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Inhibitory effect of a toxin okadaic acid, isolated from the black sponge on smooth muscle and platelets

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1 Effects of okadaic acid, a toxin isolated from marine sponges, on smooth muscle contraction and platelet activation were examined.

2 Contractions in rabbit aorta induced by high concentrations of K^+ and noradrenaline were inhibited by 0.1–1 μM okadaic acid in a concentration-dependent manner. Spontaneous rhythmic contractions as well as high K^+-induced contraction in guinea-pig taenia caeci were also inhibited by 1 μM okadaic acid.

3 High K^+-induced contraction in rabbit aorta was accompanied by increased Ca^{2+} influx measured with $^{45}Ca^{2+}$ and increased cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) measured with fura-2-Ca^{2+} fluorescence. Okadaic acid inhibited the contraction without inhibiting Ca^{2+} influx and produced only a small decrease in $[Ca^{2+}]_{cyt}$.

4 In a saponin-skinned taenia, Ca^{2+}-induced contraction was not inhibited but rather potentiated by okadaic acid.

5 Okadaic acid, 1 μM, inhibited aggregation, ATP release and increase in $[Ca^{2+}]_{cyt}$ induced by thrombin in washed rabbit platelets. Okadaic acid itself did not change the platelet activities.

6 Okadaic acid did not change the cyclic AMP content of rabbit aorta although the inhibitory effects of okadaic acid were similar to those of cyclic AMP.

7 Although the mechanism of the inhibitory effect of okadaic acid was not clarified in the present experiments, it is suggested that okadaic acid acts by inhibiting protein phosphatases resulting in an indirect activation of cyclic AMP-dependent protein phosphorylation.

Introduction

Okadaic acid is a polyether monocarboxylic acid toxin (*Mr* 802) isolated from marine sponges of the genera *Halichondria* (Tachibana *et al.*, 1981) and *Pandaros* (Schmitz *et al.*, 1981). Okadaic acid induces a contraction even in the absence of external Ca^{2+} in intact (Shibata *et al.*, 1982) and also in saponin-skinned smooth muscles (Ozaki *et al.*, 1987a). Ozaki *et al.* (1987b) have shown that okadaic acid induces phosphorylation of myosin light chain in the absence of Ca^{2+} and in the presence of calmodulin inhibitors, suggesting that the contractile effect of okadaic acid is mediated by the activation of Ca^{2+}-independent protein kinase. Okadaic acid also inhibits protein phosphatase activity (Bialojan & Takai, 1988; Ishihara *et al.*, 1989). In the present experiments, we show that okadaic acid, at concentrations lower than that needed to induce smooth muscle contraction, has an inhibitory effect on smooth muscle contraction and platelet activation.

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Methods

Smooth muscle

Thoracic aorta was isolated from male New Zealand White rabbits weighing about 3 kg and muscle strips, 2–3 mm wide and 5–6 mm long, were prepared. The adventitial layer was separated from the medial layer by gently pulling apart these two layers as described by Karaki & Urakawa (1979). Endothelium was removed by gently rubbing the intimal surface with a

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moistened finger. Taenia strips, approximately 10 mm long, were isolated from the caecum of male, white guinea-pigs weighing 300–400 g. Each strip was attached to a holder under a resting tension of 1 g for aorta and 0.2 g for taenia and equilibrated for 60–90 min in a 10 ml muscle bath. Normal physiological salt solution (pH 7.4, 37°C) contained (mM); NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5. High K^+ solution was made by increasing the concentration of KCl or by substituting NaCl with equimolar KCl. Ca^{2+}-deficient solution was made by omitting $CaCl_2$ and adding 0.5 mM ethyleneglycol *bis* (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA). These solutions were aerated with 95% O_2 and 5% CO_2 mixture. Muscle tension was recorded isometrically with a force displacement transducer.

