British Journal of Pharmacology (1997) 121, 1309–1314

Stellettamide-A, a novel inhibitor of calmodulin, isolated from a marine sponge

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1 Stellettamide A (ST-A), a novel marine toxin isolated from a marine sponge, inhibited high K^+(72.7 mM)-induced contraction in the smooth muscle of guinea-pig taenia coli with an IC_{50} of 88 μM.

2 In the taenia permeabilized with Triton X-100, ST-A inhibited Ca^{2+} (3 and 10 μM)-induced contractions with an IC_{50} of 46 μM for 3 μM Ca^{2+} and 105 μM for 10 μM Ca^{2+}. In the permeabilized taenia, calyculin-A (300 nM), a potent inhibitor of type-1 and type-2A phosphatases, induced sustained contraction in the absence of Ca^{2+}. ST-A had no effect on this contraction.

3 ST-A inhibited Mg^{2+}-ATPase activity in native actomyosin prepared from chicken gizzard with an IC_{50} of 25 μM.

4 In a reconstituted smooth muscle contractile system containing calmodulin, myosin light chain (MLC) and MLC kinase, ST-A inhibited MLC phosphorylation with an IC_{50} of 152 μM. The inhibitory effect of ST-A was antagonized by increasing the concentration of calmodulin.

5 ST-A inhibited calmodulin activity, assessed by Ca^{2+}/calmodulin-dependent enzymes, (Ca^{2+}-Mg^{2+})-ATPase of erythrocyte membrane, with an IC_{50} of 100 μM and phosphodiesterase prepared from bovine cardiac muscle with an IC_{50} of 52 μM. The inhibitory effect on phosphodiesterase activity was antagonized by increasing the calmodulin concentration.

6 Interaction between ST-A and calmodulin was demonstrated by instantaneous quenching of the intrinsic tyrosine fluorescence of calmodulin by ST-A (3–300 μM). Similar results were obtained in the presence or absence of Ca^{2+} suggesting that ST-A binds to calmodulin and that Ca^{2+} is not essential for the binding of ST-A to calmodulin.

7 These results suggest that ST-A, isolated from marine metabolites, is a novel inhibitor of calmodulin.

Keywords: Stellettamide-A; calmodulin; smooth muscle; contraction; skinned fibre; native actomyosin; phosphodiesterase; (Ca^{2+}-Mg^{2+})-ATPase; myosin light chain kinase; tyrosine fluorescence

Introduction

Stellettamide A (ST-A) was isolated from marine sponge of the genus *Stelletta* collected in Shikine-Jima Island of the Izu Archipelago, Japan (Hirota *et al*., 1990). This compound is a novel alkaloid containing a farnesyl moiety and an indolizidine (=octahydroindolizine) skeleton, which are connected through an amide bond. ST-A, having a molecular formula of $(C_{26}H_{45}N_2O)H_2PO_4$ (MW = 499) (Figure 1), has been shown to have antifungal activity against *Mortierella remannianus* and cytotoxicity against K562 epithelium cells (IC_{50} = 5.1 μg ml^{-1}) (Hirota *et al*., 1990). However, the mechanism of the effect of ST-A has not yet been studied. In the present study, we examined the effect of ST-A on the contractile system of smooth muscle and found that ST-A inhibited Ca^{2+}/calmodulin-dependent myosin light chain (MLC) phosphorylation.

Methods

Intact smooth muscle and solutions

Male guinea-pigs (300–350 g) were stunned and bled and the taenia coli was isolated. Strips of taenia, approximately 1 mm wide and 7 mm long, were prepared. Male Wistar rats (250–300 g) were stunned and bled and the thoracic aorta was dissected. After removing fat and connective tissues, the aorta was cut into helical strips approximately 2 mm in width and 8 mm in length. Endothelium was removed by gently rubbing the intimal surface with a finger moistened with physiological salt solution. The physiological salt solution (PSS) contained (in mM): NaCl 136.8, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, glucose 5.5 and ethylenediamine tetraacetic acid (EDTA) 0.01. A high K^+ solution was made by replacing NaCl with equimolar KCl. The medium was maintained at 37°C and aerated with a 95% O_2-5% CO_2 gas mixture at pH 7.4. Muscle tension was recorded isometrically with a force displacement transducer.

Permeabilized smooth muscle

Permeabilized muscle preparations were obtained according to the method described by Sparrow *et al*. (1981). A thin bundle (0.3 mm in width and 3 mm in length) of the taenia coli was prepared in the PSS and permeabilized with Triton X-100 (1%, v/v). The relaxing solution contained (in mM): KCl 50, $MgCl_2$ 4, imidazole 20, adenosine 5′-triphosphate (ATP) 2, NaN_3 1, calmodulin 0.2 μM, KH_2PO_4 3, K_2HPO_4 3 and EGTA 2 at pH 6.8 and 25°C. The free Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$ to EGTA (Ca^{2+}-EGTA buffer). The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^{-6} M at pH 6.8 (Harafuji & Ogawa, 1980). Muscle tension was measured isometrically under a resting tension of approximately 50 mg.

