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Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases

Specificity and kinetics

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The inhibitory effect of a marine-sponge toxin, okadaic acid, was examined on type 1, type 2A, type 2B and type 2C protein phosphatases as well as on a polycation-modulated (PCM) phosphatase. Of the protein phosphatases examined, the catalytic subunit of type 2A phosphatase from rabbit skeletal muscle was most potently inhibited. For the phosphorylated myosin light-chain (PMLC) phosphatase activity of the enzyme, the concentration of okadaic acid required to obtain 50% inhibition (ID_{50}) was about 1 nM. The PMLC phosphatase activities of type 1 and PCM phosphatase were also strongly inhibited (ID_{50} 0.1–0.5 μM). The PMCL phosphatase activity of type 2B phosphatase (calcineurin) was inhibited to a lesser extent (ID_{50} 4–5 μM). Similar results were obtained for the phosphorylase *a* phosphatase activity of type 1 and PCM phosphatases and for the *p*-nitrophenyl phosphate phosphatase activity of calcineurin. The following phosphatases were not affected by up to 10 μM-okadaic acid: type 2C phosphatase, phosphotyrosyl phosphatase, inositol 1,4,5-trisphosphate phosphatase, acid phosphatases and alkaline phosphatases. Thus okadaic acid had a relatively high specificity for type 2A, type 1 and PCM phosphatases. Kinetic studies showed that okadaic acid acts as a non-competitive or mixed inhibitor on the okadaic acid-sensitive enzymes.

INTRODUCTION

Okadaic acid ($C_{44}H_{66}O_{13}$) is a monocarboxylic acid extracted from common black sponges of the genus *Halichondria*. The chemical structure has been determined by Tachibana *et al.* (1981). Recently we reported that micromolar concentrations of this substance strongly inhibit the phosphorylated myosin light-chain (PMLC) phosphatase activity of smooth-muscle extract (Takai *et al.*, 1987; Bialojan *et al.*, 1987, 1988). Okadaic acid similarly inhibited a purified polycation-modulated (PCM) phosphatase from bovine aorta (DiSalvo *et al.*, 1984). So far, two endogenous phosphatase inhibitors, termed inhibitor 1 and inhibitor 2, have been isolated (Huang & Glinsmann, 1976). According to Cohen's classification (Ingebritsen & Cohen, 1983), our PCM phosphatase is a type 2 phosphatase, since it is not affected by these endogenous inhibitors (DiSalvo *et al.*, 1984). To our knowledge, okadaic acid is the first substance that has been shown to inhibit type 2 phosphatase in micromolar concentrations. Besides inhibitor 1 and inhibitor 2, only rather non-specific phosphatase inhibitors such as NaF (Morgan *et al.*, 1976) or vanadate (Searle, 1983) have been described. In the present experiments we further studied the inhibitory effect of okadaic acid on type 1, type 2A, type 2B and type 2C phosphatases as well as PCM phosphatase. The results show that okadaic acid has a relatively high specificity for type 2A and type 1 phosphatases, and that it acts as a non-competitive or mixed inhibitor for the okadaic acid-sensitive enzymes.

EXPERIMENTAL

Materials

All chemicals and reagents were purchased from Sigma Chemical Co. [γ-^{32}P]ATP was obtained from NEN. Okadaic acid, isolated from the black sponge *Halichondria okadai*, was generously given by Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan).

Preparation of proteins

Phosphorylatable myosin light chains were prepared from bovine myocardium by the method of Cummins & Lambert (1986). Myosin light-chain kinase was purifed from chicken gizzards as described by Ngai *et al.* (1984). Phosphorylase *b* and phosphorylase kinase from rabbit skeletal muscle as well as acid phosphatase and alkaline phosphatase were obtained from Sigma Chemical Co. Calmodulin from pig brain was purchased from Boehringer Mannheim. Calcineurin (= type 2B phosphatase; Klee & Krinks, 1978; Stewart *et al.*, 1982) from bovine brain was kindly provided by Professor C. Klee, National Institutes of Health, Bethesda, MD, U.S.A. The catalytic subunits of type 1 phosphatase and type 2A phosphatase (type $2A_c$) from rabbit skeletal muscle (Tung *et al.*, 1984) as well as type 2C phosphatase from rabbit liver (McGowan & Cohen, 1987) were generously given by Dr. G. Mieskes, Göttingen, Germany. Aortic PCM phosphatase was purified as described previously (DiSalvo *et al.*, 1984), involving chromatography on DEAE-Sephacel, polylysine–agarose, heparin–agarose and a Mono Q

Abbreviations used: PCM phosphatase, polycation-modulated phosphatase; PMLC, phosphorylated myosin light chain.

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HR 5/5 anion-exchange column with the Pharmacia f.p.l.c. system. SDS/polyacrylamide-gel electrophoresis of the purified enzyme revealed three polypeptides corresponding to 72, 53 and 35 kDa, confirming the previous report (DiSalvo *et al.*, 1985). According to Cohen's classification of protein phosphatases (Ingebritsen & Cohen, 1983) this PCM phosphatase is a type 2A phosphatase because it is not affected by inhibitor 1 or inhibitor 2 (DiSalvo *et al.*, 1984) and has a 5-fold higher PMLC phosphatase activity than phosphorylase *a* phosphatase activity (see the Results section).

