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Mycalolide-B, a novel and specific inhibitor of actomyosin ATPase isolated from marine sponge

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A toxin isolated from marine sponge, mycalolide-B, inhibited smooth muscle contractions without changing cytosolic Ca^{2+} levels. It also inhibited Ca^{2+}-induced contraction in permeabilized smooth muscles. In native actomyosin prepared from chicken gizzard, mycalolide-B inhibited superprecipitation and Mg^{2+}-ATPase activity stimulated by Ca^{2+} without changing myosin light chain phosphorylation. In the permeabilized muscle and native actomyosin preparation thiophosphorylated with ATPγS, mycalolide-B inhibited ATP-induced contraction and Mg^{2+}-ATPase activity, respectively, in the absence of Ca^{2+}. Mycalolide-B also inhibited Mg^{2+}-ATPase activity of skeletal muscle native actomyosin. Mycalolide-B had no effect on calmodulin-stimulated (Ca^{2+}–Mg^{2+})-ATPase activity of erythrocyte membranes. These results suggest that mycalolide-B selectively inhibits actin–myosin interaction.

Mycalolide-B; Marine sponge; Actomyosin inhibitor; Smooth muscle; Permeabilized muscle; Skeletal muscle

1. INTRODUCTION

Recently, three novel cytotoxic compounds, mycalolide-A, B and C were isolated from a marine sponge of the genus *Mycale* in the Bay of Gokasho, Kii Peninsula, Japan [1]. Mycalolide-B belongs to macrolide antibiotics and consists of tris-oxazole with chemical structure of $C_{52}H_{74}N_4O_{17}$ (M.W. = 1052) (Fig. 1) with strong cytotoxic activity (IC_{50} : 10–50 nM for growth of L1210 murine leukemia cells) [2]. In this study, we investigated the effect of mycalolide-B on contraction and biochemical parameters in smooth, skeletal and cardiac muscles, and found that this toxin selectively inhibits actin–myosin interaction.

2. MATERIALS AND METHODS

2.1. *Muscle preparation and solutions*

Thoracic aorta was isolated from male Wistar rats (200–250 g) and mesenteric artery and psoas muscle and heart were isolated from male New Zealand White rabbits (2–3 kg). Papillary muscle was removed from the heart to make permeabilized muscle preparations. Endothelium was removed from vascular muscles. Gizzard isolated from chicken (1.5–2 kg) and rabbit psoas muscle were used to make native actomyosin preparations. The physiological salt solution (PSS) contained (in mM): NaCl 136.9; KCl 5.4; $CaCl_2$ 1.5; $MgCl_2$ 1.0; $NaHCO_3$ 23.8; glucose 5.5 and enthylenediamine tetraacetic acid 0.01. A high K^+ solution was made by substituting equimolar KCl for NaCl. The Ca^{2+}-free solution was made by removing $CaCl_2$ and adding 0.5 mM EGTA.

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Abbreviations: DTE, dithioerythritol; DTT, dithiothreitol; DFP, diisopropylfluorophosphate; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; fura-2/AM, acetoxymethyl ester of fura-2; MLC, myosin light chain; PSS, physiological salt solution; TCA, trichloroacetic acid; Tris, tris[hydroxymethyl]aminomethane.

2.2. *$[Ca^{2+}]_i$ and tension measurement*

The cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) was measured simultaneously with muscle tension [3,4] using a fluorescent Ca^{2+} indicator, fura-2 [5]. Rat aorta was loaded with acetoxymethyl ester of fura-2 which was dissolved in PSS for 4 h at room temperature. The muscle strip was illuminated alternately (48 Hz) at the excitation wavelengths of 340 nm and 380 nm and the amount of 500 nm fluorescence induced by 340 nm excitation (F_{340}) and that induced by 380 nm excitation (F_{380}) was measured using a fluorimeter (CAF-100, Japan Spectroscopic, Tokyo, Japan). The ratio of F_{340} to F_{380} ($R_{340/380}$) was used as an indicator of $[Ca^{2+}]_i$. Muscle tension was recorded isometrically with a force displacement transducer.