Native actomyosin preparation

Native actomyosin, containing every kind of contractile element, was prepared from chicken gizzard according to the method described by Ozaki *et al*. (1987a). Protein concentration was determined by the method described by Bradford (1976) with bovine serum albumin as a standard.

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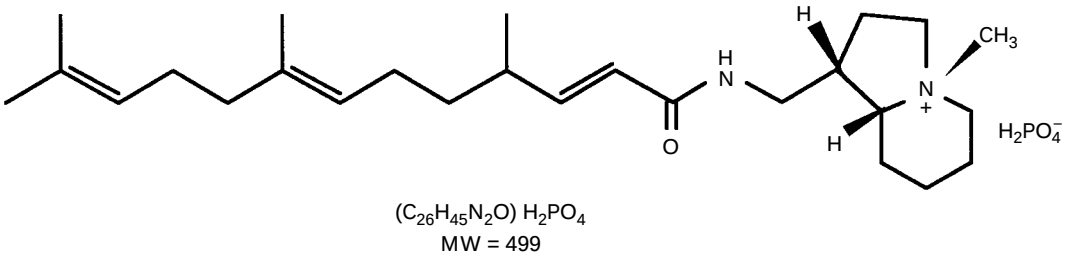

$(C_{26}H_{45}N_2O)\ H_2PO_4$
MW = 499

Figure 1 Chemical structure of stellattamide-A (ST-A) isolated from a marine sponge of the genus *Stelletta* (Hirota *et al*., 1990).

Mg^{2+}-ATPase activity

Mg^{2+}-ATPase activity of native actomyosin prepared from chicken gizzard was measured by the method described by Ikebe and Hartshorne (1985), with [γ-^{32}P]-ATP. The reaction buffer solution contained 0.5 mg ml^{-1} native actomyosin, 50 mM KCl, 8 mM $MgCl_2$, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-maleate (pH 6.8 at 25°C). Samples were treated with or without various concentrations of ST-A for 1 h. The reaction was started by adding 1 mM [γ-^{32}P]-ATP and stopped by the addition of an aliquot (0.2 ml) of the assay mixture to a stoppered plastic funnel containing 1 ml of 2% (w/w) activated charcoal, 0.5 ml of 1 N perchloric acid and 0.35 M NaH_2PO_4. The samples were mixed by a vortex mixer, kept on ice to retard the acid-catalyzed hydrolysis of ATP and filtered (facilitated by slight positive air pressure). Aliquots of the filtrate were measured by liquid scintillation counter (LSC-3500, Aloka, Tokyo, Japan). Release of inorganic phosphate (Pi) was measured 0, 1, 2, 3 and 4 min after the reaction had been started. Velocity of Pi release (μmol Pi min^{-1} mg^{-1} protein $\pm$ s.e., $n=5$ each) was calculated with a least square regression analysis.

MLC phosphorylation

Phosphorylation of 20 kDa MLC was carried out with the same reaction mixture as that for Mg^{2+}-ATPase activity. The reaction was started by adding 1 mM ATP and stopped by adding to 8 M urea after 1 min. The extent of phosphorylated 20 kDa MLC was measured by microdensitometry (CS-9300PC, Shimadzu, Tokyo, Japan) after urea-PAGE according to the method described by Pires *et al*. (1974).

MLC kinase activity

MLC kinase activity was measured by estimating ^{32}P incorporation into isolated 20 kDa MLC according to the method described by Walsh *et al*. (1983). The reaction buffer solution contained isolated 7 μM 20 kDa MLC, 100 nM MLC kinase, 100 nM calmodulin, 30 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-HCl (pH 7.5 at 25°C). The reaction was started by adding 1 mM [γ-^{32}P]-ATP and stopped by adding 5% (w/w) trichloroacetic acid (TCA) and 1% (w/w) NaH_2PO_4. Release of Pi was measured 0, 1, 2, 3 and 4 min after the reaction had started. Velocity of Pi release (μmol Pi min^{-1} mg^{-1} protein $\pm$ s.e., $n=5$ each) was calculated with a least square regression analysis.

(Ca^{2+}-Mg^{2+})-ATPase activity of erythrocyte membranes

Erythrocyte membranes were prepared from rabbit erythrocytes according to the method described by Ozaki *et al*. (1987b). The reaction buffer solution contained 0.5 mg ml^{-1} erythrocyte membranes, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 0.1 mM ouabain, 50 nM calmodulin, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-maleate (pH 6.8 at 25°C). The reaction was started by adding 2 mM [γ-^{32}P]-ATP and liberated inorganic phosphate was measured by the method described above. Release of Pi was measured 1, 3, 5, 10 and 20 min after the reaction had been started. Velocity of Pi release (μmol Pi min^{-1} mg^{-1} protein, $n=5$ each) was calculated with a least square regression analysis.

