RESEARCH COMMUNICATION

Affinity of okadaic acid to type-1 and type-2A protein phosphatases is markedly reduced by oxidation of its 27-hydroxyl group

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Okadaic acid (OA), a potent inhibitor of type-1 and type-2A protein phosphatases (PP1 and PP2A), has four hydroxyl groups at 2, 7, 24 and 27 positions (see Figure 1). By chemical treatment of OA we synthesized a derivative, in which the 27-hydroxyl group was specifically oxidized (27-dehydro-OA). The inhibitory effect of this OA derivative was examined on the activities of PP1 and PP2A, which were inhibited by intact OA with dissociation constants (K_i) of 150 nM and 32 pM respectively. We found that the affinity of OA was decreased 40-fold (K_i = 6 μM) with PP1 and 230-fold (K_i = 7.3 nM) with PP2A after oxidation of the 27-hydroxyl group. According to the model of the three-dimensional conformation of OA on the basis of X-ray analyses, the 27-hydroxyl group appears to be present in a position relatively free from intramolecular bonding formation, in comparison with the other three hydroxyl groups. The marked increases in the K_i values for PP1 and PP2A , which indicate the reduction of the absolute values of the free energy of binding by 9 kJ/mol and 14 kJ/mol respectively, may imply that the 27-hydroxyl group serves as a binding site with the phosphatase molecules.

INTRODUCTION

Okadaic acid (OA) is a polyether derivative of a C_{38} fatty acid with one carboxyl group and four hydroxyl groups (see Figure 1). This substance is now widely used as a potent and specific inhibitor of type-1 and type-2A protein phosphatases (PP1 and PP2A), on which it acts with dissociation constants (K_i) of the order of 150 nM and 30 pM respectively (Takai and Mieskes, 1991; Takai et al., 1992a). The relationship between the chemical structure of OA and its inhibitory effect on the protein phosphatases has been studied to some extent by experiments with OA derivatives. Nishiwaki et al. (1990) have examined the effect of 17 OA derivatives on the protein phosphatase (mainly PP2A) activity of a cytosolic fraction of mouse brain. They have reported that OA loses its affinity to the phosphatase activity when the carboxylic acid is either removed or esterified [see also Takai et al. (1992a)]. They have also reported that 2,7,24,27-tetramethoxy-OA, in which all the four hydroxyl groups are methoxylated, has no inhibitory effect on the phosphatase activity (Sassa et al., 1989; Nishiwaki et al., 1990). The latter observation indicates the crucial importance of the hydroxyl groups for the interaction of OA with protein phosphatases. In a recent paper we have examined the effect of some other OA derivatives, including 2-deoxy-OA and 7-deoxy-OA, on the affinity for purified preparations of PP1 and PP2A (Takai et al., 1992a). We have shown that selective removal of the hydroxyl groups at the 2 and 7 positions increases the K_i values for the interaction with PP2A 30-fold and 2-fold respectively, while it results in relatively small effects on the affinity to PP1 (see Table 1, below).

In the present experiments, we have examined the effect of specific oxidation of the 27-hydroxyl group on the affinity of OA to PP1 and PP2A, using 27-dehydro-OA, which was synthesized by chemical treatment of OA (see the Experimental section). We report here that the K_i value is increased 35-fold for PP1 and 230-fold for PP2A by the modification of the OA molecule. According to the model of the three-dimensional conformation of OA based on X-ray analyses (Tachibana et al., 1981), the 27-hydroxyl group appears to be present in a position relatively free from intramolecular bonding formation, compared with the other three hydroxyl groups. The remarkable increases in the K_i values for PP1 and PP2A, which are equivalent to the differences in the free energy of binding of 9 kJ/mol and 14 kJ/mol respectively, may imply that the 27-hydroxyl group acts as a binding site for the phosphatase molecules.

EXPERIMENTAL

Materials

OA from two different sources was used: Dr. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan) kindly gave OA, isolated from the black sponge *Halichondria okadai*, which was used for the dose–inhibition analyses. For chemical synthesis of 27-dehydro-OA (see below), we used OA which we purified by the method described previously (Yanagi et al., 1989) from *H. okadai* collected in Izu Peninsula, Japan. *p*-Nitrophenyl phosphate (*p*NPP) was a product of Sigma Chemical Co. (catalogue code, Sigma-104). All other chemical reagents were of analytical grade.