2.3 *Permeabilized muscle*

Permeabilized muscle preparations were obtained according to the method described by Sparrow et al. [6] and Sugi et al. [7]. A thin bundle (0.3 mm in width and 3 mm in length) of mesenteric artery, papillary muscle or skeletal muscle were prepared in PSS and permeabilized using Triton X-100 or glycerol. The relaxing solution for permeabilized smooth muscle contained: 20 mM imidazole (pH 7.4 at 4°C); 50 mM KCl; 4 mM $MgCl_2$; 3 mM ATP; 1 mM NaN_3; 0.2 μM calmodulin, 1 mM creatine phosphate; 10 U/ml creatine phosphokinase and 2 mM EGTA at pH 6.8 and 25°C. The free Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$ (Ca^{2+}-EGTA buffer). In permeabilized cardiac and skeletal muscles, creatine phosphate, creatine phosphokinase and calmodulin were removed from the relaxing solution. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^{-6} M at pH 6.8 [8]. Muscle tension was measured isometrically under the resting tension of approximately 50 mg.

In some experiments, MLC was thiophosphorylated by adding 1

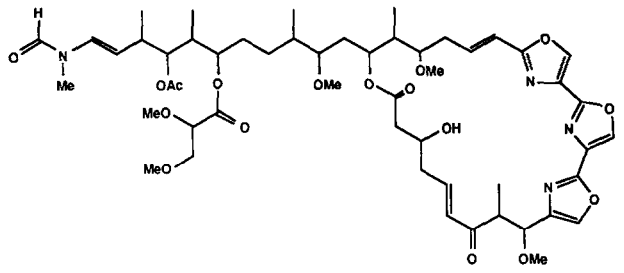

Fig. 1. Chemical structure of mycalolide-B isolated from marine sponge of the genus *Mycale*.

μM Ca^{2+} and 1 mM ATPγS in the absence of ATP for 10 min [9]. Sequential addition of ATP caused a sustained contraction in the absence of Ca^{2+} [9,10].

2.4. *Native actomyosin preparation*

Native actomyosin was prepared from chicken gizzard or rabbit skeletal muscle according to the method described by Ozaki et al. [10,11]. Protein concentration was determined by the Lowry method [12] using bovine serum albumin as a standard.

2.5. *Superprecipitation*

Superprecipitation of native actomyosin was measured by modifying the method reported by Ebashi [13]. The reaction buffer solution contained 1 mg/ml native actomyosin, 50 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA and 20 mM Tris-maleate (pH 6.8 at 4°C) and the reaction was started by adding 1 mM ATP. Superprecipitation was measured by the optical density of actomyosin suspension at 690 nm using a spectrophotometer (UVDEC-460, Japan Spectroscopic) at 25°C stirring continuously.

2.6. *Mg^{2+}-ATPase activity*

Mg^{2+}-ATPase activity of native actomyosin prepared from chicken gizzard or skeletal muscle were measured by the method described previously [10,11]. The reaction buffer was of the same content as that of superprecipitation. The reaction was started by 1 mM ATP and stopped by 5% TCA. Liberated inorganic phosphate was determined by the method of Martin and Doty [14].

Thiophosphorylated native actomyosin preparations were prepared by the following protocol. Native actomyosin was treated with reaction buffer containing 10 μM Ca^{2+} and 1 mM ATPγS in the absence of ATP and EGTA for 15 min. Sequential addition of 2 mM EGTA was carried out for chelating free Ca^{2+}. The reaction was started by addition of 1 mM ATP in the absence of Ca^{2+}.

2.7. *Myosin light chain phosphorylation*

Phosphorylation of the 20 kDa myosin light chain (MLC) was carried out using the same reaction mixture as that for superprecipitation. The reaction was started by adding 1 mM ATP and stopped by adding urea to 8 M. The extent of phosphorylated MLC after 1 min was measured by microdensitometry after urea-PAGE [15].