Phosphodiesterase activity

Phosphodiesterase activity was measured by a modification of the method of Teo *et al*. (1973). The reaction buffer solution contained 0.032 u ml^{-1} phosphodiesterase isolated from bovine heart (calmodulin free; Boehringer Mannheim-Yamanouchi, Tokyo, Japan), 50 nM calmodulin, 0.5 u ml^{-1} 5′-nucleotidase (Sigma, St. Louis, MO, U.S.A.), 20 mM imidazole, 10 mM $MgCl_2$, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-maleate (pH 6.8 at 25°C). The reaction was started by adding 2 mM [γ-^{32}P]-cyclicAMP (adenosine 3′:5′-cyclic monophosphate) and liberated inorganic phosphate was measured by the method described above. Release of Pi was measured 0, 1, 5, 10 and 15 min after starting the reaction. Velocity of Pi release (μmol Pi min^{-1} mg^{-1} protein, $n=5$ each) was calculated with a least square regression analysis.

Tyrosine fluorescence of calmodulin

Experiments were performed with a fluorescence spectrophotometer (Jasco FP2060, Tokyo, Japan). The reaction buffer solution (100 μl) contained 20 μM calmodulin, 20 mM Tris-HCl (pH 6.8 at 25°C), 40 mM KCl and 0.5 mM $CaCl_2$ or 5 mM EGTA. Emission spectra between 295 and 330 nm, excited at 280 nm, were monitored to measure the intrinsic tyrosine fluorescence of calmodulin. The maximum quenching of tyrosine fluorescence was obtained 10 min after addition of ST-A in the preliminary experiments. Based on these experiments, ST-A (3–300 μM) was cumulatively added to the buffer and the emission spectra were measured 10 min after addition of each concentration of ST-A. Autofluorescence of ST-A was measured at the same emission spectrum. The amount of fluorescent intensity of ST-A alone at 300 μM was approximately 20% of the tyrosine fluorescence of 20 μM calmodulin with 0.5 mM Ca^{2+}. This autofluorescence did not change with or without Ca^{2+} in the medium. In all the experiments, the autofluorescence of ST-A was deducted to evaluate a real fluorescent change of the intrinsic tyrosine fluorescence of calmodulin.

Chemicals

The chemicals used were: 1-noradrenaline bitartrate, imidazole, heparin, calyculin-A (Wako Pure Chemicals, Tokyo, Japan), ethylenediaminetetraacetic acid (EDTA), glycol-ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EGTA), adenosine-5′-triphosphate (ATP), Triton X-100, activated charcoal, ouabain, 5′-nucleotidase (Sigma Chemical Co., St. Louis, MO, U.S.A.), dithioerythritol (DTE), dithiothreitol (DTT), diisopropylfluorophosphate (DFP), trichloroaceticacid (TCA) (Nacalai Tesque, Kyoto, Japan), [γ-^{32}P]-ATP (Amersham, Buckinghamshire, U.K.), calmodulin-free phosphodiesterase isolated from bovine cardiac muscle (Boehringer Mannheim-Yamanouchi, Tokyo, Japan), [^{32}P]-cyclicAMP (Toho Biochemicals, Tokyo, Japan). Calmodulin was purified from swine

testis by the method described by Walsh *et al.*(1983). ST-A was purified from sea sponge (genus *Stelletta*) (Hirota *et al.*, 1990). MLC and MLC kinase purified from chicken gizzard were donated by Dr M. Ito (University of Mie) and Dr T. Kanoh (University of Mie), respectively.

Statistics

The results of the experiments are expressed as mean ± s.e. mean. EC_{50} was calculated by analysing concentration-response curve by use of Macintosh software, Delta Graph (Delta Point Inc., U.S.A.). The curve was fitted to the following equation; $y = aK_D^{n_H}/(x^{n_H} + K_D^{n_H}) + d$ where y: response, x: concentration of ST-A, d: background, a + d: maximum response, n_H Hill slope and K_D dissociation constant.

Results

Intact smooth muscle

In the guinea-pig taenia coli, high K^+(72.7 mM) induced a sustained contraction. Cumulative addition of ST-A (30–300 μM) inhibited the contraction in a concentration-dependent manner (Figure 2). The concentration needed to induce 50% inhibition (IC_{50}) was calculated to be 88 μM. ST-A also inhibited the high K^+ (72.7 mM)-induced contraction and noradrenaline (1 μM)-induced contraction in the rat aorta denuded of endothelium with IC_{50}s of 29 μM and 66 μM, respectively ($n = 4$).