$[Ca^{2+}]_{cyt}$ was measured simultaneously with muscle contraction in fura-2-loaded rabbit aorta as reported by Ozaki *et al.* (1987c) and Sato *et al.* (1988). Muscle strips were loaded with 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) (Grynkiewicz *et al.*, 1985) for 3 h in the presence of 0.02% cremophor EL at room temperature (23–25°C) and then placed in a tissue bath at 37°C. The muscle strip was illuminated alternately (48 Hz) with 340 nm and 380 nm light, and emission at 500 nm was detected with a spectrophotometer (CAF-100, Japan Spectroscopic Co., Tokyo). At the end of the experiment, the maximum and minimum fluorescence levels were determined using 5 μM ionomycin and 4 mM EGTA, respectively, as reported previously (Ozaki *et al.*, 1987c; Sato *et al.*, 1988). However, the absolute value for $[Ca^{2+}]_{cyt}$ was not calculated because the dissociation constant for fura-2 and Ca^{2+} in cytoplasm may be different from that obtained *in vitro* (Konishi *et al.*, 1988). Instead, the ratio of these two fluorescence ($R_{340/380}$) was used as an indicator of relative $[Ca^{2+}]_{cyt}$ (Ozaki *et al.*, 1987c; Sato *et al.*, 1988).

To prepare the saponin-skinned taenia preparation, a thin bundle of taenia (0.2 mm in width and 1.5 mm in length) was treated with 100 $\mu g\,ml^{-1}$ saponin for 30–40 min in a solution containing (mM): KCl 136.9, $MgCl_2$ 5.0, EGTA 2.0, Tris maleate 20.0 (pH 6.8) and Na_2ATP 5, at 24°C. The Ca^{2+} concentration of this solution was changed by use of Ca^{2+}-EGTA buffers.

$^{45}Ca^{2+}$ uptake was measured as described by Karaki & Weiss (1979). Rabbit aortic strips were incubated in a solution containing $^{45}Ca^{2+}$ (1 $\mu Ci\,ml^{-1}$) for 5 min. Okadaic acid was added 60 min before the addition of $^{45}Ca^{2+}$, and 65.4 mM K^+ was added simultaneously with $^{45}Ca^{2+}$. After the $^{45}Ca^{2+}$ incubation, muscle strips were washed for 30 min with an ice-cold lanthanum solution containing 73.8 mM $LaCl_3$, 5.5 mM glucose and 24 mM Tris adjusted to pH 6.9 at 0°C. $^{45}Ca^{2+}$ in the strips was extracted overnight with 1 ml of 20 mM EGTA solution. Scintillation mixture (ACS II, Amersham Japan, Tokyo, 1 ml) was added to the extract and radioactivity was counted with a liquid scintillation spectrometer (Packard Tri-Carb 3380, U.S.A.).

The adenosine 3' : 5'-cyclic monophosphate (cyclic AMP) content of rabbit aorta was measured as described previously (Abe & Karaki, 1988). After a 60 min incubation with 1 μM okadaic acid, muscle strips were removed from the bath, rapidly frozen in liquid N_2 and homogenized for 30 s in 6% trichloroacetic acid. Cyclic AMP in the extract was measured by radioimmunoassay using a commercial assay kit (Yamasa Shoyu, Tokyo).

Platelets

Fresh blood was obtained from male rabbits and mixed immediately with 1/10 volume of trisodium citrate solution (3.8 w/v%). The citrated blood was centrifuged at 90 *g* for 15 min to obtain platelet-rich plasma (PRP). PRP was then washed by centrifugation at 800–900 *g* for 10 min and the supernatant was removed. The pellet was resuspended in normal solution. This washing procedure was repeated three times. Normal solution contained (mM): NaCl 145, KCl 5, $MgSO_4$ 1.0, NaH_2PO_4 0.5, glucose 5.0, EDTA 0.001 and HEPES 10.0 adjusted to pH 7.4 with NaOH. The platelet suspension was restored to a final density of approximately 3×10^5 cells ml^{-1}. Aggregation was assessed by a standard turbidimetric technique using a spectrophotometer equipped with a stirrer (UVDEC-460, Japan Spectroscopic Co., Tokyo). Three min after the addition of 1.0 mM $CaCl_2$ at 37°C, thrombin was applied for 5 min and aggregation was measured. Okadaic acid was added simultaneously with $CaCl_2$. At the end of the incubation, the amount of ATP released in the external medium was measured with a luciferin–luciferase method (Strehler & McElroy, 1957) using a photomultiplier (TD-4000, Lab-Science, Tokyo).