Enzyme assays

All enzymes were tested at 30 °C. The assays were made in a buffer containing 20 mM-Tris/HCl, 0.5 mM-dithiothreitol and 1 mg of bovine serum albumin/ml, pH 7.4. The reaction mixtures for the assays of phosphorylase *a* phosphatase activity contained 5 mM-caffeine in addition. For standard assays, either ^{32}P-labelled PMLC or ^{32}P-labelled phosphorylase *a* was used as substrate. Reactions were started by injecting the ^{32}P-labelled substrate into the reaction mixture, and stopped by addition of ice-cold trichloroacetic acid (20%, w/v) and 6 mg of bovine serum albumin/ml. Assessment of released P_i was performed by standard procedures (DiSalvo *et al.*, 1982). The enzyme activity of type 1 phosphatase was measured in buffer containing 2 mM-$MnCl_2$. For the assay of type 2C phosphatase 11 mM-magnesium acetate was included in the buffer. The activity of calcineurin was measured with either *p*-nitrophenyl phosphate or PMLC as substrate. The reaction mixtures contained 40 mM-Tris/HCl, 0.1 M-KCl, 0.1 mg of bovine serum albumin/ml, 6 mM-$MgCl_2$, 0.25 mM-dithiothreitol, 0.1 μM-calmodulin and 0.1 mM-$CaCl_2$, pH 8.0. *p*-Nitrophenyl phosphate phosphatase activity of calcineurin was measured by recording the initial rate of increase in absorbance at 400 nm.

In the dose–inhibition analyses, the concentration of enzymes was such that less than 5% of the radioactive substrate was consumed during the reaction time of 3–10 min. The consumption of *p*-nitrophenyl phosphate was less than 1% of the initial concentration. In the enzyme-kinetic studies, we allowed consumption of the substrate to a maximum of 15% of the initial concentration, so as to make the measurements at the lower substrate concentration range sufficiently accurate. The effect of accumulating product (dephosphorylated protein and P_i) was examined for the protein phosphatases as follows. The rate of dephosphorylation was measured with different concentrations of substrate (1 μM-, 2 μM- and 5 μM-PMLC; 2 μM-, 5 μM- and 10 μM-phosphorylase *a*). For this purpose, the reaction was allowed to proceed until about 5% of the substrate added was depleted. The rate was not significantly changed when the reaction was started in the presence of dephosphorylated products (myosin light chains or phosphorylase *b* plus P_i) equivalent to 15% of the initial substrate concentration. Therefore the effect of accumulating product appeared to be negligible under the present experimental conditions. The effect of depletion of substrate can be mathematically corrected (see below).

Alkaline phosphatases (from bovine intestinal mucosa and pig placenta) and acid phosphatases (from milk and potato) were tested by standard procedures in alkaline and citrate buffer (Sigma kit) respectively. *p*-Nitrophenol phosphate (0.1 M) was used as a substrate and the rate of decrease in the absorbance at 400 nm was measured.

Dr. E. Ulug and Dr. S. Courtneidge from the European Molecular Biology Laboratory, Heidelberg, Germany, kindly performed the phosphotyrosine-specific phosphatase assays. The assays were carried out by incubating crude cell lysates from mouse NIH 3T3 cells with various ^{32}P-labelled phosphotyrosine-containing substrates such as enolase, poly(Glu-Tyr) and '*in-vitro*'-autophosphorylated cell lysates.

Assessment of inositol trisphosphate phosphatase was performed in Professor A. P. Somlyo's and Professor Y. E. Goldman's laboratories in Philadelphia, PA, U.S.A., together with Dr. J. Walker from the National Institute for Medical Research, London N.W.7, U.K. Both a crude membrane and a cytosolic fraction of rabbit main pulmonary artery were incubated with [^{3}H]inositol trisphosphate, and the initial rate of breakdown of inositol trisphosphate was measured as described by Walker *et al.* (1987).

Okadaic acid was dissolved in pure dimethyl sulphoxide and added to the experimental solution. Control solutions contained the same concentration (0.5%, v/v) of dimethyl sulphoxide.

Protein was measured by the method of Lowry *et al.* (1951).

Analysis of kinetic data

When an enzyme reaction is monitored over an extended time, the velocity of the reaction progressively slows down as a result of depletion of substrate and accumulation of product. In the present experiments, accumulation of product was limited, so that its effect on the reaction velocity could be neglected (see above). The slowing down resulting from depletion of substrate was corrected for by the following mathematical procedure.

Let $v(s)$ be the initial steady-state velocity at a given substrate concentration s (= [S]). When the reaction obeys the Michaelis–Menten kinetics:

$$v(s) = \frac{V \cdot s}{K_m + s} \quad (1)$$

where K_m is the Michaelis constant and V is the maximal velocity of the reaction. The value of $v(s)$ can be estimated by measuring the mean rate of the increase of product, which is equal to the decrease of substrate, i.e.:

$$\hat{v}(s) = \frac{s - s(t)}{t} \quad (2)$$

where $\hat{v}(s)$ is the estimate of $v(s)$ and $s(t)$ is the concentration of substrate remaining in the reaction mixture at time t [$s(0) = s$].