Permeabilized smooth muscle

To determine whether ST-A directly inhibits the contractile elements, we further examined its effect on guinea-pig taenia coli permeabilized with Triton X-100. In the permeabilized taenia, Ca^{2+} (3 and 10 μM) induced sustained contraction. Cumulative addition of ST-A inhibited the Ca^{2+}-induced contraction in a concentration-dependent manner with an IC_{50} of 46 μM (Figure 3a). We also examined the effect of ST-A on the contraction induced by a higher concentration (10 μM) of Ca^{2+}. ST-A also inhibited this contraction in a concentration-dependent manner, although the inhibitory effect of ST-A became weaker (IC_{50}: 105 μM) (Figure 3a).

It has been shown that calyculin-A, an inhibitor of type 1 and type 2A phosphatases (Ishihara *et al.*, 1989a), induces contraction in the absence of Ca^{2+} (Ishihara *et al.*, 1989b). This contraction is considered to be due to the MLC phosphorylation through Ca^{2+}-independent MLC kinase. The contraction induced by calyculin-A (300 nM) in the absence of Ca^{2+} was not affected by ST-A (300 μM) (Figure 3b).

Native actomyosin

In the native actomyosin prepared from chicken gizzard, Mg^{2+}-ATPase activity was increased by adding 100 μM Ca^{2+}. ST-A (3–300 μM) inhibited this Mg^{2+}-ATPase activity in a concentration-dependent manner with an IC_{50} of 25 μM (Figure 4).

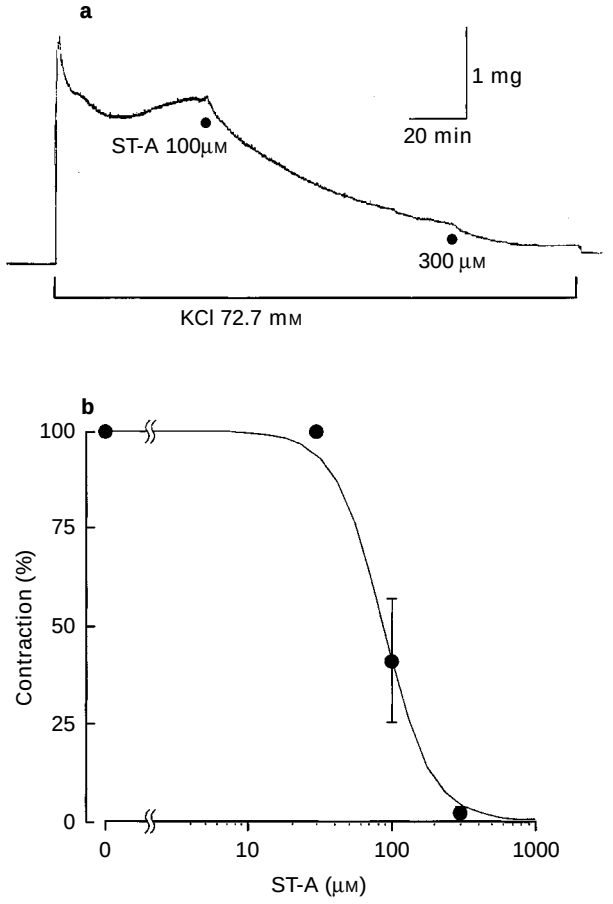

Figure 2 Effect of ST-A on high K^+-induced contraction in guinea-pig taenia coli. (a) Typical recording of the effect of ST-A (100 and 300 μM) on 72.7 mM KCl-induced contraction. (b) Concentration-response relationship for the effect of ST-A on high KCl-induced contraction; 100% represents the contractile tension due to 72.7 mM KCl. Mean values for four experiments are given and s.e.mean is shown by vertical lines ($n = 4$ each).

