Estimation of the rate constants associated with the inhibitory effect of okadaic acid on type 2A protein phosphatase by time-course analysis

Akira TAKAI,*† Yasumasa OHNO,* Takeshi YASUMOTO‡ and Gottfried MIESKES§

*Department of Physiology, School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan,

‡Laboratory of Food Hygiene, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amemiyamachi,

Aoba-ku, Sendai 981, Japan, and §Abteilung Klinische Biochemie, Zentrum für Innere Medizin, Universität Göttingen, Robert-Koch-Straße 40, 3400 Göttingen, Federal Republic of Germany

As is often the case with tightly binding inhibitors, okadaic acid produces its inhibitory effect on type 2A protein phosphatase (PP2A) in a time-dependent manner. We measured the rate constants associated with the binding of okadaic acid to PP2A by analysing the time-course of the reduction of the *p*-nitrophenyl phosphate (*p*NPP) phosphatase activity of the enzyme after application of okadaic acid. The rate constants for dissociation of okadaic acid from PP2A were also estimated from the time-course of the recovery of the activity from inhibition by okadaic acid after addition of a mouse IgG1 monoclonal antibody raised against the inhibitor. Our results show that the rate constants for the binding of okadaic acid and PP2A are of the order of $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, a typical value for reactions involving relatively large molecules, whereas those for their dissociation are in the range 10^{-4} – 10^{-3} s^{-1} . The very low values of the latter seems to be the determining factor for the exceedingly high affinity of okadaic acid for PP2A. The dissociation constants for the interaction of okadaic acid with the free enzyme and the enzyme–substrate complex, estimated as the ratio of the rate constants, are both in the range 30–40 pM, in agreement with the results of previous dose–inhibition analyses.

INTRODUCTION

Okadaic acid is a polyether derivative of a C_{38} fatty acid (Tachibana *et al.*, 1981) which is thought to be produced by some dinoflagellates (plankton) and to accumulate in other marine organisms such as sponges or shellfish as a result of filter feeding (Yasumoto *et al.*, 1987; Yasumoto, 1990). The substance is now widely used as a potent inhibitor of protein phosphatases [for review see Takai (1988) and Cohen *et al.* (1990)].

The specificity of the inhibitory effect has been studied with purified enzymes (Hescheler *et al.*, 1988; Bialojan & Takai, 1988; Haystead *et al.*, 1989; see also Cohen *et al.*, 1990). It is well known that okadaic acid exhibits an exceedingly high affinity for type 2A protein phosphatase (PP2A), although it also inhibits type 1 and type 2B enzymes with much lower potency [for classification of protein phosphatases, see Cohen (1989)]. Our dose–inhibition analyses have shown that the value of the dissociation constant K_i for PP2A is of the order of 30 pM (see Takai & Mieskes, 1991). We have studied the characteristics of okadaic acid as a tightly binding inhibitor (Henderson, 1972) by means of a sensitive spectrophotometric method using *p*-nitrophenyl phosphate (*p*NPP) as substrate (Takai & Mieskes, 1991). In the previous paper we discussed the limitation of steady-state analyses for determining the kinetic constants for the interaction of okadaic acid with PP2A.

During further experiments with the spectrophotometric method, we noticed that the inhibition of PP2A by okadaic acid was markedly time-dependent when the inhibitor was applied after the reaction was started. As pointed out by Cha (1975, 1976), such time-dependence is a general feature of the action of tightly binding inhibitors, kinetic analyses of which often have to be done at enzyme concentrations comparable with the very low dissociation constant [see also Cha *et al.* (1975) and Williams & Morrison (1979)].

Cha (1976) has described a method for utilizing the information contained in the pre-steady-state time-course to obtain values for the rate constants associated with the binding and dissociation of an enzyme and its inhibitor. With his assumptions, which can be applied to most reactions inhibited by a tightly binding inhibitor, mathematical analysis of the time-dependent process is reasonably simplified. However, the method requires some tedious replotting procedures and is not very sensitive for determining the rate constants for the dissociation of inhibitor from enzyme because of the inherent nature of the rate equation used (Cha, 1976; see the Theory section of this paper).