Ca^{2+} signals in platelets were measured with fura-2. The PRP was centrifuged and the pellet was resuspended in a normal solution containing 1 μM fura-2/AM and incubated for 10 min at 37°C. Platelets were then washed twice with a normal solution to remove extracellular fura-2/AM. Changes in fura-2-Ca^{2+} fluorescence were measured with a spectrofluorometer (FP-2060, Japan Spectroscopic). Platelets were excited at a wavelength of 340 nm and emission was measured at 500 nm. At the end of the experiments, the maximum and minimum fluorescence levels were determined using 0.2% Triton X-100 and 10 mM EGTA at pH 8.5, respectively, and the $[Ca^{2+}]_{cyt}$ was calculated as reported previously (Pollock *et al.*, 1986). However, we used only relative $[Ca^{2+}]_{cyt}$ in the present results because of the uncer-

tainty of the intracellular dissociation constant of fura-2 for Ca^{2+} (Konishi *et al.*, 1988).

Statistics

Experiments were repeated on at least 4 preparations obtained from 4 different animals. Results of the experiments are expressed as mean ± s.e.mean. Student's *t* test was used for statistical analysis of the results.

Drugs and chemicals

Okadaic acid was isolated from *Halichondria okadai* as described by Tachibana *et al.* (1981) and dissolved in 100% ethanol. (±)-Noradrenaline bitartrate (Wako Pure Chemicals, Tokyo, Japan), thrombin (Sigma, St. Louis, MO, U.S.A.), ionomycin and forskolin (Calbiochem, San Diego, CA, U.S.A.), Tris (Sigma), EGTA (Wako), saponin (ICN, Cleveland, OH, U.S.A.), cremophor EL (Nakarai Chemicals, Kyoto, Japan), fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) and $^{45}CaCl_2$ (Amersham Japan, Tokyo) were used.

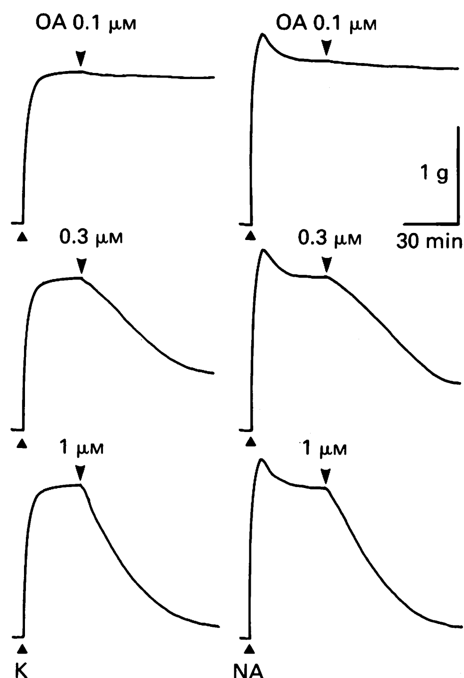

Figure 1 Inhibitory effect of 0.1–1 μM okadaic acid (OA) on the sustained contractions induced by 65.4 mM KCl (K, left) or 1 μM noradrenaline (NA, right) in rabbit aorta.

Results

Smooth muscle contraction

Addition of 0.1–1 μM okadaic acid did not change the resting tone whereas 100 μM okadaic acid induced a sustained contraction in rabbit aorta as has been reported (Shibata *et al.*, 1982). As shown in Figure 1, addition of 0.1 to 1 μM okadaic acid during the high K^+- or noradrenaline-induced sustained contraction slowly decreased muscle tension. The cumulative concentration-response curves for KCl and noradrenaline in rabbit aorta were shifted to the right and downwards in the presence of 0.1–1 μM okadaic acid (Figure 2a and b). In a Ca^{2+}-free, high K^+ solution, cumulative addition of $CaCl_2$ induced a concentration-dependent contraction. This contraction was also inhibited by okadaic acid (Figure 2c).

In a Ca^{2+}-free solution, noradrenaline induced a transient contraction which is due to release of intracellular Ca^{2+} (Karaki, 1987). The peak tension level of the noradrenaline-induced transient contraction was 65.2 ± 5.4% ($n = 4$) of that in the presence of 1.5 mM Ca^{2+}. After a 120 min resting period in normal solution, the second application of noradrenaline in a Ca^{2+}-free solution induced a transient contraction with similar magnitude as that of the first transient contraction (68.8 ± 4.8%, $n = 4$). In the aorta pretreated with 1 μM okadaic acid for 60 min, the peak tension level decreased to 9.0 ± 3.3% ($n = 6$, $P < 0.01$), as shown in Figure 3.

In guinea-pig taenia, addition of 1 μM okadaic acid inhibited the spontaneous rhythmic contractions. The high K^+ (45.4 mM)-induced contraction was also inhibited by 1 μM okadaic acid to 5.2 ± 3.1% of the contraction before the addition of okadaic acid ($n = 6$, $P < 0.01$) (Figure 4).