Because of depletion of substrate, $\hat{v}(s) < v(s)$. We define the correction factor, f, by the following equation:

$$v(s) = \hat{v}(s)/f \quad (3)$$

Inserting eqns. (1) and (2) into eqn. (3), and rearranging, we have:

$$f = \frac{(K_m + s)[s - s(t)]}{s \cdot V \cdot t} \quad (4)$$

The integrated form of eqn. (1) is:

$$V \cdot t = s - s(t) + K_m \cdot \ln\left(\frac{s}{s(t)}\right) \quad (5)$$

(see Cornish-Bowden, 1977; Dixon & Webb, 1979).

Eliminating $V \cdot t$ in eqn. (4) by eqn. (5), and rearranging, we obtain:

$$f = f(s, K_m, p) = \frac{\frac{s}{K_m} + 1}{\frac{s}{K_m} - \frac{\ln(1-p)}{p}} \quad (6)$$

where $p = [s - s(t)]/s$. The enzyme-kinetic results presented have been corrected for substrate depletion by using this correction factor (see also eqn. 3). As for the present data, the values of f were not less than 0.9, i.e. $0.9 < f < 1$.

Dose–inhibition relation

In the following description, the apparent values of K_m and V in the presence of inhibitor (okadaic acid) are denoted as K_m' and V' respectively.

In general, the concentration of inhibitor required to obtain 50% inhibition (ID_{50}) is a function of substrate concentration. This fact is important when the values of ID_{50} are compared. The function can be mathematically derived if the dependence of initial steady-state velocity on the substrate concentration is described in the form of the Michaelis–Menten equation both in the absence and in the presence of inhibitor, and if the dose–inhibition relation is given in the form of the Hill function (see Bialojan *et al.*, 1988). The following equations are relevant to the present results.

(i) When $K_m' > K_m$ and $V' < V$ (mixed inhibition):

$$ID_{50}(s) = K_2\left(\frac{\frac{s}{K_m} + 1}{\left(\frac{K_2}{K_1}\right)^h \cdot \frac{s}{K_m} + 1}\right)^{\frac{1}{h}} \quad (7)$$

where $ID_{50}(s)$ is the value of ID_{50} at a given substrate concentration s, h is the Hill coefficient, and K_1 and K_2 are the constants that give the upper and lower limits of $ID_{50}(s)$ respectively. The values of K_1 and K_2 are experimentally determined by using the relations:

$$K_1 = \left(\frac{V}{V'} - 1\right)^{-\frac{1}{h}} \cdot I \quad (8)$$

and

$$K_2 = \left(\frac{K_m'}{K_m} \cdot \frac{V}{V'} - 1\right)^{-\frac{1}{h}} \cdot I \quad (9)$$

(ii) When $K_m' = K_m$ and $V' < V$ (non-competitive inhibition):

$$ID_{50} = K_1 = K_2 \text{ (constant)} \quad (10)$$

(see eqns. 7, 8 and 9).

The results presented have been corrected for a higher extent of substrate utilization at lower inhibitor concentrations by using the correction factor described above (see eqns. 3 and 6). The increase of the values of h produced by the correction procedure was less than 0.05 in the present results.

Statistics

The kinetic constants of enzyme reactions were determined by the direct-linear-plot method (Eisenthal & Cornish-Bowden, 1974), and evaluation of their difference by a non-parametric method (Hollander & Wolfe, 1973) was made in accordance with Porter & Trager (1977) with the use of computer programs. The values of the constants obtained were presented with 95% confidence limits. The other numerical data were described as means ± S.E.M., and differences were assessed by the Student's t test. The dose–inhibition relations were fitted by the linear least-squares method to the linear form of the Hill function:

$$\ln[(100 - E)/E] = h \cdot \ln I + \ln K_h$$

where E is the percentage enzyme activity, I is the concentration of inhibitor, h is the Hill coefficient and K_h is the association constant. The values in the range $10\% < E < 90\%$ were used for the fitting. The values of ID_{50} and Hill coefficient were compared by a method of co-variance analysis (Snedecor & Cochran, 1980). Very similar values of ID_{50} and Hill coefficient were obtained when the fitting was made by a non-linear least-squares method (Snedecor & Cochran, 1980). In every case, differences were evaluated as statistically significant when a two-sided probability of less than 0.05 was obtained.

RESULTS

Dose–inhibition relations

Fig. 1(*a*) shows the dose–inhibition relations for the PMLC phosphatase activity of various types of phosphatase. For type $2A_c$, PCM and type 1 phosphatases the