In this paper we have introduced an improved procedure for determining the rate constants from time-course data, which utilizes a sequestering agent against the inhibitor. The rate constants for the association of a tightly binding inhibitor with an enzyme are more easily obtained by this procedure than by the method of Cha (1976). The use of an inhibitor sequesteror makes it possible to observe the time-course of the recovery of enzyme activity from suppression by the inhibitor and thereby directly estimate the rate constants for the dissociation process. We have used the procedure to analyse the time-course of interaction of okadaic acid with PP2A. We have used as the sequestering agent a monoclonal antibody which binds to okadaic acid with a dissociation constant of about 100 pM (Usagawa *et al.*, 1989).

The results show that the rate constants for binding of okadaic acid and PP2A are of the order of $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, a typical value for reactions involving relatively large molecules, whereas those for their dissociation are in the range 10^{-4} – 10^{-3} s^{-1} . The very low values of the latter seem to be the determining factor for the exceedingly high affinity of okadaic acid for PP2A. The dissociation constants for the interaction of okadaic acid with the free enzyme and the enzyme–substrate complex, estimated as

Abbreviations used: PP2A, type 2A protein phosphatase; *p*NPP, *p*-nitrophenyl phosphate.

† To whom correspondence should be addressed.

the ratio of the rate constants, are both in the range 30–40 μM , in agreement with previous dose–inhibition analyses.

EXPERIMENTAL

Materials

Okadaic acid, isolated from the black sponge (*Halichondria okadae*), was kindly given by Dr. Yasumasa Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan). Mouse IgG1 monoclonal antibody against okadaic acid, prepared by the method of Usagawa *et al.* (1989), was a gift from Ube Industries Ltd. (Ube, Japan). *p*NPP was purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Preparation and assay of PP2A

The catalytic subunit of PP2A was prepared from rabbit skeletal muscle by the method of Tung *et al.* (1984). The molar concentration of PP2A was determined by the titration procedure with okadaic acid as described previously (Takai & Mieskes, 1991; Takai *et al.*, 1992).

The activity of PP2A was measured with *p*NPP as substrate essentially by the method described previously (Takai & Mieskes, 1991; Takai *et al.*, 1992); for the *p*NPP phosphatase activity of PP2A, see also Goris *et al.* (1989) and Cayla *et al.* (1990).

All assays were carried out at 25 °C. Reaction mixtures contained 40 mM-Tris, 34 mM-MgCl₂, 4 mM-EDTA and 4 mM-DL-dithiothreitol (pH 8.4, adjusted with HCl at 25 °C). Liberation of the reaction product (*p*-nitrophenol) was continuously monitored by tracing the increase in absorbance at 400 nm with a U1100 spectrophotometer (Hitachi Co., Tokyo, Japan) connected to a pen recorder. Digital output from the spectrophotometer was simultaneously recorded with a PC9801 computer (NEC, Tokyo, Japan) for mathematical analyses.

THEORY

Symbols and model

The symbols used are defined in Table 1.

Ordinary inhibition mechanisms are generally represented by Scheme 1. The following relations exist between the rate constants and the kinetic constants:

$$K_m = (k_{-1} + k_{+2})/k_{+1}$$

$$V_{\max} = k_{+2}E_t$$

$$K_0 = k_{-3}/k_{+3} \quad (1)$$

$$K_1 = k_{-4}/k_{+4} \quad (2)$$

and

$$K_m' = (K_1/K_0)K_m = k_{-5}/k_{+5} \quad (3)$$

Competitive and uncompetitive inhibitions are treated as special cases where $k_{+4} = 0$ and $k_{+3} = 0$ respectively (Cleland, 1963). In this paper, the terms 'non-competitive inhibition' and 'mixed inhibition' are used for inhibitions where $K_0 = K_1$ and $K_0 < K_1$ respectively, as defined by Dixon & Webb (1979a,b).

Time-course of inhibition: determination of k_{+3} and k_{+4}

According to Cha (1976), we adopt the following assumptions, which are reasonable under the present experimental settings.

(a) Steady state conditions are instantaneously reached between $E + S$ and ES and between $EI + S$ and ESI .

(b) Prolonged non-steady state conditions exist between $E + I$ and EI and between $ES + I$ and ESI .