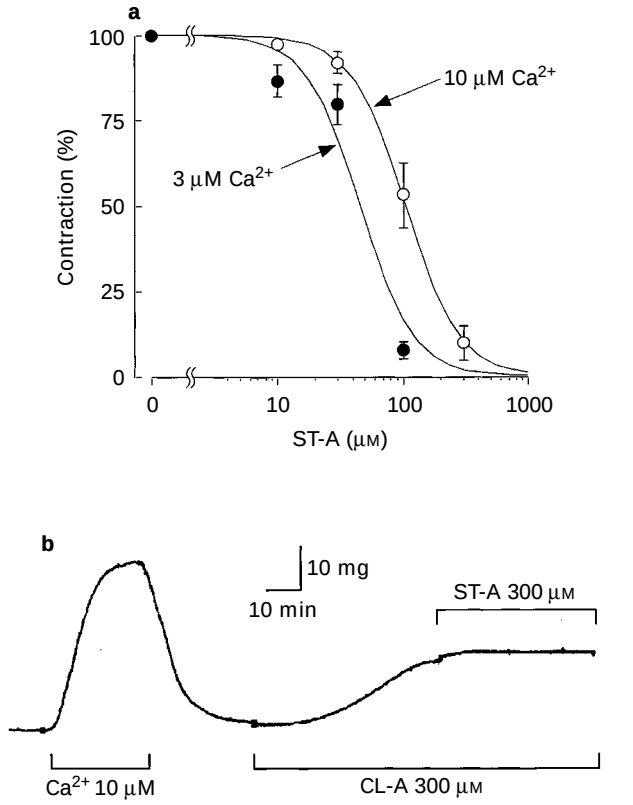

Figure 3 Effects of ST-A on Ca^{2+}-induced contraction (a) and calyculin-A(CL-A)-induced contraction in the absence of Ca^{2+} (b) in permeabilized taenia with Triton X-100. In (a) 100% represents the contractile tension due to 3 μM and 10 μM Ca^{2+}, respectively ($n = 4$ each). Mean values for four experiments are given and s.e. mean is shown by vertical lines. In (b), after contractility of the permeabilized muscle was checked by application of 10 μM Ca^{2+}, calyculin-A (300 nM) was added to induce sustained contraction in the absence of Ca^{2+}. ST-A (300 μM) was added after the contraction had reached a steady level.

MLC kinase activity (reconstituted system)

To determine the mechanism of the inhibition of MLC phosphorylation by ST-A, we used a reconstituted contractile system containing calmodulin, MLC and MLC kinase. In a reconstituted contractile system containing 100 nM calmodulin, 7 μM 20 kDa MLC and 100 nM MLC kinase, ST-A (3–300 μM) inhibited MLC phosphorylation in a concentration-dependent manner with an IC_{50} of 152 μM (Figure 5). This inhibitory effect of ST-A was antagonized by increasing the concentration of calmodulin to 300 nM (Figure 5).

Calmodulin activity

The effect of ST-A on calmodulin activity was examined in two different Ca^{2+}/calmodulin-dependent enzyme systems, (Ca^{2+}-Mg^{2+})-ATPase of rabbit erythrocyte membranes and phosphodiesterase of bovine heart. ST-A (3–300 μM) inhibited these enzyme activities in a concentration-dependent manner (Figure 6a and b). The IC_{50} values on (Ca^{2+}-Mg^{2+})-ATPase and phosphodiesterase were calculated to be 100 μM and 52 μM, respectively. We also examined the effect of ST-A on phosphodiesterase activity at different concentrations of calmodulin (10–300 nM). The inhibition by ST-A (100 μM) of phosphodiesterase activity was greater at a lower concentration of calmodulin (Figure 7).

Tyrosine fluorescence of calmodulin

Interaction between ST-A and calmodulin was determined by monitoring intrinsic tyrosine fluorescence of calmodulin (Figure 8). In the absence of Ca^{2+} with 5 mM EGTA, tyrosine fluorescence of calmodulin was quenched concentration-dependently by ST-A (3–300 μM) without a significant shift in the emission maximum (Figure 8a). In the presence of Ca^{2+}, in-

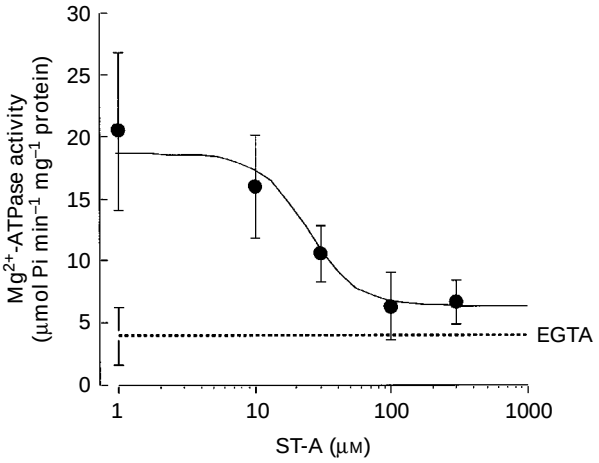

Figure 4 Effects of ST-A on Mg^{2+}-ATPase activity of native actomyosin prepared from chicken gizzard. Preparations were treated with ST-A for 60 min. The reaction buffer solution contained 0.5 mg ml^{-1} native actomyosin, 50 mM KCl, 8 mM $MgCl_2$, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-maleate (pH 6.8). The reaction was started by adding 1 mM [γ-^{32}P]-ATP. Dotted line represents enzyme activity in the absence of Ca^{2+}. ($n=5$ each). Vertical lines show s.e.mean.