OA and 27-dehydro-OA 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.

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

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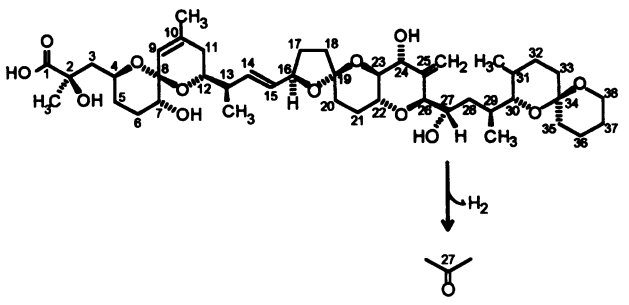

Figure 1 Chemical synthesis of 27-dehydro-OA from OA

27-Dehydro-OA was synthesized by chemical oxidation of OA (see the Experimental section). The backbone carbon atoms of OA are numbered (1–38).

Preparation and assay of protein phosphatases

The catalytic subunits of PP2A and PP1 were prepared from rabbit skeletal muscle by the method of Tung et al. (1984). Myosin light chains were purified by using essentially the same method as described for isolation of cardiac light chains by Cummins and Lambert (1986), and they were ^{32}P-phosphorylated with chicken gizzard myosin light-chain kinase isolated by the method of Ngai et al. (1984).

The methods for the assay of protein phosphatases were the same as described in a previous paper (Takai et al., 1992a).

Preparation of 27-dehydro-OA

27-Dehydro-OA was synthesized by chemical oxidation of OA as follows (see Figure 1): to prevent cleavage of the carboxyl group during the oxidation procedure, OA was first esterified by treating with *p*-bromophenacyl bromide and triethylamine in acetone at room temperature for 24 h, followed by recrystallization from CH_3CN. The resulting *p*-bromophenacyl-OA (10 μmol) was dissolved in CH_2Cl_2 (400 μl) and mixed with pyridinium dichromate (40 μmol) and pyridinium trifluoroacetate (4 μmol) dissolved in C_6H_6 (100 μl) to start the oxidation. The reaction was terminated 5 h later by adding isopropanol (100 μl). After removing chromate salts by filtering through a column packed with anhydrous $MgSO_4$ (80 mg), the mixture was applied to h.p.l.c. with an ODS column (Cosmosil ODS5C18-AR, internal diam. 10 mm × 250 mm; Nacalai Tesque, Kyoto, Japan) using CH_3CN/water (9:1, v/v) as mobile phase at a flow rate of 2 ml/min. 27-Dehydro-OA ester was eluted at 32.4 ml (yield, 12.1 %), while the elution peak of unreacted *p*-bromophenacyl-OA appeared at 26.4 ml. Fast-atom-bombardment mass spectroscopy (f.a.b.-m.s.) of the oxidized ester revealed peaks at m/z 999 and 1001 (corresponding to the protonated molecular species containing the natural bromine isotopes ^{79}Br and ^{81}Br respectively), compared with those for *p*-bromophenacyl-OA at m/z 1001 and 1003. This confirms removal of two hydrogen atoms.

For hydrolysis of the ester bond, the 27-dehydro-OA ester was then treated with 4 mM Na_2CO_3 in diethylene glycol dimethyl ether/water (5:1, v/v) at 60 °C for 2 h. (Because of instability of the ester in either reductive or basic conditions, ordinary methods for hydrolysis such as those with Zn in acetic acid or with alkaline methanol were unsuccessful). After neutralization with 2.3 % (v/v) trifluoroacetic acid in ethanol, the hydrolysate was chromatographed over a reversed-phase column (Cosmosil 5C18-AR, internal diam. 10 mm × 250 mm; Nacalai Tesque Co., Kyoto, Japan) with CH_3CN/water (90:10, v/v) supplemented with 0.01 % (v/v) acetic acid as mobile phase at a flow rate of 2 ml/min, to give elution of 27-dehydro-OA at 20 ml (yield, 94 %). F.a.b.-m.s. of the sample obtained showed a peak corresponding to a protonated ion at m/z 803, in comparison with that for OA at m/z 805. The ^{1}H-n.m.r. spectroscopy (400 MHz; in C^2HCN) confirmed disappearance of the signals related to the 27-hydroxyl group and adjacent 27-H, which are observed with OA at 2.97 p.p.m. and 4.07 p.p.m. respectively.