(c) The substrate concentration is sufficiently high that the depletion of free substrate by binding to the enzyme and that by conversion into product are both negligible.

(d) The concentration of product is negligibly small.

Now let $E_t = [E] + [ES]$ and $E_i = [EI] + [ESI]$; it then follows from the assumptions that

$$E_t = xE_i \quad (4)$$

$$[E] = E_t/(1 + s) \quad (5)$$

$$[ES] = E_t s/(1 + s) \quad (6)$$

and

$$[EI] = E_i/(1 + s') \quad (7)$$

$$[ESI] = E_i s'/(1 + s') \quad (8)$$

The conservation equations for the enzyme and the inhibitor are

$$E_t = E_t + E_i \quad (9)$$

and

$$I_t = [I] + [EI] + [ESI] \quad (10)$$

The time course of changes of E_t is given by the following differential equation (see Scheme 1):

$$\frac{dE_t}{dt} = -k_{+3}[E][I] - k_{+4}[ES][I] + k_{-3}[EI] + k_{-4}[ESI] \quad (11)$$

Table 1. Symbols used and their definitions

Symbol	Definition
t	Time
K_m	Michaelis constant
K_m'	Dissociation constant for interaction of enzyme–inhibitor complex with substrate
$V_{\max}$	Maximal velocity
E	Enzyme
E_t	Total concentration of enzyme in reaction mixture
I	Inhibitor
I_t	Total concentration of inhibitor in reaction mixture
S and S'	Substrate and its concentration
s	S/K_m
s'	S/K_m'
P	Product
$P(t)$	Concentration of product produced during the time interval (0, t)
k_{+n} and k_{-n}	Rate constants for forward (+) and backward (–) reactions; the number, n , is assigned as shown in Schemes 1 and 2
v_0	Reaction velocity in the absence of inhibitor
v_i	Reaction velocity in the presence of inhibitor
x [or $x(t)$]	Fractional activity, v_i/v_0 (at t)
K_i	Dissociation constant for enzyme–inhibitor interaction
K_0	Dissociation constant for interaction of inhibitor with free enzyme
K_1	Dissociation constant for interaction of inhibitor with enzyme–substrate complex
A and A'	Antibody (as inhibitor–sequestrator) and its total concentration in the reaction mixture
K_a	Dissociation constant for inhibitor–antibody interaction
α	Apparent second-order rate constant associated with binding of inhibitor to enzyme
β	Apparent first-order rate constant associated with dissociation of inhibitor from enzyme

Scheme 1

Eliminating $[E]$, $[I]$, $[ES]$, $[EI]$, $[ESI]$ and E_t in eqn. (11) by eqns. (3)–(10) and rearranging, we find that time-dependent changes of the partial activity, x , in the presence of an inhibitor are described by a simple Riccati equation:

$$\frac{dx}{dt} + \alpha[E_t x^2 - (E_t - I_t - K_i)x - K_i] = 0 \quad (12)$$

Note that this equation can be applied for tightly binding inhibitor as well as for classical inhibitors.

For non-competitive inhibition and mixed inhibition, the apparent second-order rate constant, α , is given by the general form:

$$\alpha = \alpha(s) = (k_{+3} + k_{+4}s)/(1 + s) \quad (13)$$

If $k_{+3} = k_{+4}$, then

$$\alpha = k_{+3} = k_{+4} \text{ (constant)} \quad (14)$$

For competitive inhibition where $k_{+4} = 0$, eqn. (13) reduces to

$$\alpha(s) = k_{+3}/(1 + s) \quad (15)$$

Note that the plot of the reciprocal of $\alpha(s)$ against s (or S) becomes linear in this case (see Fig. 2 below).

For uncompetitive inhibition where $k_{+3} = 0$, eqn. (13) becomes

$$\alpha(s) = k_{+4}s/(1 + s) \quad (16)$$

Thus, the mode of inhibition can be estimated by examining the dependence of α on s .