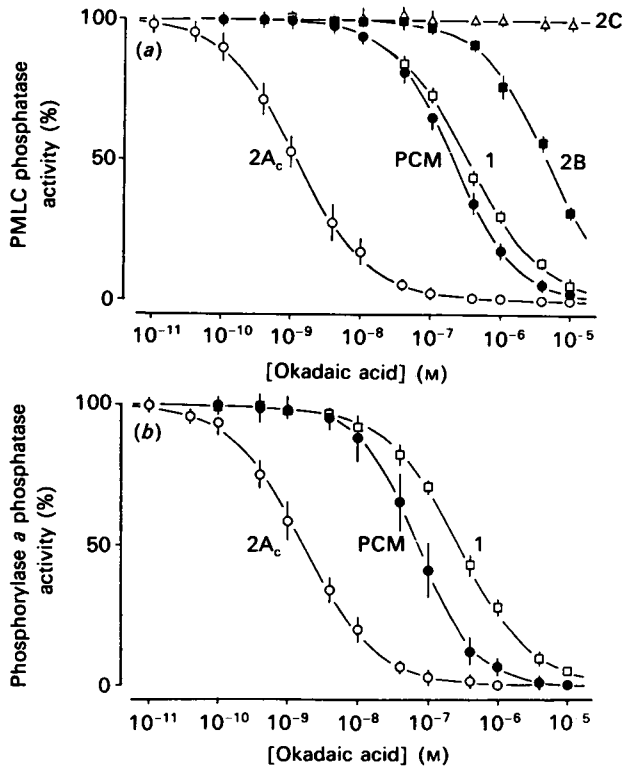

Fig. 1. Effect of okadaic acid on protein phosphatases

The inhibitory effect of okadaic acid on various types of protein phosphatase was examined with PMLC (4 μM) (*a*) and phosphorylase *a* (10 μM) (*b*) as substrates. ○, Type $2A_c$ phosphatase; ●, PCM phosphatase; □, type 1 phosphatase; ■, type 2B phosphatase; △, type 2C phosphatase. Tris buffer, pH 7.4, was used. The enzyme activities are given as percentages of the control values (see Table 1). The values of ID_{50} and Hill coefficients are given in Table 1. See the text for further explanation. Vertical bars indicate S.E.M. (n = 4–6).

Table 1. Parameters of the dose–inhibition relations

Enzyme activities were measured with 4 μM-PMLC, 10 μM-phosphorylase *a* or 5 mM-*p*-nitrophenyl phosphate as substrate. The control activities are the average of 12–16 values. See the text for explanation.

Phosphatase	Substrate	Control activity (units/mg of protein)	ID_{50} (nM)	*h*
Type $2A_c$	PMLC	8800±160	1.2	0.84
Type $2A_c$	Phosphorylase *a*	1750±50	1.6	0.84
PCM	PMLC	1550±120	205	0.95
PCM	Phosphorylase *a*	300±23	72	0.97
Type 1	PMLC	47.2±3.9	315	0.80
Type 1	Phosphorylase *a*	22.6±2.0	272	0.82
Type 2B	PMLC	1.4±0.2	4530	0.97
Type 2B	*p*-Nitrophenyl phosphate	62.1±8.1	3600	1.04

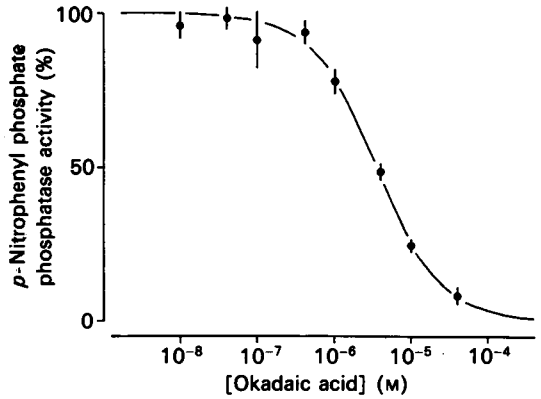

Fig. 2. Inhibition of the *p*-nitrophenyl phosphate phosphatase activity of calcineurin by okadaic acid

The *p*-nitrophenyl phosphate phosphatase activity of calcineurin was measured in Tris buffer, pH 8.0. The activity is presented as a percentage of the control activity (see Table 1). The values of ID_{50} and Hill coefficient are given in Table 1. See the text for further explanation. Vertical bars indicate S.E.M. ($n = 4$–5).

dose–inhibition relations were also examined for their phosphorylase *a* phosphatase activity (Fig. 1*b*). Parameters of the inhibition curves are given in Table 1.

When 4 μM-PMLC was used as substrate, the catalytic subunit of the skeletal-muscle type 2A phosphatase (type $2A_c$) was most potently inhibited (ID_{50} 1.2 nM). Okadaic acid also strongly inhibited PCM phosphatase (ID_{50} 205 nM) and type 1 phosphatase (ID_{50} 315 nM). Calcineurin (type 2B phosphatase) was inhibited to a lesser extent (ID_{50} 4350 nM). Type 2C phosphatase was not affected by up to 10 μM-okadaic acid (Fig. 1*a*; for the dependence of ID_{50} on the substrate concentration see below.)

When 10 μM-phosphorylase *a* was used as substrate, results were similar, except for PCM phosphatase, for which the value of ID_{50} (72 nM) was considerably smaller than that for its PMLC phosphatase activity (Table 1). This seems to be related to the enzyme-kinetic nature of the phosphorylase *a* phosphatase activity of PCM phosphatase (see below).