Determination of the dissociation constants

To determine K_i values for the interaction of PP2A with OA and 27-dehydro-OA, the steady-state dose–inhibition relationships were fitted by the theoretical function

$$x = \frac{(E_t - I_t - K_i) + \sqrt{(E_t - I_t - K_i)^2 + 4E_tK_i}}{2E_t} \quad (1)$$

where x is the fractional activity (i.e. the ratio of the activity in the presence of the inhibitor to that in its absence), and E_t and I_t are the total concentrations of the enzyme and the inhibitor respectively. This function is derived with steady-state assumptions, considering the reduction of the free inhibitor concentration as a result of binding with the enzyme (Takai and

Table 1 Inhibition of PP2A and PP1 by OA and 27-dehydro-OA

The dissociation constants K_i estimated by the dose–inhibition analyses are listed. The values for PP2A determined by the non-parametric regression procedure are presented with 95% confidence limits (in parentheses). The K_i values for 7-deoxy-OA and 2-deoxy-OA obtained in the previous experiments are included for comparison. k_i is the ratio of the K_i value of derivative to that of OA. n denotes the total number of experiments. $\Delta G^{0'}$ stands for the standard free-energy change of binding, which is calculated by the conversion equation $\Delta G^{0'} = RT\ln K_i$. $\delta\Delta G^{0'}$ signifies the difference of the $\Delta G^{0'}$ value for derivative and that for OA; i.e. $\delta\Delta G^{0'} = RT\ln k_i$. PP1/PP2A is the ratio of the K_i value for PP1 to that for the *p*NPP phosphatase activity of PP2A. [cf. Table 1 of Takai et al. (1992a)]

		PP2A					PP1					
OA and derivatives	Substrate	K_i (pM)	k_i	n	$\Delta G^{0'}$ (kJ/mol)	$\delta\Delta G^{0'}$ (kJ/mol)	K_i (nM)	k_i	n	$\Delta G^{0'}$ (kJ/mol)	$\delta\Delta G^{0'}$ (kJ/mol)	PP1/PP2
OA	*p*NPP	32 (28–37)	1	88	−60	0	150	1	46	−39	0	4700
7-Deoxy-OA	*p*NPP/PMLC*	69 (31–138)	2.2	33	−58	2	220	1.5	36	−38	1	3200
2-Deoxy-OA	*p*NPP	900 (760–1040)	30	40	−52	8	870	6	42	−35	4	970
27-Dehydro-OA	*p*NPP	7300 (6800–7800)	230	187	−46	14	6200	41	34	−30	9	850
	PMLC	–	–		–	–	5800	39	30	−30	9	800

* The effect of 7-deoxy-OA has been examined for the *p*NPP phosphatase activity of PP2A and the phosphorylated myosin light chain (PMLC) phosphatase activity of PP1.

Mieskes, 1991; Takai et al., 1992a,b). To fit eqn. (1) to data, we applied the non-parametric regression procedure which has been used in determination of the K_i values for interaction of PP2A with OA and some OA derivatives (Takai et al., 1992a). The K_i values obtained are presented (Table 1) as 'median (95% confidence limits)'.

To describe the dose–inhibition relationships for PP1 we used the Hill function

$$x = [1 + (I_t/K_i)^h]^{-1}$$

where h is the Hill coefficient. This model function was fitted to the data by non-linear least-squares regression using as weight the reciprocal of the square of the standard error of x at each inhibitor concentration [see Carroll and Ruppert (1988)].