The dissociation constant, K_i , is also a function of s :

$$K_i = K_0(1 + s)/[1 + (K_0/K_1)s] \quad (17)$$

Suppose that the inhibitor is added at $t = 0$ after a steady-state velocity (v_0) is measured. Then, imposing on eqn. (12) the boundary condition that $x = 1$ at $t = 0$, we get

$$x = x(t) = x(\infty) + (Q/E_t)\gamma/[\exp(\lambda t) - \gamma] \quad (18)$$

where

$$Q = \sqrt{[(E_t - I_t - K_i)^2 + 4E_t K_i]} = \sqrt{[(E_t + I_t + K_i)^2 - 4E_t I_t]} \quad (19)$$

$$x(\infty) = (E_t - I_t - K_i + Q)/2E_t \quad (20)$$

$$\gamma = (E_t + I_t + K_i - Q)/(E_t + I_t + K_i + Q) \quad (21)$$

and

$$\lambda = \alpha Q$$

[For derivation of eqn. (18) from eqn. (12), see e.g. Tenenbaum & Pollard (1963)].

Eqn. (20) describes the dose-inhibition relationship in a steady state where $dx/dt = 0$ [compare with eqns. (4) and (5) of Takai & Mieskes (1991)]. Rate equations as used by Cha (1976) and Williams *et al.* (1980) for analysis of time-dependent inhibitory processes can be obtained by solving eqn. (12) under different boundary conditions.

Integrating eqn. (18) and remembering the definition of $P(t)$, we obtain

$$P(t) = v_0 \int_0^t x(z) dz = v_0 x(\infty)t + [v_0/(\alpha E_t)] \ln[1 - \gamma \exp(-\lambda t)]/(1 - \gamma) \quad (22)$$

Now, we define Δ by

$$\Delta = \lim_{t \rightarrow \infty} [P(t) - v_0 x(\infty)t] = [v_0/(\alpha E_t)] |\ln(1 - \gamma)| \quad (23)$$

Rearranging eqn. (23), we have

$$\alpha = [v_0/E_t]/\Delta |\ln(1 - \gamma)| \quad (24)$$

We use this relation to experimentally estimate the value of α for a given value of s because Δ can easily be determined from progress curves (see Fig. 1 below). The values of k_{+3} and k_{+4} are then estimated by fitting eqn. (13) to a set of (s, α) data. Generally,

Scheme 2

the coefficient γ is a function of s [see eqns. (17), (19) and (21)]. In order to simplify analyses, we use the condition that $I_t > E_t \gg K_i$, where $\gamma = E_t/I_t$.

Note that eqn. (12) and rate equations derived from it contain the rate constants for the dissociation process, k_{-3} and k_{-4} , only implicitly. This is the basic reason why the Cha (1976) method is not very sensitive in estimating these rate constants. Because they can be more directly determined by the method described below, the information concerning the dissociation process is sacrificed in the above procedure for making estimation of k_{+3} and k_{+4} easy.

Time-course of recovery: determination of k_{-3} and k_{-4}

Scheme 2 represents the mechanism by which the inhibitory effect of an inhibitor on an enzyme is reversed by sequestration by an antibody. In addition to the above assumptions, we assume here that a steady-state condition is instantaneously reached between $I + A$ and IA , and that $[IA]$ is negligibly smaller than $[A]$ (i.e. $A \div [A]$). These additional assumptions are reasonable when the concentration of antibody is sufficiently high.

Under these assumptions, the time-course of recovery of activity after application of antibody is described by the following differential equation which is similar in form to eqn. (12):

$$\frac{dx}{dt} + \alpha[\epsilon E_t x^2 - (\epsilon E_t - \epsilon I_t - K_i)x - K_i] = 0 \quad (25)$$

where $\epsilon = 1/[1 + (A/K_a)]$. Note that $K_a = k_{-6}/k_{+6}$.

By arranging eqn. (25), we have:

$$\frac{dx}{dt} + \beta x - \beta + \alpha \epsilon [E_t x^2 - (E_t - I_t)x] = 0 \quad (26)$$

where β is the apparent first-order rate constant which is defined by

$$\beta = \alpha K_i \quad (27)$$

It can be shown from eqns. (3), (13) and (17) that

$$\beta = [1 + (k_{+4}/k_{+3})s]/[k_{-3}^{-1} + (k_{+4}/k_{+3})k_{-4}^{-1}s] \quad (28)$$

Note that $\beta = k_{-3}$ (constant) for competitive inhibition where $k_{+4} = 0$.