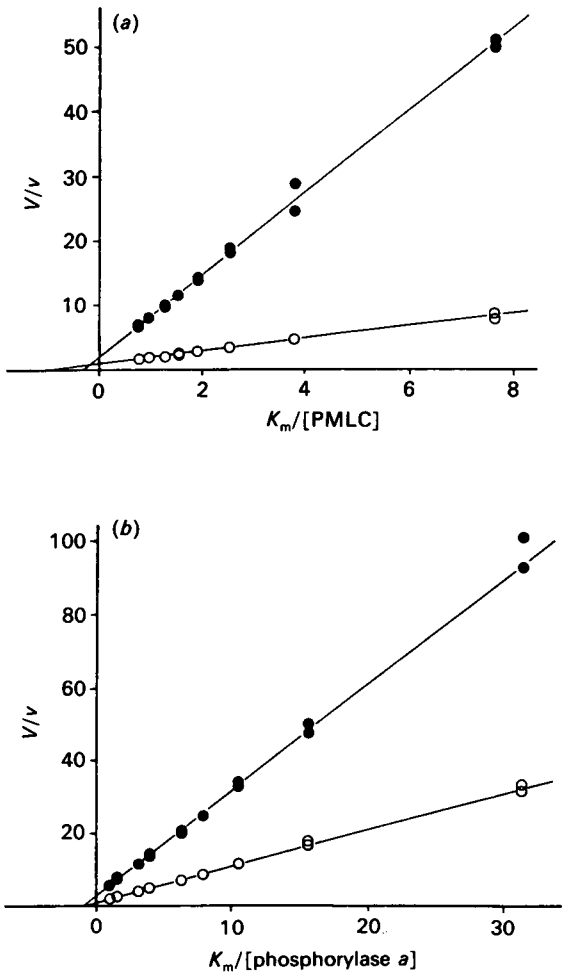

Fig. 3. Enzyme kinetics of the inhibition of type $2A_c$ phosphatase by okadaic acid

The dependence of initial steady-state velocity, *v*, on substrate concentration was examined for the PMLC phosphatase activity (*a*) and phosphorylase *a* phosphatase activity (*b*) of type $2A_c$ phosphatase. ○, Control; ●, okadaic acid (*a*, 5 nM; *b*, 2 nM). The values are normalized to the kinetic constants determined by the direct-linear-plot method (Table 2). See the text for further explanation.

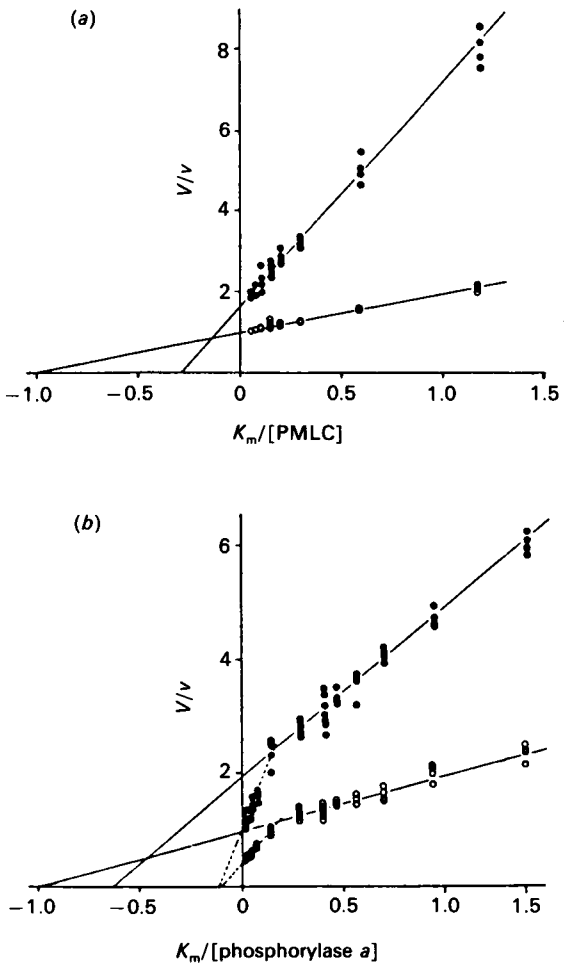

Fig. 4. Enzyme kinetics of the inhibition of PCM phosphatase by okadaic acid

The experiments are similar to those illustrated in Fig. 3. The PMLC phosphatase activity (*a*) and phosphorylase *a* phosphatase activity (*b*) of PCM phosphatase were measured. ○, Control; ●, okadaic acid (*a*, 300 nM; *b*, 100 nM). The values are normalized to the kinetic constants determined by the direct-linear-plot method (Table 2). In (*b*) the values of K_m and V at higher substrate concentrations (10–80 μM; broken lines) were both different from those at lower concentrations (0.5–5 μM; continuous lines); the constants for the substrate concentration range 0.5–5 μM were used for normalization. See the text for further explanation.

As for phosphatase 2B, the effect of okadaic acid was also tested for its *p*-nitrophenyl phosphate phosphatase activity in Tris buffer, pH 8.0 (Fig. 2). The control activity (Table 1) was similar to the value [120 units (nmol of P_i/min)/mg of protein] obtained in Professor C. Klee's laboratory (personal communication). The dose–inhibition relation was similar to that obtained for the PMLC phosphatase activity of the enzyme in Tris buffer, pH 7.5 (see also Table 1). When the PMLC phosphatase activity was measured in Tris buffer, pH 8.0, the control activity (1.4 units/mg of protein) was lower than that obtained in Tris buffer, pH 7.5, but the values of ID_{50} (5130 nM) and Hill coefficient (0.95) were not significantly changed.

The values of the Hill coefficient *h* tended to be smaller than 1 (Table 1), although the data were corrected for a higher extent of substrate utilization at lower inhibitor concentrations (see the Experimental section).

