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Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin

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Smooth muscle contraction depends on the state of myosin phosphorylation and hence on the balance of myosin light chain kinase and phosphatase activity. Effects of okadaic acid isolated from black sponge on both enzyme activities and contractility were studied in chemically skinned fibers from guinea pig taenia coli. The toxin strongly inhibits myosin phosphatase and enhances tension development.

Okadaic acid; Myosin dephosphorylation; Muscle contractility; (Skinned fiber)

1. INTRODUCTION

Okadaic acid (OA), a toxin isolated from black sponges [1], has marked contractile effects on the vascular and intestinal smooth muscles [2] and the heart muscle [3]. OA increases the slow inward current in the heart muscle [3] whereas it does not affect the ATPase activities of the Na^+-K^+ pump and smooth muscle myosin [2]. Recently, it was reported that OA enhanced the contraction of guinea pig taenia coli fibers skinned with Triton X-100 at low levels of Ca^{2+} [4]. These findings strongly indicate that the effect of OA on smooth muscle tissue are produced by its direct interaction with intracellular regulatory mechanisms that activate contraction via phosphorylation of myosin light chains. As the state of phosphorylation depends on the balance of activities of calmodulin-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase, we have examined the effect of OA on these enzymic activities as well as on tension development of skinned fibers of guinea pig taenia coli. We report that OA has a specific inhibitory action on myosin phosphatase thereby abolishing its activity at micromolar concentrations.

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2. MATERIALS AND METHODS

Guinea pig taenia coli was skinned in a 1% Triton X-100 solution and stored in a glycerol solution [5]. The skinned fibers were used within five days after skinning either for mechanical experiments or for enzyme assays.

Both solutions for mechanical experiments and the reaction mixture for enzyme analyses contained (in mM): imidazole (30), EGTA (4), KH_2PO_4 (6) and dithioerythritol (DTE; 2); adjusted to pH 6.7 with KOH. The free Ca^{2+} concentration was varied by changing the ratio of EGTA to Ca-EGTA, assuming the apparent dissociation constant of 1.6 μM [6]. Except for the measurement of protein phosphatase activity, the solution also contained 0.2 μM calmodulin. For mechanical experiments, 7.5 mM adenosine 5'-triphosphate (ATP), 10 mM creatine phosphate and 50 U/ml creatine kinase were supplemented. In enzyme analyses, 1 mg/ml bovine serum albumin (BSA) was added to the reaction mixture, and tempera-

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ture was maintained at 30°C. The mechanical experiments were performed at room temperature (about 23°C). OA was dissolved in dimethylsulfoxide (DMSO) and added to the solutions. The same amount of DMSO (0.1–0.5% vol.) was added to the control solutions, too. The mechanical response was not altered when ethanol was used instead of DMSO. The isometric tension was recorded using an AME 801 force transducer (SensoNor, Horten, Norway) [7].

In the enzyme analyses, the fiber was homogenized in a solution (5 ml/g) containing (in mM): imidazole (30), DTE (2), phenylmethylsulfonyl fluoride (PMSF; 0.1) and KCl (20). The homogenate was centrifuged for 15 min at 17 500 × g, and resulting supernatant was used for the enzyme analyses of protein phosphatase and MLCK. Bovine aortic myosin phosphatase was extracted and purified as described [8].

Protein phosphatase activity was measured by a modification of the isotope method [9], using isolated bovine heart light chains [7] which had been ^{32}P-phosphorylated [10] by chicken gizzard MLCK [11] as substrate. MLCK activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the bovine heart light chains [11]. Calcium/calmodulin-dependent phosphodiesterase (PDE) activity was measured by modification of the method described for calmodulin measurement [12]. Briefly, adenosine 3′,5′-cyclic monophosphate (cAMP) was degraded to inosine with 3 mU/ml PDE, 30 U/ml alkaline phosphatase (AP) and 4 U/ml adenosine deaminase (ADA) in the presence of 0.2 μM calmodulin and various concentrations of calcium, and the rate of decrease in absorbance was measured at 265 nm. Protein was determined by the Lowry method [13] using BSA as the standard.

Numerical data were presented as means ± SE, and the difference was evaluated by the Student's t-test. Concentration-response relations were fitted by the Hill function with the least-square method, and the difference was examined by a method of covariance analysis [14]. In both cases, probabilities smaller than 0.05 were evaluated as significant.