In saponin-skinned taenia, addition of 0.1–10 μM okadaic acid during the sustained contraction induced by 3 μM Ca^{2+} did not change the muscle tension but rather potentiated it, as reported previously (Ozaki *et al.*, 1987a).

The effects of okadaic acid on contraction and $[Ca^{2+}]_{cyt}$ in rabbit aorta are shown in Figure 5. High K^+ (27.8 mM) induced a sustained increase in both muscle tension and $[Ca^{2+}]_{cyt}$. Okadaic acid (1 μM) gradually and strongly inhibited the high K^+-induced contraction. The peak tension level of the high K^+-induced contraction measured 18 min after the addition of 1 μM okadaic acid was 18.2 ± 5.3% ($n = 4$) of that before the addition of okadaic acid. However, okadaic acid inhibited the high K^+-stimulated $[Ca^{2+}]_{cyt}$ only to 77.1 ± 5.3% ($n = 4$).

In rabbit aorta, high K^+ increased $^{45}Ca^{2+}$ uptake

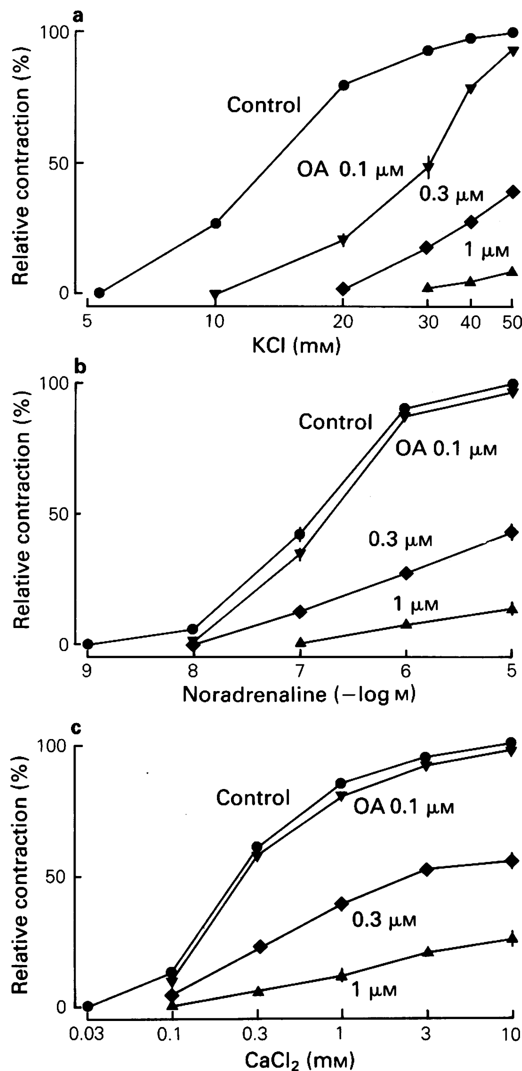

Figure 2 Cumulative concentration-response curves for KCl (a), noradrenaline (b) or $CaCl_2$ (c) in the absence or presence of okadaic acid in rabbit aorta. In (c), muscle strips were pretreated for 15 min with a Ca^{2+}-free, 55.4 mM KCl solution. Okadaic acid (OA; 0.1, 0.3 or 1 μM) was added 60 min before the cumulative addition of the stimulants. Each curve represents mean of 6 experiments and s.e.mean is shown by vertical lines.

(during 5 min) from a resting level of $78.7 \pm 2.4\,\text{nmol g}^{-1}$ ($n = 6$) to $144.7 \pm 6.6\,\text{nmol g}^{-1}$ ($n = 6$, $P < 0.01$). Okadaic acid (1 μM) changed neither the resting $^{45}Ca^{2+}$ uptake ($82.7 \pm 7.0\,\text{nmol g}^{-1}$, $n = 6$) nor the high K^+-stimulated $^{45}Ca^{2+}$ uptake ($154.3 \pm 4.3\,\text{nmol g}^{-1}$, $n = 6$).