Enzyme kinetics

Figs. 3, 4 and 5 show the results of the enzyme-kinetic study. The data are presented as double-reciprocal plots. The values are normalized to the kinetic constants determined by the direct-linear-plot method (Table 2).

Type $2A_c$ phosphatase. For type $2A_c$ phosphatase (catalytic subunit of the skeletal-muscle type 2A phosphatase) the double-reciprocal plots were linear over the substrate concentration range for both PMLC phosphatase activity and phosphorylase *a* phosphatase activity of the enzyme. For the PMLC phosphatase activity the values of K_m and V were estimated, as described in the Experimental section, to be 3.8 μM and 17200 units/mg of protein respectively (Table 2). These were significantly changed to 12 mM ($= K_m'$; $P < 0.008$) and 8550 units/mg of protein ($= V'$; $P < 0.016$) by 5 nM-okadaic acid, indicating that okadaic acid acts as a mixed inhibitor. From the values of V'/V (0.50) and K_m'/K_m (3.2), the upper and lower limits of the change of ID_{50} produced by changing the substrate concentration were estimated, by using eqns. (8) and (9), to be 5 nM ($= K_1$) and 0.7 nM ($= K_2$) respectively.

For the phosphorylase *a* phosphatase activity the K_m was 8.2-fold larger whereas the V was 2.3-fold smaller than those for the PMLC phosphatase activity (Table 2). This qualitatively agrees with the previous report by Ingebritsen & Cohen (1983). Okadaic acid (2 nM) significantly decreased the value of V whereas it did not affect the value of K_m, i.e. the inhibition was non-competitive. As expected from eqn. (10), the value of ID_{50} of the dose–inhibition relation was not altered when the substrate concentration was changed from 10 μM to 2 μM and 20 μM.

PCM phosphatase. When PMLC was used as substrate, the double-reciprocal plot was linear over the concentration range (0.5–10 μM) of PMLC examined (Fig. 4*a*). Both K_m and V were significantly ($P < 0.002$) changed by 300 nM-okadaic acid (Table 2), indicating that okadaic acid acts as a mixed inhibitor. From the change of the kinetic constants, the lower and upper limits of the variation of ID_{50} produced by changing the substrate concentration were estimated, by using eqns. (7) and (8), to be 60 nM and 460 nM respectively.

For the phosphorylase *a* phosphatase activity of PCM phosphatase the double-reciprocal plot had two components (Fig. 4*b*), one below and one above a substrate concentration of about 8 μM (K_m/[phosphorylase *a*] = 0.14–0.18). The values of K_m and V above 8 μM substrate were both significantly different ($P < 0.002$) from those below this critical concentration. In the lower substrate concentration range (0.5–5 μM) both K_m and V were changed by 100 nM-okadaic acid ($P < 0.002$), i.e. the inhibition was a mixed type. In the higher concentration range (10–80 μM) V was decreased ($P < 0.002$) whereas K_m was not significantly changed by okadaic acid, i.e. the inhibition was a non-competitive type. As a consequence the dependence of ID_{50} on the substrate concentration was somewhat complicated (Fig. 6). In the range 0.5–8 μM the ID_{50} increased, as expected from eqn. (7) (K_1 = 50 nM and K_2 = 115 nM), as the concentration of

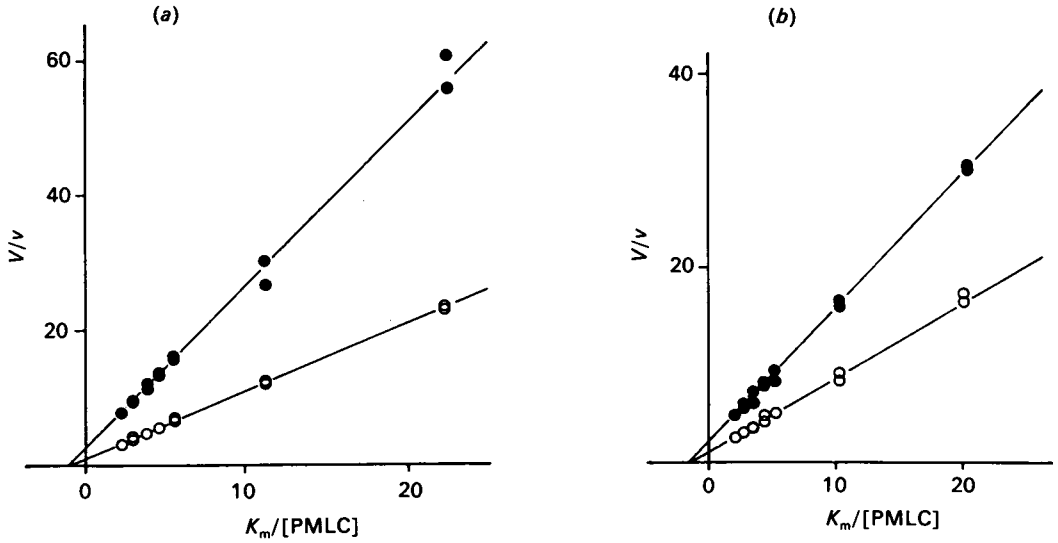

Fig. 5. Enzyme kinetics of the inhibition of type 1 phosphatase and calcineurin by okadaic acid

The experiments are similar to those illustrated in Fig. 3. The initial steady-state velocity, v, of the dephosphorylation of PMLC (0.5–5 μM) by type 1 phosphatase (*a*) and calcineurin (*b*) was measured. ○, Control; ●, okadaic acid (*a*, 315 nM; *b*, 5000 nM). See Table 2 for the kinetic constants. See the text for further explanation.