Let $A \rightarrow \infty$; then $\epsilon \rightarrow 0$ and therefore eqn. (26) reduces to:

$$\frac{dx}{dt} + \beta x - \beta = 0 \quad (29)$$

Suppose that the antibody is added after establishment of a steady state among E , S and I . Then, imposing on eqn. (29) the boundary condition that $x(0) = (E_t - I_t - K_i + Q)/2E_t$ [cf. eqn. (20)], we obtain:

$$x(t) = 1 - \zeta \exp(-\beta t) \quad (30)$$

where $\zeta = (E_t + I_t + K_i - Q)/2E_t$.

Integrating eqn. (30), we see that $P(t)$ is now given by:

$$P(t) = v_0 t - v_0 (\zeta/\beta) [1 - \exp(-\beta t)] \quad (31)$$

We can determine β by fitting of eqn. (31) to data. The magnitude of k_{-3} and k_{-4} is then estimated by fitting eqn. (28) to a set of (s, β) data with the use of the values of k_{+3} and k_{+4} obtained by the above procedure.

Fig. 1. Time-course of the inhibition of PP2A by okadaic acid

Accumulation of *p*-nitrophenol produced by dephosphorylation of *p*NPP by PP2A (2 nM) was recorded as the increase in absorbance at 400 nm. After the control activity (v_0) had been measured (not shown), okadaic acid (5 mM) was applied ($t = 0$) and the time-course of the ensuing decrease in activity was recorded. The results obtained with different concentrations of substrate were superimposed. In order to make comparison of the curves easy, the residual linear component $v_0 x(\infty)t$ has been subtracted from $P(t)$. The time-course was well fitted by the theoretical function [eqn. (22); dashed lines] with the use of the values of Δ which were determined as the plateau level of each curve [see eqn. (23)].

It is important to consider how high the concentration of antibody, A , should be, so that the approximate equation (30) can be used. A sufficient condition for this is:

$$|\beta x - \beta| \gg |\alpha e[E_t x^2 - (E_t - I_t)x]| \quad (32)$$

[see eqn. (26)]. Now let $I_t \geq E_t$; then $0 \leq E_t x^2 - (E_t - I_t)x \leq I_t$ because $0 \leq x \leq 1$. Therefore, since $\alpha > 0$, $\beta > 0$ and $K_a/A > \epsilon > 0$, by recalling eqn. (27), we obtain a usable condition under which relationship (32) is satisfied:

$$(1 \geq) 1 - x \gg (I_t/K_t)/(A/K_a) (\geq 0) \quad (33)$$

Data fitting and statistics

In the present paper, the values of α were calculated by using eqn. (24) with the use of the values of Δ directly measured from experimentally obtained progress curves (see the Results section). For analysis of the data from the experiments with antibody, a non-linear least-squares method without weighting (Snedecor & Cochran, 1980, *a,b*) was applied to fit the theoretical function (31) to progress curves to give the estimates of β . The rate constants were determined by fitting eqns. (13) and (28) to the sets of observations (s, α) and (s, β) respectively (see above). The fitting procedure used was essentially the same as that described previously for determination of E_t and K_t (Takai *et al.*, 1992). The procedure is a modification of the direct linear plot (Porter & Trager, 1977; Cornish-Bowden *et al.*, 1978), by which the kinetic constants were determined. Ranks corresponding to 95 % confidence limits were calculated with correction for replicated observations, as described by Kendall (1970). Other numerical data are presented as means $\pm$ S.E.M.

RESULTS

Time-course of inhibition of PP2A activity by okadaic acid

Fig. 1 shows the time-course of the decrease in the *p*NPP phosphatase activity of PP2A after application of okadaic acid. The reaction was started by addition of the substrate to the buffer containing PP2A (2 nM), and after the steady-state velocity (v_0) had been measured, okadaic acid (5 nM) was applied ($t = 0$).

Fig. 2. Dependence of the apparent second-order rate constant on substrate concentration

The reciprocals of the apparent second-order rate constant, α , obtained from experiments such as those shown in Fig. 1 were plotted against substrate concentration, S ($= [pNPP]$). The curve given by eqn. (13) was drawn (solid line) by using the values of k_{+3} and k_{+4} , which were determined by the non-parametric statistical procedure (see Table 2). Graphs of eqn. (13) for $k_{+4} = 0$ (competitive inhibition) and $k_{+3} = k_{+4}$ are also shown for comparison (dashed lines) [see eqns. (14) and (15)].