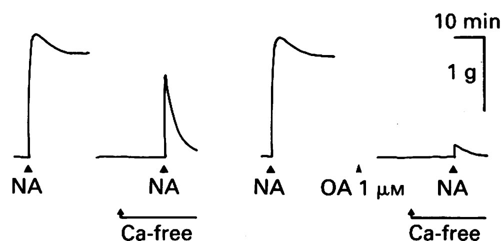

Figure 3 Inhibitory effect of 1 μM okadaic acid (OA) on 1 μM noradrenaline (NA)-induced transient contraction in the absence of external Ca^{2+} in rabbit aorta. Okadaic acid was added 60 min before the addition of noradrenaline and Ca^{2+} was removed 15 min before the addition of noradrenaline.

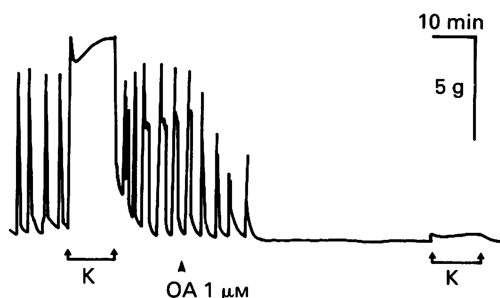

Figure 4 Inhibitory effect of 1 μM okadaic acid (OA) on guinea-pig taenia. K: 45.4 mM KCl.

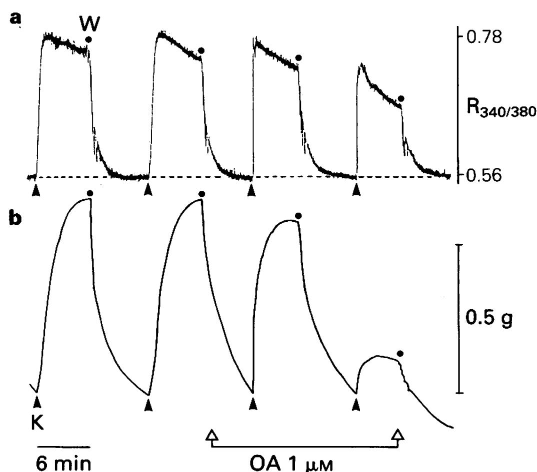

Figure 5 Inhibitory effect of 1 μM okadaic acid (OA) on relative $[Ca^{2+}]_{cyt}$ (a; shown by $R_{340/380}$, see Methods) and contraction (b) induced by 27.8 mM KCl (K) in rabbit aorta. High K^+ was applied at ▲ and removed at ●.

The cyclic AMP content of rabbit aorta was $121.9 \pm 16.0\,\mathrm{pmol\,g^{-1}}$ wet weight ($n = 6$). After a 60 min incubation with 1 μM okadaic acid, this value did not change significantly (105.3 ± 7.2, $n = 6$, $P > 0.05$) although 3 μM forskolin, which almost completely inhibited the 1 μM noradrenaline-induced contraction, increased the cyclic AMP content to $459.1 \pm 69.1\,\mathrm{pmol\,g^{-1}}$ wet weight ($n = 6$, $P < 0.01$).

Platelets

Figure 6 shows the effects of okadaic acid on platelet aggregation and $[Ca^{2+}]_{cyt}$. Thrombin, $1\,\mathrm{u\,ml^{-1}}$, induced aggregation and increased $[Ca^{2+}]_{cyt}$. Okadaic acid (1 μM) itself did not change the platelet activities but strongly inhibited both the thrombin-induced aggregation (to $8.1 \pm 1.4\%$, $n = 6$) and $[Ca^{2+}]_{cyt}$ (to $17.2 \pm 2.2\%$, $n = 6$). Okadaic acid (1 μM) also strongly inhibited the thrombin-induced ATP-release (to $20.7 \pm 7.4\%$, $n = 7$). A lower concentration (0.1 μM) of okadaic acid was almost ineffective.

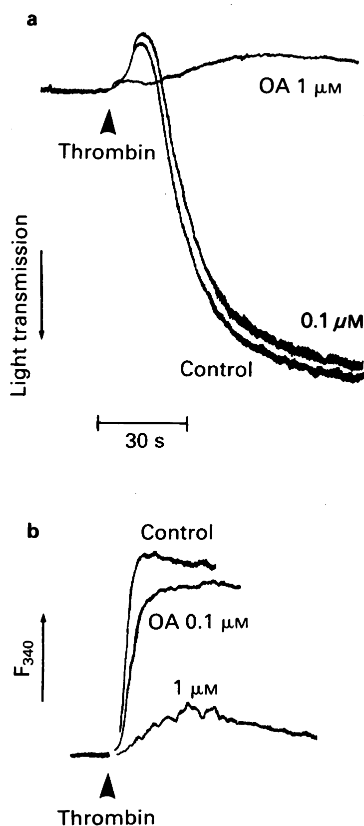

Figure 6 Inhibitory effect of okadaic acid (OA, 0.1 or 1 μM) on aggregation (a, shown by light transmission) and $[Ca^{2+}]_{cyt}$ (b, shown by F_{340}, see Methods) in rabbit platelets stimulated by thrombin ($1\,\mathrm{u\,ml^{-1}}$).