RESULTS

Figure 2 shows the relationship between the concentration of 27-dehydro-OA applied and the steady-state activity of PP2A against *p*NPP (5 mM) as substrate. The molar concentration of PP2A in the reaction mixture was kept constant at 2 nM on the basis of the value of E_t determined by fitting eqn. (1) to the dose–inhibition data for the standard inhibitor OA (0.5–5 nM) [results not shown; for a typical steady-state dose–inhibition curve for inhibition of PP2A by OA, see Figure 1(a) of Takai et al. (1992a)]. Eqn. (1) was also fitted well to the dose–inhibition data with 27-dehydro-OA by the non-parametric regression procedure (Figure 2; solid line), which gave the estimate of the K_i values with 95% confidence limits (see Table 1).

To examine the effect of 27-dehydro-OA on PP1, which has a relatively weak activity with *p*NPP (Takai and Mieskes, 1991), measurements were done with phosphorylated myosin light chains (4 μM) as well as with *p*NPP (5 mM) as substrate. The Hill function ($h = 0.90$–0.94) fitted well to the dose–inhibition data by the non-linear least-squares regression procedure (results not shown).

Table 1 lists the values of K_i obtained from the dose–inhibition analyses. The data of the previous experiments with 2-deoxy-OA and 7-deoxy-OA are included for comparison. The values of the standard free-energy change of binding formation, calculated by the conversion equation $\Delta G^{0'} = RT\ln K_i$, are also presented together with the difference in the $\Delta G^{0'}$ values for the OA derivatives and OA, $\delta\Delta G^{0'}$.

The K_i values for OA agreed well with those reported for *p*NPP phosphatase activities of PP2A (30 pM) and PP1 (145 nM) in our previous paper (Takai et al., 1992a). 27-Dehydro-OA had a 230-fold larger K_i with PP2A than OA did (Table 1, k_i). This increase in the K_i value is equivalent to the decrease in the absolute value of $\Delta G^{0'}$ of 14 kJ/mol (Table 1, $\delta\Delta G^{0'}$). Although the K_i for interaction with PP1 was increased 35-fold by oxidation of the 27-hydroxyl group, the change in K_i value, which is equivalent to an increase in the $\Delta G^{0'}$ value of 9 kJ/mol, was considerably smaller than that observed with PP2A.

We have shown in the previous experiments with other OA derivatives (Takai et al., 1992a) that the ratio of the K_i value for PP1 to that for PP2A (PP1/PP2A) tends to be smaller for derivatives with lower affinity to PP1 and PP2A. A similar tendency was also found within the OA derivatives in which one of the four hydroxyl group was modified (Table 1). The ratio of the PP1/PP2A value for 27-dehydro-OA was about one-sixth of that for OA (Table 1).

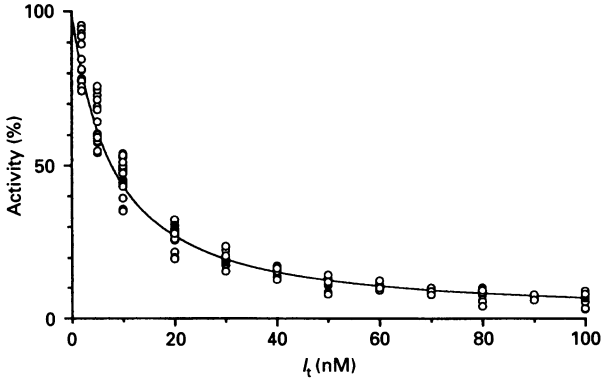

Figure 2 Inhibition of PP2A by 27-dehydro-OA

A dose–inhibition relationship is illustrated. The inhibitory effect of 27-dehydro-OA on 2 nM PP2A was examined with 5 mM *p*NPP was substrate. The activities are presented as percentages of the values in the absence of the inhibitor. The curve given by eqn. (1) was drawn using the value of K_i (see Table 1) obtained by non-parametric regression. Note that the abscissa (I_t = [27-dehydro-OA]) is linearly scaled. See the text for further explanations.