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

Erythrocyte membranes were prepared from rabbit erythrocytes [10,11,16]. The reaction buffer solution contained 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 20 mM Tris-maleate (pH 6.8 at 25°C), 0.1 mM ouabain, 30 nM calmodulin, 1 mg protein/ml erythrocyte membranes and Ca^{2+}-EGTA buffer (pCa 5). The reaction was started by adding 1 mM ATP and stopped by adding 5% TCA. Liberated inorganic phosphate after 30 min was measured by the Martin–Doty method.

2.9. *Chemicals*

Drugs used were imidazole (Wako Pure Chemical, Tokyo, Japan), Tris-HCl, DFP, creatine phosphate, phosphocreatine kinase, ATP, ATPγS, Triton X-100 (Sigma, St. Louis, MO), DTE, DTT, cremophor EL (Nacalai Tesque, Tokyo, Japan), and fura-2/AM, EDTA, EGTA (Dojindo Laboratories, Kumamoto, Japan).

3. RESULTS

In fura-2-loaded rat aorta, high K^+ (72.4 mM) increased $[Ca^{2+}]_i$ and muscle tension. Mycalolide-B (10 μM), added during a high K^+-induced sustained contraction, decreased force without changing $[Ca^{2+}]_i$ (Fig. 2A). Mycalolide-B (10 μM) completely inhibited the contractions induced by high K^+ or norepinephrine (NE; 0.01–1 μM) with a similar time course (time to 50% relaxation; 8–10 min). Concentrations to induce 50% inhibition (IC_{50}) of contractions induced by high K^+ or NE (0.01, 0.1 and 1 μM) were 40, 110, 210 and 220 nM, respectively ($n = 4$ each). Caffeine (20 mM) caused transient increases in $[Ca^{2+}]_i$ and force in the Ca^{2+}-free solution (with 0.5 mM EGTA). Mycalolide-B (30 μM) inhibited the caffeine-induced contraction without affecting the change in $[Ca^{2+}]_i$ (Fig. 2B).

In permeabilized rabbit mesenteric artery, Ca^{2+} (1 μM) induced sustained contraction. Mycalolide-B (10 μM) completely inhibited this contraction (Fig. 3A).

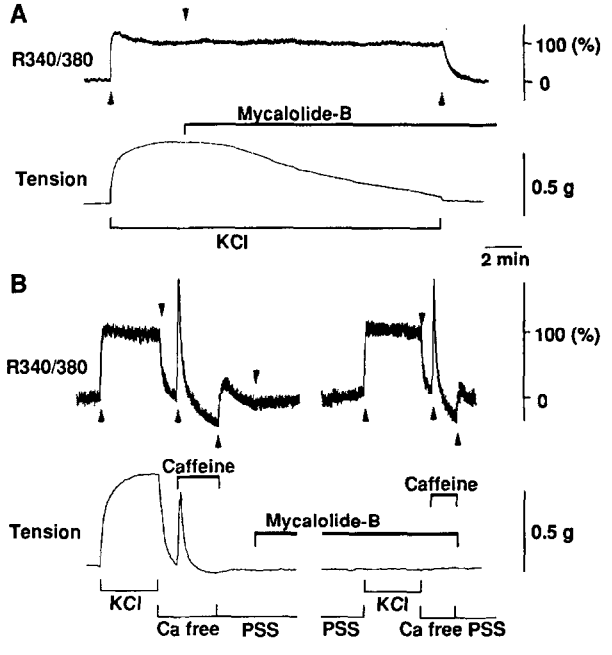

Fig. 2. Effect of mycalolide-B on $[Ca^{2+}]_i$ and contraction in fura-2-loaded rat aorta. In panel A, mycalolide-B (10 μM) was applied 5 min after the addition of high K^+ (72.7 mM). In panel B, high K^+ was added followed by the addition of caffeine (20 mM) which induced transient increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution (0.5 mM EGTA). After observing the control responses, muscle strip was treated with mycalolide-B (30 μM) for 30 min and high K^+ and caffeine were added. *R*340/380; an indicator of $[Ca^{2+}]_i$. 100% represents the steady level of high K^+-induced increase in $[Ca^{2+}]_i$.