Discussion

Present results showed that 0.1–1 μM okadaic acid inhibited contractions in intact vascular and intestinal smooth muscles. However, the same concentration of okadaic acid did not inhibit the Ca^{2+}-induced contraction in skinned smooth muscle (Ozaki *et al.*, 1987a). Previously, it was shown that higher concentrations of okadaic acid (10 μM or higher) induced contraction in smooth muscle of rabbit aorta and guinea-pig taenia (Shibata *et al.*, 1982) which was attributable to the Ca^{2+}-independent phosphorylation of myosin light chain (Ozaki *et al.*, 1987b). These results indicate that okadaic acid has a dual effect on smooth muscle contraction that is dependent on the concentration of okadaic acid; lower concentrations inhibit contractions and this is not attributable to a direct effect on the contracile elements, whereas higher concentrations directly activate the contracile elements.

Contraction of smooth muscle is mediated by an increase in $[Ca^{2+}]_{cyt}$ followed by the activation of the Ca^{2+}/calmodulin-myosin light chain kinase system. This enzyme phosphorylates myosin light chain to induce contraction. When $[Ca^{2+}]_{cyt}$ is decreased, kinase activity is decreased and the phosphorylated myosin light chain is dephosphorylated by a phosphatase resulting in relaxation of smooth muscle (Hartshorne & Mrwa, 1982). Ca^{2+} is supplied by influx of extracellular Ca^{2+} by the opening of Ca^{2+} channels or by the release of Ca^{2+} from storage sites (Karaki & Weiss, 1988). The inhibitory effect of okadaic acid was accompanied by a small decrease in $[Ca^{2+}]_{cyt}$ but not by a decrease in $^{45}Ca^{2+}$ uptake, indicating that the decrease in $[Ca^{2+}]_{cyt}$ is only partially responsible for the inhibition.

A possible mechanism for the okadaic acid-induced inhibition is the decrease in Ca^{2+}-sensitivity of the contracile elements. It has been shown that cyclic AMP-dependent protein kinase phosphorylates myosin light chain kinase, inhibits the activity of this enzyme and inhibits smooth muscle contractions (Pfitzer *et al.*, 1985). In vascular smooth muscle, forskolin, an activator of adenylate cyclase, inhibits contraction with a small decrease in $[Ca^{2+}]_{cyt}$ (Abe & Karaki, 1989) and no change in $^{45}Ca^{2+}$ uptake (Hwang & Van Breemen, 1987). These effects are quite similar to those of 1 μM okadaic acid. However, okadaic acid does not seem to activate directly adenylate cyclase nor inhibit phosphodiesterase because the concentration of cyclic AMP was not altered by the okadaic acid

treatment. Recently, it was found that okadaic acid inhibits various phosphatases at different concentrations (Bialojan & Takai, 1988; Ishihara *et al.*, 1989). It is possible that okadaic acid inhibits the phosphatase which dephosphorylates the cyclic AMP-dependent phosphorylation and thus augments the cyclic AMP-dependent phosphorylation. Since cyclic GMP has similar inhibitory effects to cyclic AMP on smooth muscle contraction and $[Ca^{2+}]_{cyt}$ (Karaki *et al.*, 1988) and myosin light chain phosphorylation (Pfitzer *et al.*, 1986), okadaic acid may also augment cyclic GMP-dependent phosphorylation by inhibiting the phosphatase activity.

Activation of platelets also involves Ca^{2+}-dependent pathways: the Ca^{2+} and calmodulin-dependent myosin light chain and protein kinase C (Feinstein *et al.*, 1985). Okadaic acid inhibited the thrombin-induced aggregation, ATP release and the increase in cellular Ca^{2+} induced by thrombin. These effects are again similar to those of cyclic AMP (Feinstein *et al.*, 1985) suggesting the participation of similar mechanisms as those in smooth muscle. Further experiments are necessary to know the precise mechanism of the inhibitory action of okadaic acid.

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