Table 2. Kinetic constants of the phosphatase inhibition by okadaic acid

The values of K_m and V determined by the direct-linear-plot method for the results shown in Figs. 3, 4 and 5. They are presented with the 95% confidence limits (in parentheses). [S] denotes the substrate concentration.

Phosphatase	Substrate	Range of [S] (μM)	[Okadaic acid] (nM)	K_m (μM)	V (units/mg of protein)
Type $2A_c$	PMLC	0.5–5	0	3.8 (3.4–4.2)	17200 (16200–18200)
Type $2A_c$	PMLC	0.5–5	5	12 (10–15)	8550 (7240–10800)
Type $2A_c$	Phosphorylase *a*	1–30	0	31 (28–36)	7350 (6720–7930)
Type $2A_c$	Phosphorylase *a*	1–30	2	32 (28–38)	2590 (2300–2920)
PCM	PMLC	0.5–10	0	0.6 (0.5–0.7)	1830 (1790–1910)
PCM	PMLC	0.5–10	300	2.0 (1.6–2.6)	1100 (990–1250)
PCM	Phosphorylase *a*	1–5	0	1.4 (1.0–1.7)	340 (310–365)
PCM	Phosphorylase *a*	1–5	100	2.2 (1.8–2.6)	180 (165–195)
PCM	Phosphorylase *a*	10–80	0	14 (11–16)	710 (650–770)
PCM	Phosphorylase *a*	10–80	100	12 (9–16)	290 (250–320)
Type 1	PMLC	0.5–5	0	11 (9–13)	177 (155–207)
Type 1	PMLC	0.5–5	315	10 (7–14)	68 (50–83)
Type 2B	PMLC	0.5–5	0	7.9 (5.9–9.1)	4.2 (3.7–4.7)
Type 2B	PMLC	0.5–5	5000	6.5 (3.4–9.3)	2.1 (1.1–2.6)

phosphorylase *a* concentration was increased, whereas it abruptly decreased to a constant value when the phosphorylase *a* concentration exceeded 8 μM, as expected from eqn. (10) ($K_1 = K_2 = 64$ nM). At the critical concentration (8–10 μM) the co-variance analysis of the dose–inhibition relation gave a significantly larger residual variance ($P < 0.01$) than for both sides of this concentration. [Note the relatively large S.E.M. values in the dose–response relation for this enzyme activity (Fig. 1*b*).] The range of variation of ID_{50} produced by changing substrate concentration was estimated to be 50–100 nM (Fig. 6). This falls within the variation range of ID_{50} estimated for the PMLC phosphatase activity of the enzyme (see above).

The control value of V was 5.4-fold higher for the PMLC phosphatase activity than for the phosphorylase *a* phosphatase activity of the enzyme (Table 2). In this respect the substrate specificity of our PCM phosphatase has a similarity to that of type 2A phosphatase (cf. Ingebritsen & Cohen, 1983).

Type 1 phosphatase and calcineurin. For these types of enzyme, kinetic studies were made with PMLC as substrate (Fig. 5). The values of K_m were one order of magnitude larger whereas the values of V expressed in terms of the specific activity were considerably smaller than those of the PMLC phosphatase activity of PCM phosphatase (Table 2). In the range of substrate concen-

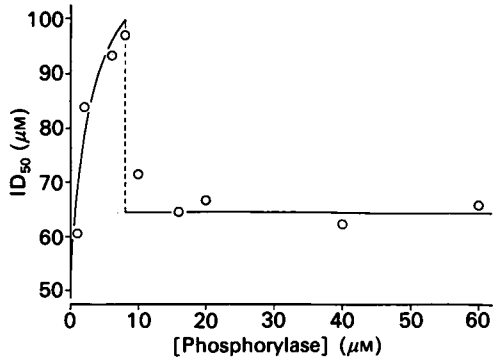

Fig. 6. Dependence of ID_{50} on substrate concentration

The effect of okadaic acid on the phosphorylase *a* activity of PCM phosphatase was examined with various concentrations (1–60 μM) of phosphorylase *a*. The values of ID_{50} of the dose–inhibition relation were plotted against the concentration of phosphorylase *a*. The curve and horizontal line show the relation expected from eqns. (7) and (10) (see the Experimental section); the kinetic constants for the lower and higher concentration ranges of phosphorylase *a* given in Table 2 were used for computation. The Hill coefficient ($h = 0.96$–0.98) was not significantly changed when the substrate concentration was varied. See the text for further detail.

trations examined the inhibition was non-competitive for both enzymes; okadaic acid changed only the values of V ($P < 0.02$). In accordance with eqn. (10), we found that the values of ID_{50} of the dose–inhibition relations were not significantly altered when the substrate concentration was changed from 4 μM to 1 μM and 10 μM.