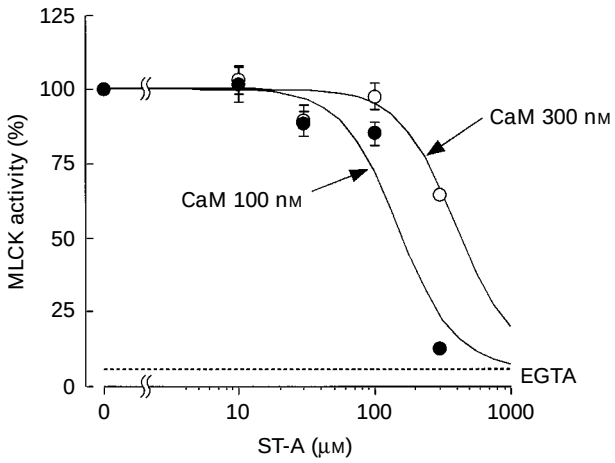

Figure 5 Effect of ST-A on myosin light chain kinase (MLCK) activity in the reconstituted system; 100% represents MLCK activity in the presence of 100 μM Ca^{2+}. Dotted line represents enzyme activity in the absence of Ca^{2+}. Preparations were treated with ST-A for 60 min. The reaction buffer solution contained 7 μM isolated 20 kDa MLC, 100 nM MLCK, 100 nM or 300 nM calmodulin (CaM), 30 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-HCl (pH 7.5). The reaction was started by adding 1 mM [γ-^{32}P]-ATP. The curve for 300 nM calmodulin was generated by assuming that the curve was shifted in parallel from that for 100 nM calmodulin ($n=5$ each). Vertical lines show s.e.mean.

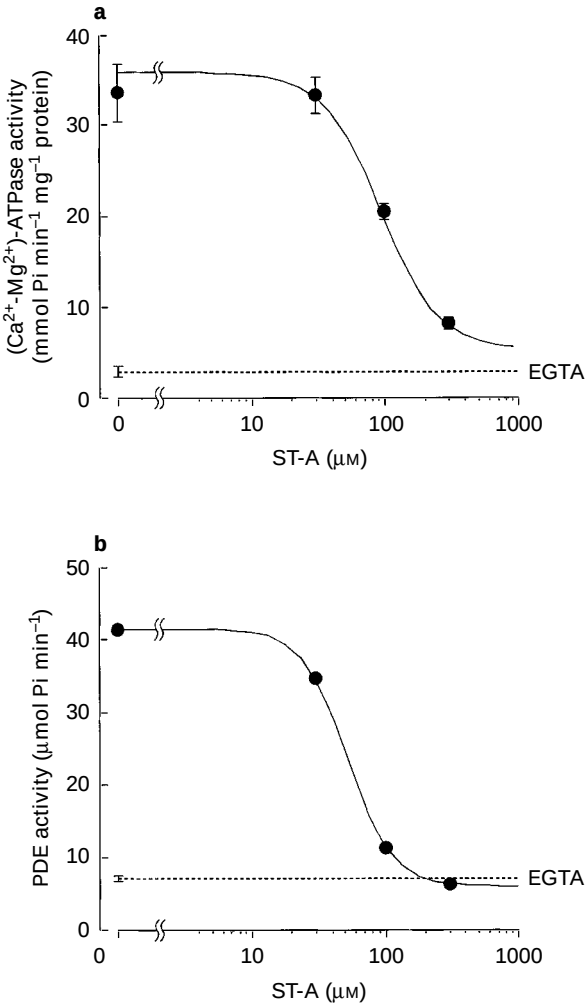

Figure 6 Effects of ST-A on the activities of two Ca^{2+}/calmodulin-dependent enzymes, (Ca^{2+}-Mg^{2+})-ATPase of rabbit erythrocyte membranes (a) and phosphodiesterase of bovine heart (b). Dotted lines represent activities of respective enzymes in the absence of Ca^{2+}. Preparations were treated with ST-A for 60 min. In (a), the reaction buffer solution contained 0.5 mg ml^{-1} erythrocyte membranes, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 0.1 mM ouabain, 50 nM calmodulin, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-maleate (pH 6.8). The reaction was started by adding 2 mM [γ-^{32}P]-ATP. In (b), the reaction buffer solution contained 0.032 u ml^{-1} phosphodiesterase (PDE) (calmodulin free), 50 nM calmodulin, 0.5 u ml^{-1} 5′-nucleotidase, 20 mM imidazole, 10 mM $MgCl_2$, 2 mM EGTA or 100 μM Ca^{2+} and 20 mM Tris-maleate (pH 6.8). The reaction was started by adding 2 mM [^{32}P]-cyclicAMP. Vertical lines show s.e.mean ($n=5$ each).