The time-course of the ensuing inhibition was observed with various concentrations of the substrate ($S = [pNPP]$). The values of the apparent second-order rate constant, α , were calculated by eqn. (24) using the values of Δ , which were determined as the plateau level of the progress curves obtained (Fig. 1; solid lines). This simple procedure seems to be adequate because eqn. (22) was well fitted to the data by using the values of α obtained thereby (dashed lines).

Fig. 2 shows the dependence of α on the substrate concentration, S . The reciprocals of the values of α obtained by the above procedure were plotted against S . Table 2 gives the values of k_{+3} and k_{+4} estimated by fitting eqn. (13) to the results. Both the rate constants were significantly larger than zero and

Table 2. Rate constants for interaction of okadaic acid with PP2A

The rate constants obtained by the time-course analyses are listed with the kinetic constants. The values are given with the 95% confidence limits (in parentheses).

Kinetic constant	Value
k_{+3}	$2.4 (2.1-2.8) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$
k_{+4}	$7.3 (5.3-9.2) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$
k_{-3}	$9.4 (7.1-11.8) \times 10^{-4} \text{ s}^{-1}$
k_{-4}	$2.5 (1.7-3.2) \times 10^{-4} \text{ s}^{-1}$
K_m	$5.3 (4.3-6.2) \text{ mM}$
$V_{\max}$	$54 (50-60) \text{ mol} \cdot \text{s}^{-1} \cdot (\text{mol of PP2A})^{-1}$

$k_{+3} > k_{+4}$, indicating that the inhibition is either a non-competitive type or a mixed type [cf. eqns. (13)–(16)]. In Fig. 2, the graphs of eqn. (13) for $k_{+4} = 0$ (competitive inhibition) and $k_{+3} = k_{+4}$ are also shown for comparison (dashed lines) [see eqns. (14) and (15)].

Time-course of recovery of the activity after application of the monoclonal antibody against okadaic acid

As shown in Fig. 3, the pNPP phosphatase activity of PP2A suppressed by okadaic acid was recovered by application of the monoclonal antibody (IgG1) in a highly time-dependent manner. The reaction was started by addition of the substrate (1–20 mM) to the buffer containing PP2A (2 nM) and okadaic acid (2 nM). The monoclonal antibody (about 2 μM) was added after the steady-state velocity in the presence of okadaic acid [$= v_0 x(0)$] had been measured. The time-course of the resulting recovery of the activity was very slow. The fractional activity x was restored only to 0.3–0.5 even 600 s after application of the antibody. This is not because the amount of the antibody added was too small to sequester okadaic acid in the reaction mixture. Okadaic acid exerted very little inhibitory effect on the phosphatase activity when it was applied after the reaction was started in the presence of the same amount of the antibody (results not shown).

Usagawa *et al.* (1989) have shown by enzyme immunoassay that the value of the dissociation constant for the interaction of

the antibody with okadaic acid is of the order of 100 pM. Our dose-inhibition analyses with okadaic acid and PP2A have shown that the value of K_i is about 30 pM, which remains more or less the same over a relatively wide range of substrate concentration (Takai & Mieskes, 1991). On the basis of these previous results, the value of $(I_i/K_i)/(A/K_m)$ under the present experimental conditions is estimated at 10^{-2} or less. Therefore, the inequality (33), a sufficient condition under which the approximate differential equation (29) can be used, seems to be fulfilled. Indeed, the time-course of recovery observed with the different substrate concentrations appeared to be well fitted by eqn. (31) which is derived from eqn. (29) (see Fig. 3). For substrate concentrations of 1, 5, 10 and 20 mM, the values of β were estimated, as the rate constants of the recovery process, to be $9.5 (\pm 0.9) \times 10^{-4}$, $5.8 (\pm 1.2) \times 10^{-4}$, $4.4 (\pm 0.8) \times 10^{-4}$ and $3.9 (\pm 0.5) \times 10^{-4} \text{ s}^{-1}$ ($n = 3-4$) respectively. The rate constants k_{-3} and k_{-4} obtained by fitting eqn. (28) to the data are listed in Table 2.