OA, isolated from the black sponge *Halichondria okadai*, was a generous gift from Dr Y. Tsukitani (Fujisawa Pharmaceutical Company, Tokyo, Japan). Calmodulin (from porcine brain), PDE (from bovine heart), AP and ADA were obtained from Boehringer Mannheim, FRG.

3. RESULTS

3.1. *Mechanical effect*

It was confirmed that OA has a contractile effect on the chemically skinned taenia coli fiber (fig.1). The fiber was first immersed in calcium-free solution (Ca^{2+} < 1 nM) and then maximally contracted by applying a solution containing 30 μM Ca^{2+} and 0.2 μM calmodulin. When a steady level was approached, Ca^{2+} concentration was reduced to 1.5 μM to induce a partial relaxation, and when a plateau was reached, 5 μM OA were added. OA produced an immediate increase in tension, which was completely reversed when OA was washed out. No response was observed when OA was applied to the fiber relaxed in calcium-free solution (not shown).

Fig.2 shows the dose-response relationship of the effect. The response was normalized to the maximal contraction produced by 30 μM Ca^{2+} (control 0.6 ± 0.1 mN (n = 22)). The data are presented as percentages of the response with 10 μM OA which was 80 ± 6% of the control (n = 5). From this curve the concentration for 50% response (ED_{50}) of 0.67 μM OA was obtained.

3.2. *Inhibition of protein phosphatase*

As shown in fig.3, OA produced a dose-dependent inhibition of the phosphatase activity. The measurement was carried out in calcium-free

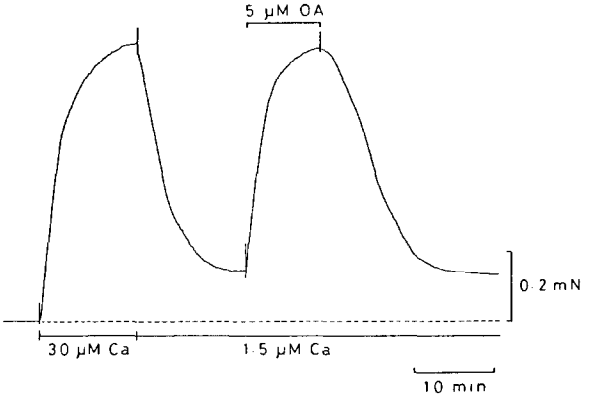

Fig.1. Mechanical effect of okadaic acid (OA) on the Triton X-100 skinned fiber. See text for explanation.

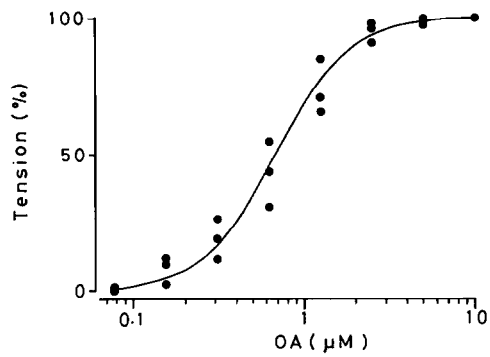

Fig.2. Dose-response relationship of mechanical response. Data were obtained by the same type of experiments as in fig.1. Various concentrations of okadaic acid (OA) were applied at the constant Ca^{2+} concentration of 1.5 μM.

solution in order to suppress the MLCK activity (see below). The specific activity was 1.42 ± 0.07 nmol P/min per mg protein (n = 6) when measured using 4 μM ^{32}P-labelled light chains as substrate (= 100% activity), and this was almost completely inhibited by 5–10 μM OA. The dose response was well fitted by the theoretical function with a Hill coefficient of 0.88, and from this the concentration for 50% inhibition (ID_{50}) of 14 nM was obtained. The purified aortic phosphatase with a specific activity of 1600 nmol P/min per mg protein was inhibited by 98 ± 2% (n = 6) in the presence of 5 μM OA. The Michaelis constant K_m of 0.8 μM was obtained for the purified enzyme from the dependence of initial velocity on substrate concentration (0.4–8 μM phosphorylated light chains).

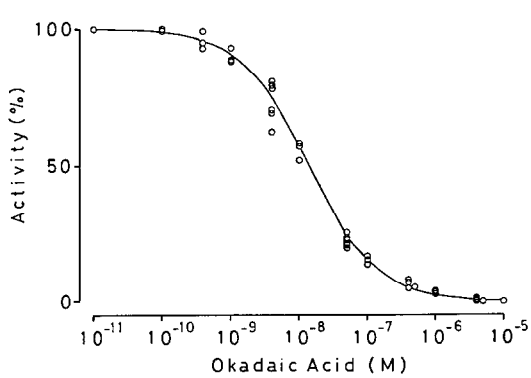

Fig.3. Inhibition of protein phosphatase activity in the taenia coli extract by okadaic acid. A dose-inhibition relationship.