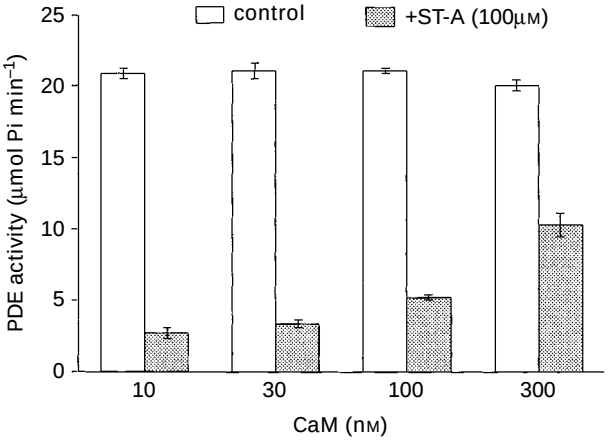

Figure 7 Effects of increasing calmodulin (CaM) concentration on the inhibitory effect of ST-A on phosphodiesterase activity. Phosphodiesterase activity was assayed at different concentrations of calmodulin (10, 30, 100 and 300 nM) in the absence and presence of 100 μM ST-A. The experimental protocol was the same as described in Figure 6 ($n=5$ each).

trinsic tyrosine fluorescence was increased by approximately 1.7 times, suggesting a conformation change of the calmodulin molecule. In the presence of Ca^{2+}, cumulative addition of ST-A (3–300 μM) also decreased the tyrosine fluorescence in a concentration-dependent manner without shifting the maximum emission spectra (Figure 8b). The inhibitory effects of ST-A on tyrosine fluorescence with or without Ca^{2+} were similar and the concentrations of ST-A to induce 50% quenching were about 85 μM or 70 μM, respectively. ST-A (300 μM) did not change the fluorescence of tyrosine (1 μM) (data not shown).

Discussion

In the guinea-pig isolated taenia coli, high K^+ solution induces contraction which is attributable to the increase in cytosolic Ca^{2+} level and the following MLC phosphorylation. ST-A inhibited the contraction induced by high K^+ in a concentration-dependent manner. In the permeabilized taenia with Triton X-100, ST-A inhibited the Ca^{2+}-induced contractions. Calyculin-A, a potent inhibitor of phosphatases, induced sustained contraction in the permeabilized taenia which is attributable to the Ca^{2+}/calmodulin-independent MLC kinase (Ozaki *et al*., 1987a; Ishihara *et al*., 1989b). In contrast to the Ca^{2+}/calmodulin-dependent contraction, the calyculin-A-induced contraction was not affected by ST-A. These results suggest that ST-A directly inhibits contractile elements by acting on a Ca^{2+}-dependent regulatory mechanism but not on the interaction between actin and phosphorylated myosin.

In native actomyosin prepared from chicken gizzard, ST-A inhibited Mg^{2+}-ATPase activity and MLC phosphorylation. Furthermore, ST-A inhibited MLC phosphorylation in the reconstituted system containing calmodulin, MLC and MLC kinase. The inhibitory effect of ST-A on contractile responses in the permeabilized muscle and on MLC kinase activity were antagonized by increasing the concentration of Ca^{2+} and/or calmodulin. All of these results suggest that the inhibitory effect of ST-A on smooth muscle contraction would be due to the inhibition of calmodulin. To examine this possibility, we investigated the effects of ST-A on calmodulin activity by monitoring the effects on two Ca^{2+}/calmodulin-dependent enzymes and found that ST-A inhibited both of the activities. Furthermore, the inhibitory effect of ST-A on phosphodiesterase activity was antagonized by increasing the concentration of calmodulin.

Measurement of intrinsic tyrosine fluorescence of calmodulin has been widely used to evaluate the interaction between the drugs and calmodulin. For instance, it has been shown that