DISCUSSION

In the present time-course analyses, the rate constants for the binding of okadaic acid to PP2A (k_{+3} and k_{+4} ; Table 2) have been estimated to be of the order of $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. Such values are typical for reactions involving a relatively large molecule. For example, the rate constants for the binding of an enzyme and its substrate lie mostly in the range $10^6-10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (see e.g. Mahler & Cordes, 1969). On the other hand, the rate constants for dissociation of okadaic acid from PP2A (k_{-3} and k_{-4}) have very low values which fall in the range $10^{-3}-10^{-4} \text{ s}^{-1}$. The exceedingly high affinity of okadaic acid for PP2A seems to be attributed to very slow dissociation rather than to very fast binding.

The values of k_{+3} and k_{+4} are both significantly larger than zero, indicating that the inhibitor interacts with both the free enzyme and the enzyme-substrate complex. Therefore, the mode of the inhibition is unlikely to be competitive or uncompetitive (see the Theory section). The values of K_0 and K_1 calculated as the ratios of the rate constants (see eqns. 1 and 2) fall in the range 30–40 pM. This is in agreement with the results of our previous dose-inhibition study (Takai & Mieskes, 1991), which have

Fig. 3. Time-course of recovery of the PP2A activity suppressed with okadaic acid by application of the monoclonal antibody

PP2A (2 nM) was first incubated with okadaic acid (2 nM), and the pNPP (1 mM or 20 mM) was added to start the reaction (not shown). The monoclonal antibody (IgG1; 320 $\mu\text{g}/\text{ml} \div 2 \mu\text{M}$) was applied ($t = 0$) after the basal activity in the presence of okadaic acid [$= v_0 x(0)$] had been recorded. The basal linear component, $v_0 x(0)t$, has been subtracted from $P(t)$. The time-course of recovery of the activity after application of the antibody was well fitted by eqn. (30) (dashed lines). See the text for further explanation.

shown that the dissociation constant K_i for interaction of okadaic acid and PP2A is of the order of 30 μM and is not substantially changed over a relatively wide range of substrate concentrations [cf. eqn. (9)]. Given the level of the experimental errors of the rate constants obtained in the present experiments, however, we cannot exclude the possibility that there may be a small difference between K_0 and K_i . Further experiments are necessary to establish whether okadaic acid acts as a non-competitive inhibitor or a mixed inhibitor.

Using the value of K_i (30 μM) estimated from previous dose-inhibition studies (Takai & Mieskes, 1991; Takai *et al.*, 1992), which is supported by the present time-course analyses, the standard free-energy change for the binding of okadaic acid and PP2A is estimated by the equation $\Delta G^\circ = RT \ln K_i$ (where K_i is measured in mol/l) at -60 kJ/mol. Since the standard free-energy change of formation of single hydrogen bonds or electrostatic bonds is in the range -10 to -30 kJ/mol (see e.g. Rawn, 1989), it appears that there are more than one intermolecular interactions in the binding of okadaic acid and PP2A. The fact that the values of k_{+3} and k_{+4} do not approach $10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, the maximal value estimated for a purely diffusion-controlled process, may be interpreted in terms of a time-requiring fit in the interaction sites, which involves the process of orientation of the inhibitor on the enzyme surface and conformational changes of the enzyme.

Calyculin A (Ishihara *et al.*, 1989) and microcystin-LR (MacKintosh *et al.*, 1990) have been reported to be strong inhibitors of protein phosphatases. The published dose-inhibition relationships for these toxins indicate that they also belong to the category of tightly binding inhibitors, for which kinetic analysis based on the Michaelis-Menten equation is not applicable. It should be pointed out that the method described in this paper can be applied in principle to any tightly binding inhibitor which reacts with an enzyme in a time-dependent manner if a strong and specific sequester of the inhibitor is available. For the purpose of applying the method to calyculin A or microcystin-LR, some types of antibody against them may be useful as the inhibitor-sequestering agents required.