DISCUSSION

The first experimental evidence for the importance of the four hydroxyl groups of OA for its inhibitory effect on protein phosphatases was obtained by Sassa et al. (1989), who reported that 2,7,24,27-tetramethoxy-OA, in which all the four hydroxyl groups were methoxylated, had no inhibitory effect on the protein phosphatase (mainly PP2A) activity in a cytosolic fraction of mouse brain [see also Nishiwaki et al. (1990)]. One of the objectives of our previous (Takai et al., 1992a) and present papers has been to evaluate the contribution of each of the four hydroxyl groups to the interaction of OA with PP1 and PP2A. We have shown in the previous dose–inhibition study with highly purified enzymes (Takai et al., 1992a) that selective replacement of the hydroxyl groups at the 2 and 7 positions with a hydrogen atom (2-deoxy-OA and 7-deoxy-OA) increases the K_i values for the interaction with PP2A 30-fold and 2-fold respectively, whereas it results in relatively small effects on the affinity to PP1 (Table 1). The present results show that oxidation (dehydrogenation) of the 27-hydroxyl group increases the K_i values 40-fold with PP1 and 230-fold with PP2A (Table 1). Thus, of the specific modifications of the hydroxyl groups examined, oxidation of the 27-hydroxyl group causes the most marked reduction of the affinity to the phosphatases. In contrast with methoxylation of all four hydroxyl groups (see above), however, even this modification does not completely abolish the inhibitory effect. The results obtained so far suggest that the 24-hydroxyl group also plays an important role in the interaction with the phosphatases. A study involving specific modification of the 24-hydroxyl group will be highly interesting.

It is now well known that OA exhibits much higher affinity to PP2A than it does to PP1 (Bialojan and Takai, 1988; Hescheler et al., 1988; Haystead et al., 1989; Takai et al., 1992a). The ratio of the K_i value for PP1 to that for PP2A (PP1/PP2A) is as large as 4700 for OA (Table 1). This is one of the conspicuous natures of OA, compared with other protein phosphatase inhibitors like calyculin A (Ishihara et al., 1989) or microcystin-LR (MacKintosh et al., 1990) which act on PP1 and PP2A with similar potency. We notice that all three OA derivatives listed in Table 1 give smaller PP1/PP2A ratios than OA does. This means that each of the three hydroxyl groups makes some contribution to the exceedingly high affinity of OA for PP2A compared with that for PP1. However, the PP1/PP2A ratio is still no less than 800 for 27-dehydro-OA, which gives the smallest value. [The

PP1/PP2A ratio tends to be smaller with OA derivatives with lower affinity (Table 1). A similar tendency has been observed among other types of OA derivatives (Takai et al., 1992a).] Further experiments are necessary to determine the major structural factors responsible for the characteristic specificity of OA to PP1 and PP2A.

From the K_i values for interaction of intact OA with PP1 (150 nM) and PP2A (32 pM), the values of the standard free-energy change ($\Delta G^{0'}$) of binding are estimated, by the conversion equation $\Delta G^{0'} = RT\ln K_i$, as -39 kJ/mol and -60 kJ/mol respectively (Table 1). As the $\Delta G^{0'}$ of formation of single hydrogen bonds or electrostatic bonds is typically in the range -10 to -20 kJ/mol [see e.g. Rawn (1989)], it appears that more than one intramolecular interaction exists in the binding of OA with PP1 or PP2A. There may be a special implication in the fact that the changes of the $\Delta G^{0'}$ for binding with PP1 and PP2A resulting from oxidation of the 27-hydroxyl group (Table 1, $\delta\Delta G^{0'}$) are comparable in magnitude with the $\Delta G^{0'}$ of formation of hydrogen bonds or electrostatic bonds. According to the model of the three-dimensional conformation based on X-ray analyses (Tachibana et al., 1981), one end (C-25–C-38) of the OA molecule, which bears the 27-hydroxyl group, resides outside the cavity of the circular component formed by the other end (C-1–C-24) of the molecule. The 27-hydroxyl group thus appears to be present in a position relatively free from intramolecular bond formation, in comparison with the other three hydroxyl groups, which are thought to form hydrogen bonds either with the carboxyl group or with the cyclic-ether oxygens to stabilize the circular conformation [see also Schmitz et al. (1981) and Quinn et al. (1993)]. Therefore, it seems reasonable to speculate that the 27-hydroxyl group serves as one of the multiple binding sites, forming a hydrogen bond with some electron-rich portion of the PP1 and PP2A molecules.

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