* (1989) have reported that 2,7,24,27-tetramethoxy-OA, in which all the hydroxyl groups are methoxylated, has no inhibitory effects on PP2A and PP1 (a study involving specific oxidation of the 24-hydroxyl group could be interesting). These observations suggest that the circular conformation plays an important role in the inhibitory action of OA.

Removal of the 7-hydroxyl group (7-deoxy-OA) caused a relatively slight (2.3-fold) decrease in the affinity, whereas its esterification with a fatty acid (7-*O*-palmitoyl-OA) resulted in a more than 3000-fold increase in the $K_i$ value. The inhibitory effect of OA on PP2A is reversed by application of the monoclonal antibody against OA (see the Results section), which has been shown by enzyme immunoassay to recognize chemical modifications of the 7-hydroxyl group (Usagawa *et al.*, 1989). These results suggest that the part of OA molecule near the 7-hydroxyl group may directly take part in interaction with the phosphatase molecule.

The sequence of the affinity of OA and its derivatives is essentially the same for PP1 and PP2A (Table 1), although the absolute values of $K_i$ are very different for these enzymes (see below). Therefore the above arguments concerning the relationship between the configuration and inhibitory effect of OA may also qualitatively apply for PP1.

The contribution of the remaining part of the OA molecule (C-25–C-38) to the inhibitory action of OA on PP2A and PP1 is not clear from the present results. The C-15–C-38 fragment of OA has no effect on PP2A. Methylation of the C-35 position ($DTX_1$) results in a slight but significant decrease in the $K_i$ value for PP2A. This result may suggest that hydrophobicity of this component makes some contribution to the affinity of OA to PP2A.

Recently, strong inhibitory activities have been demonstrated for calyculin A (Ishihara *et al.*, 1989) and microcystin-LR (MacKintosh *et al.*, 1990). It is interesting that these toxins also have chemical structures consisting of a circular component and an extended aliphatic chain (Kato *et al.*, 1986; MacKintosh *et al.*, 1990). Such a conformation is possibly related to their inhibitory action on protein phosphatases.

It is now well known that OA exhibits much higher affinity to PP2A than it does to PP1 (Hescheler *et al.*, 1988; Bialojan & Takai, 1988; Haystead *et al.*, 1989; Takai & Mieskes, 1991). This is one of the interesting characteristics of OA in comparison with calyculin A and microcystin-LR which reportedly show similar potency with PP1 and PP2A (Ishihara *et al.*, 1989; MacKintosh *et al.*, 1990). The OA derivatives, as well as OA, give much lower $K_i$ values with PP2A than with PP1. However, the PP1/PP2A ratio of the $K_i$ values, which lies in the range $10^3$–$10^4$, tends to be smaller for derivatives with lower affinity to the enzymes (Table 1). It means that the affinity of OA for PP2A is more liable to be impaired by modifications of the molecular structures of OA than is that for PP1. This seems reasonable, because the exceedingly high affinity of OA for PP2A is probably related to the conformation of OA in a more delicate manner.

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