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REFERENCES

Bialojan, C. & Takai, A. (1988) *Biochem. J.* **256**, 283–290

- Cayla, X., Goris, J., Hermann, J., Jessus, C., Hendrix, P. & Merlevede, W. (1990) *Adv. Enzyme Regul.* **30**, 265–285
- Cha, S. (1975) *Biochem. Pharmacol.* **24**, 2177–2185
- Cha, S. (1976) *Biochem. Pharmacol.* **25**, 2695–2702
- Cha, S., Agarwal, R. P. & Parks, R. E. (1975) *Biochem. Pharmacol.* **24**, 2187–2197
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 173–187
- Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453–508
- Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102
- Cornish-Bowden, A., Porter, W. R. & Trager, W. F. (1978) *J. Theor. Biol.* **74**, 163–175
- Dixon, M. & Webb, E. C. (1979a) *The Enzymes*, 3rd edn., pp. 65–67, Longman Group, London.
- Dixon, M. & Webb, E. C. (1979b) *The Enzymes*, 3rd edn., pp. 339–341, Longman Group, London
- Goris, J., Hermann, J., Hendrix, P., Ozon, R. & Merlevede, W. (1989) *FEBS Lett.* **245**, 91–94
- Haystead, A. J., Sim, A. T. R., Carling, R. C., Honnor, R. C., Tsukitani, Y., Cohen, P. & Hardie, D. G. (1989) *Nature (London)* **337**, 78–81
- Henderson, P. (1972) *Biochem. J.* **127**, 321–333
- Hescheler, J., Mieskes, G., Rüegg, J. C., Takai, A. & Trautwein, W. (1988) *Pflügers Arch.* **412**, 248–252
- Ishihara, H., Martin, B. L., Brautigan, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. & Hartshorne, D. J. (1989) *Biochem. Biophys. Res. Commun.* **159**, 871–877
- Kendall, M. G. (1970) *Rank Correlation Methods*, 4th edn., pp. 50–52, Griffin, London
- MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. & Codd, G. A. (1990) *FEBS Lett.* **264**, 187–192
- Mahler, H. R. & Cordes, E. H. (1969) *Biological Chemistry*, 2nd edn., pp. 273–276, Harper & Row Publishers, New York
- Porter, W. R. & Trager, W. F. (1977) *Biochem. J.* **161**, 293–302
- Rawn, J. D. (1989) *Biochemistry*, pp. 106–108, Neil Patterson Publishers, Burlington, NC
- Snedecor, G. W. & Cochran, W. G. (1980a) *Statistical Methods*, 7th edn., pp. 334–343, Iowa State University Press, Ames
- Snedecor, G. W. & Cochran, W. G. (1980b) *Statistical Methods*, 7th edn., pp. 407–409, Iowa State University Press, Ames
- Tachibana, K., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., van Eugen, D., Clardy, J., Gopichand, Y. & Schmitz, F. J. (1981) *J. Am. Chem. Soc.* **103**, 2469–2471
- Takai, A. (1988) *J. Musc. Res. Cell. Motility* **9**, 563–565
- Takai, A. & Mieskes, G. (1991) *Biochem. J.* **275**, 233–239
- Takai, A., Murata, M., Torigoe, K., Isobe, M., Mieskes, G. & Yasumoto, T. (1992) *Biochem. J.* **284**, 539–544
- Tenenbaum, M. & Pollard, H. (1963) *Ordinary Differential Equations*, pp. 97–98, Harper & Row Publishers, New York
- Tung, H. Y. L., Resink, T. J., Hemmings, B., Shenolikar, S. & Cohen, P. (1984) *Eur. J. Biochem.* **138**, 635–641
- Usagawa, T., Nishimura, M., Itoh, Y., Uda, T. & Yasumoto, T. (1989) *Toxicol.* **27**, 1323–1330
- Williams, J. W. & Morrison, J. F. (1979) *Methods Enzymol.* **63**, 437–467
- Williams, J. W., Morrison, J. F. & Duggleby, R. D. (1980) *Biochemistry* **18**, 2567–2573
- Yasumoto, T. (1990) in *Toxic Marine Phytoplankton* (Graneli, E., Sundström, D., Edler, L. & Anderson, D. M., eds.), pp. 3–8, Elsevier, New York
- Yasumoto, T., Seino, N., Murakami, Y. & Murata, M. (1987) *Biol. Bull.* **172**, 128–131

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