3.3. *No effect on MLCK and PDE*

OA had no effects on the MLCK activity (fig.4). The MLCK activity in the presence of 30 μM Ca^{2+} was 46 ± 3 nmol P/min per mg protein (n = 8), and all data presented are given as percentages of this control activity. In calcium-free solution, the MLCK activity was 0.56 ± 0.07% of the control (n = 16). This small but significant ($p < 0.0001$) residual activity, which was not affected by 5 μM OA, has been subtracted from all data. The Ca^{2+} concentration-activity relationship was not altered by 5 μM OA. In order to minimize the interference by protein phosphatase activity, which was 32-times lower than that of MLCK (see above), the concentration of phosphorylated light chain was not allowed to exceed 0.1 μM even at the end of the

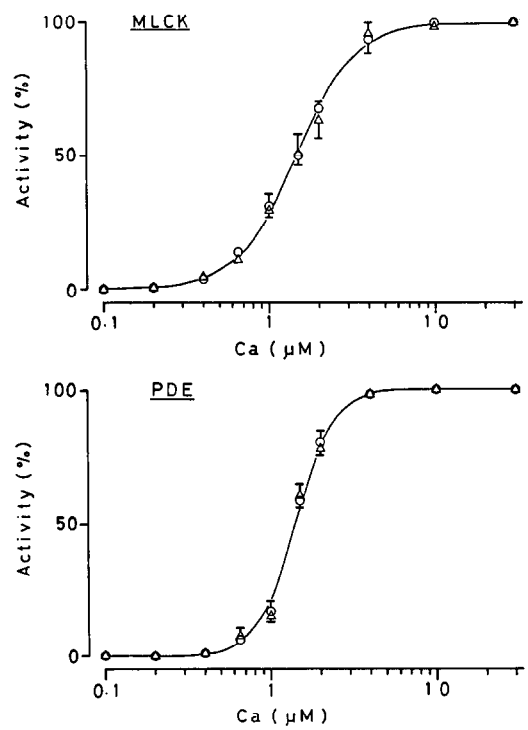

Fig.4. Ca^{2+} concentration-activity relationship of myosin light chain kinase (MLCK; upper panel, n = 8) and phosphodiesterase (PDE; lower panel, n = 3) in the absence (○) and in the presence (△) of 5 μM okadaic acid. Vertical bars indicating SE are omitted when they are within the symbols.

reaction time of 5 min by diluting the extract 300 times (at the final mixture).

5 μM OA had no effect on the calcium/calmodulin-dependent activity of PDE over the range of Ca^{2+} concentrations examined, indicating that OA does not affect calmodulin (fig.4). The activity is presented as a percentage of the activity in the presence of 30 μM Ca^{2+}. In calcium-free solution, the activity was undetectably low.

4. DISCUSSION

According to the phosphorylation theory of smooth muscle activation, contractile force depends on the state of myosin light chain phosphorylation which is dependent on the balance of myosin phosphatase and calcium/calmodulin-dependent myosin light chain kinase. Myosin phosphatase inhibitors against endogenous myosin light chain phosphatases of high molecular masses have not been described so far [15]. The present experiments show that OA has a powerful inhibitory effect on myosin phosphatase but no effect on myosin light chain kinase and calmodulin. Furthermore, the effect is not limited to the protein phosphatase(s) of the taenia extract because the purified aortic phosphatase was similarly inhibited. The results also confirm an earlier study [5] showing that OA increases force development of submaximally activated chemically skinned taenia coli fibers. Maximal mechanical response and a nearly complete inhibition of myosin phosphatase are both observed with 5 μM OA suggesting that these two phenomena are causally related. Indeed, the contractile response of the skinned taenia coli fibers to OA was accompanied by an increase in light chain phosphorylation (from $29 \pm 3\%$ to $53 \pm 2\%$ with 5 μM OA in the presence of 1 μM Ca^{2+}; $n = 4$), and relaxation by calcium removal was strikingly slowed in the presence of OA (unpublished observations).

To our knowledge, OA is the first potent and reversible myosin phosphatase inhibitor, and it may, in the future, serve as a unique tool for analyzing phosphorylation-dependent activation or inhibition mechanisms in muscle contraction and cell motility.

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