Effect on other phosphatases

Neither cytosolic nor particulate-bound inositol trisphosphate phosphatase from smooth muscle was inhibited by up to 50 μM-okadaic acid. No effect was found with tyrosine-specific phosphatase(s) from cell lysates. Alkaline phosphatases (from bovine intestinal mucosa and pig placenta) and acid phosphatases (from milk and potato) tested with *p*-nitrophenyl phosphate as substrate were not affected by 10 μM-okadaic acid. However, a three-subunit phosphatase isolated from dog heart (Kranias & DiSalvo, 1986) was strongly inhibited by okadaic acid (E. G. Kranias & J. DiSalvo, personal communication). The enzyme expressed phosphatase activity against phospholamban and phosphorylase *a*; both activities were completely inhibited by okadaic acid in micromolar concentration.

DISCUSSION

In previous papers we have reported that micromolar concentrations of okadaic acid potently inhibit the PMLC phosphatase activity of smooth-muscle extract and of the aortic PCM phosphatase (Takai *et al.*, 1987; Bialojan *et al.*, 1987, 1988). The present results show that okadaic acid inhibits type 2A, type 1 and type 2B phosphatases as well as the PCM phosphatase. On the other hand, the following phosphatases are not affected by up to 10 μM-okadaic acid: type 2C phosphatase, phosphotyrosyl phosphatase, inositol trisphosphate phosphatase, acid phosphatases and alkaline phosphatases (the present work). We have also shown that okadaic acid does not affect the Ca^{2+} + calmodulin-dependent activity of myosin light-chain kinase or phosphodiesterase (Takai *et al.*, 1987). Thus the inhibitory action of okadaic acid appears to be specific for some restricted types of protein phosphatase.

The type of substrate has little effect on the inhibitory action of okadaic acid. Essentially similar dose–inhibition relations are obtained for the PMLC phosphatase activity and the phosphorylase *a* phosphatase activity of type $2A_c$, PCM and type 1 phosphatases (Fig. 1). The *p*-nitrophenyl phosphate phosphatase activity of type 2B phosphatase (calcineurin) has nearly the same susceptibility to okadaic acid as its PMLC phosphatase activity (see also Fig. 2). These results support the idea that the inhibitory action of okadaic acid is enzyme-directed.

The affinity of okadaic acid differs remarkably among the okadaic acid-sensitive phosphatases. The catalytic subunit of skeletal-muscle type 2A phosphatase (type $2A_c$) is about 200 times more strongly inhibited by okadaic acid than is that of type 1 phosphatase, although there is a striking sequence homology between these catalytic subunits (Berndt *et al.*, 1987). The three-dimensional conformation of the enzyme molecule may be an important factor for the binding of okadaic acid. The aortic PCM phosphatase is a polymolecular enzyme consisting of three subunits, and belongs to the category of type 2A phosphatases (see the Experimental section). This phosphatase is considerably less susceptible to okadaic acid than is type $2A_c$ phosphatase (Fig. 1). The reason for this difference is not clear from the present results. It may imply that the aortic PCM phosphatase has a different catalytic subunit from that of the skeletal-muscle type 2A phosphatase. Another possibility is that the non-catalytic subunits make the catalytic subunit less accessible to okadaic acid.

The present kinetic study has shown that okadaic acid acts as a non-competitive or mixed inhibitor of the okadaic acid-sensitive enzymes, suggesting that the binding site for okadaic acid is different from that for the substrate. However, the reaction mechanism is likely to be more complicated. The Hill coefficients of dose–inhibition relations tend to be smaller than 1 even after the correction for the higher extent of substrate consumption at lower inhibitor concentrations (see the Experimental section). When the Hill coefficient is not 1, the inhibition is not 'linear' according to Cleland's (1963) classification of enzyme inhibition (see Bialojan *et al.*, 1988). In the model of ordinary (linear) inhibition, the constants K_1 and K_2 denote the dissociation constants of inhibitor for free enzyme and enzyme–substrate respectively (see Dixon & Webb, 1979). If this scheme qualitatively applies for the present results concerning the PMLC phosphatase activity of the PCM phosphatase, where the Hill coefficient (0.95) was close to 1, the larger value of K_1 than that of K_2 may suggest that the affinity of okadaic acid for the phosphatase is considerably diminished when the substrate binds to the enzyme.

The kinetic data give an explanation for the complicated nature of the dependence of ID_{50} observed for the phosphorylase *a* phosphatase activity of the PCM phosphatase (see the Results section). The kinetics rather abruptly change at a critical concentration, and the affinity of okadaic acid for the enzyme apparently becomes unstable at this concentration. This fact must be noted especially when phosphorylase *a* is used as a substrate of PCM phosphatase for studies of inhibitors.

The phenomenon is not observed for the phosphorylase *a* phosphatase activity of type $2A_c$ phosphatase. Therefore the change of kinetics is likely to be due to a property of the PCM phosphatase rather than to that of phosphorylase *a*.

To our knowledge, okadaic acid is the first-described potent substance that inhibits type 2 phosphatases as well as type 1 phosphatase. The inhibition of phosphatase is reversible, as judged from its effects on myosin light-chain phosphorylation (Bialojan *et al.*, 1988). Therefore okadaic acid may serve as a unique tool for analysing phosphorylation-regulated systems.

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