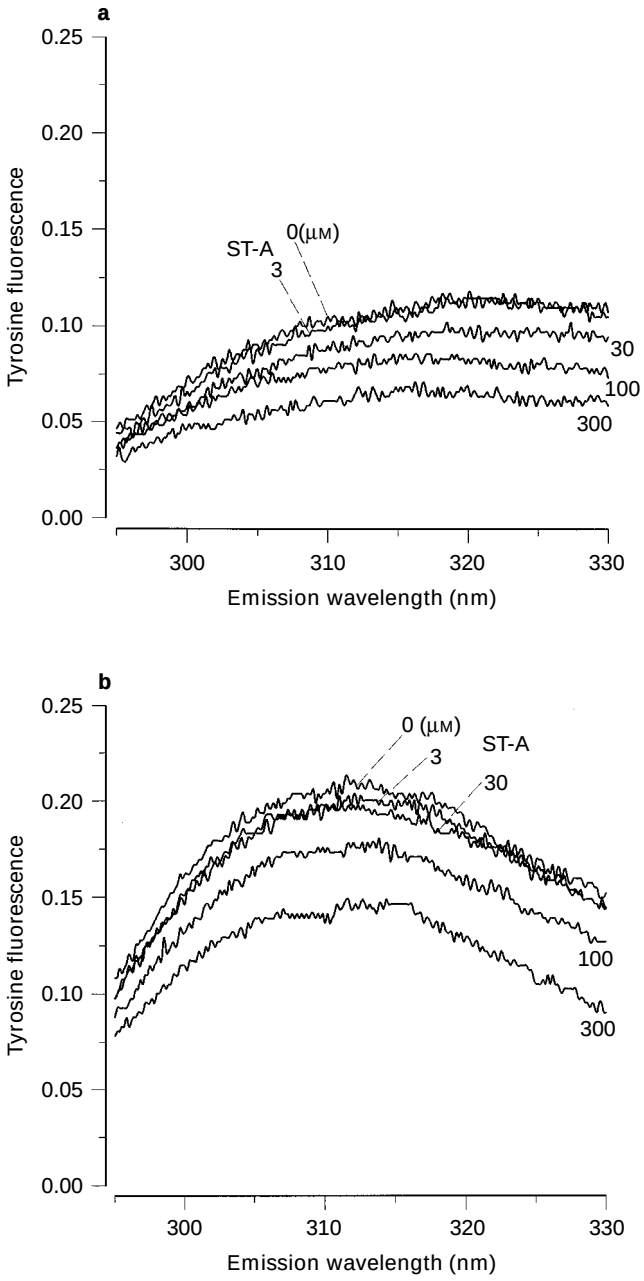

Figure 8 Effect of ST-A on intrinsic tyrosine fluorescence of calmodulin in the presence of EGTA (a) or Ca^{2+} (b). The results shown are representative of 3 experiments. Emission spectrum (290–330 nm) excited at 280 nm were measured. Each spectra was recorded 10 min after the addition of each concentration of ST-A.

addition of Ca^{2+} increases, whereas many calmodulin inhibitors, such as W-7 and trifluoperazine, decrease the intrinsic tyrosine fluorescence of calmodulin. (Walsh & Stevens, 1977; Richman, 1978; Laporte *et al*., 1980; Chao *et al*., 1984). To evaluate the possible direct interaction between ST-A and calmodulin, we measured the changes in intrinsic tyrosine fluorescent of calmodulin. The results showed that ST-A inhibited the intrinsic tyrosine fluorescence in a concentration-dependent manner. This finding strongly supports the contention that ST-A binds to calmodulin to inhibit Ca^{2+}/calmodulin-dependent enzyme activities.

It has been suggested that binding of Ca^{2+} to calmodulin results in the exposure of a hydrophobic domain of calmodulin (Laporte *et al*., 1980). Such a conformational change in calmodulin molecule can be detected by measuring the increase in intrinsic tyrosine fluorescence (Chao *et al*., 1984) (also see

Figure 8). It has also been found that binding of hydrophobic drugs to this domain antagonizes the interaction between the Ca^{2+}/calmodulin complex and a Ca^{2+}/calmodulin-dependent enzyme (Laporte *et al*., 1980). Weiss *et al*., (1980) have shown that the interaction between the calmodulin complex and the drug involves an ionic bond between a positively charged nitrogen on the drug and the negatively charged residues on calmodulin. Since ST-A has a positively charged nitrogen in the molecule, calmodulin may bind to this region. In this study, the quenching effect of ST-A on tyrosine fluorescence of calmodulin was similar in the presence and absence of Ca^{2+}. These results suggest that binding of Ca^{2+}, which induces the exposure of a hydrophobic domain of calmodulin, may not be essential for the binding of ST-A to the calmodulin molecule. Further study will be necessary to clarify this point.

Various calmodulin inhibitors, such as W-7, trifluoperazine and calmidazolium, have been synthesized and used for biological studies. However, these calmodulin inhibitors inhibit not only calmodulin but also other calmodulin-independent processes, such as Ca^{2+} influx via voltage-dependent Ca^{2+} channels (Johnson *et al*., 1983; Flaim *et al*., 1985; Greenberg *et al*., 1987), protein kinase C (Mori *et al*., 1980; Schatzman *et al*., 1983; Mazzei *et al*., 1984) and phospholipase A_2 (Watanabe *et al*., 1986). The selectivity of ST-A against calmodulin needs to be elucidated in future studies.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture and Science, Japan and Terumo Life Science Foundation. We thank Drs M. Ito and T. Kanoh (University of Mie, Tsu, Japan) for supplying us with MLC and MLC kinase, respectively.

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(Received February 7, 1997
Revised April 18, 1997
Accepted April 29, 1997)