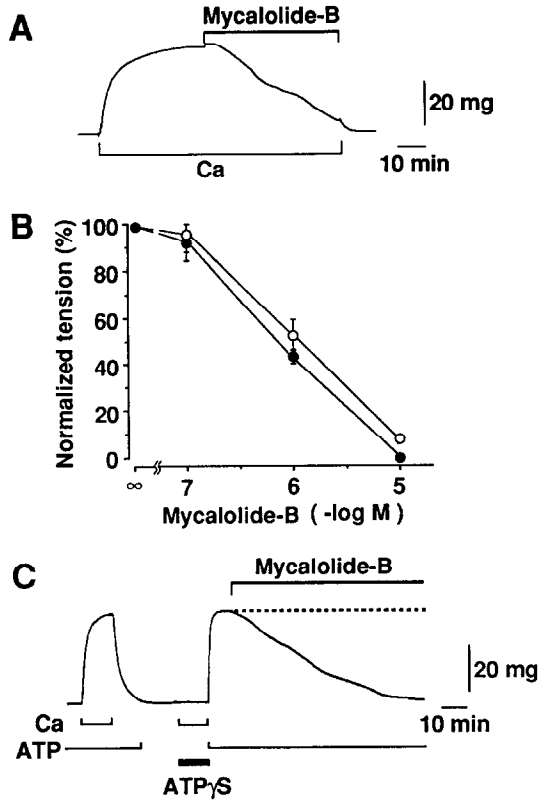

Fig. 3. Inhibitory effect of mycalolide-B on contractions in permeabilized rabbit mesenteric artery. In panel A, 10 μM mycalolide-B was added after the contraction induced by 1 μM Ca^{2+} reached a plateau. Panel B shows the concentration–response relationship for the inhibitory effects of mycalolide-B on contractions induced by 1 μM (○) or 3 μM (●) Ca^{2+}. Each point represents the mean values ± S.E.M. of 4–6 experiments measured 60 min after the addition of mycalolide-B. Panel C shows the effects of mycalolide-B (10 μM) on Ca^{2+}-independent contraction obtained in thiophosphorylated muscle. Dotted line represents contraction in the absence of mycalolide-B.

Concentration–response curves for the inhibitory effects of mycalolide-B on the contractions induced by Ca^{2+} (1 and 3 μM) are shown in Fig. 3B. The inhibitory effect of mycalolide-B was not modified by changing the Ca^{2+} concentration. Mycalolide-B (10 μM) also completely inhibited the contraction of permeabilized cardiac and skeletal muscles induced by 3 μM Ca^{2+} (n = 4). Mycalolide-B (10 μM) completely inhibited the Ca^{2+}-independent contraction in the thiophosphorylated permeabilized smooth muscle (Fig. 3C).

In the presence of 3 or 10 μM Ca^{2+}, ATP (1 mM) increased the turbidity of the native actomyosin suspension (superprecipitation) prepared from chicken gizzard. Mycalolide-B (3–100 μM) inhibited the superprecipitation in a concentration-dependent manner (Fig. 4A; IC_{50}, 9.6 μM in the presence of 3 μM Ca^{2+}, and 8.2 μM in the presence of 10 μCa^{2+}). However, mycalolide-B (10 μM) had no effect on the MLC phosphorylation at any Ca^{2+} level (Fig. 4B). Fig. 4C shows the effect of mycalolide-B on Mg^{2+}-ATPase activity of phosphorylated or thiophosphorylated native actomyosin. Mg^{2+}-ATPase activity increased 1.4 times in the presence of 3 μM Ca^{2+} and 1 mM ATP. In the thiophosphorylated native actomyosin, 1 mM ATP increased Mg^{2+}-ATPase activity to 1.6 times in the absence of Ca^{2+} (with 2 mM EGTA). Mycalolide-B inhibited the increases in Mg^{2+}-ATPase activity in a concentration-dependent manner. The IC_{50} values in phosphorylated and thiophosphorylated Mg^{2+}-ATPase activity were 3.9 μM and 4.7 μM, respectively. In skeletal muscle native acto-

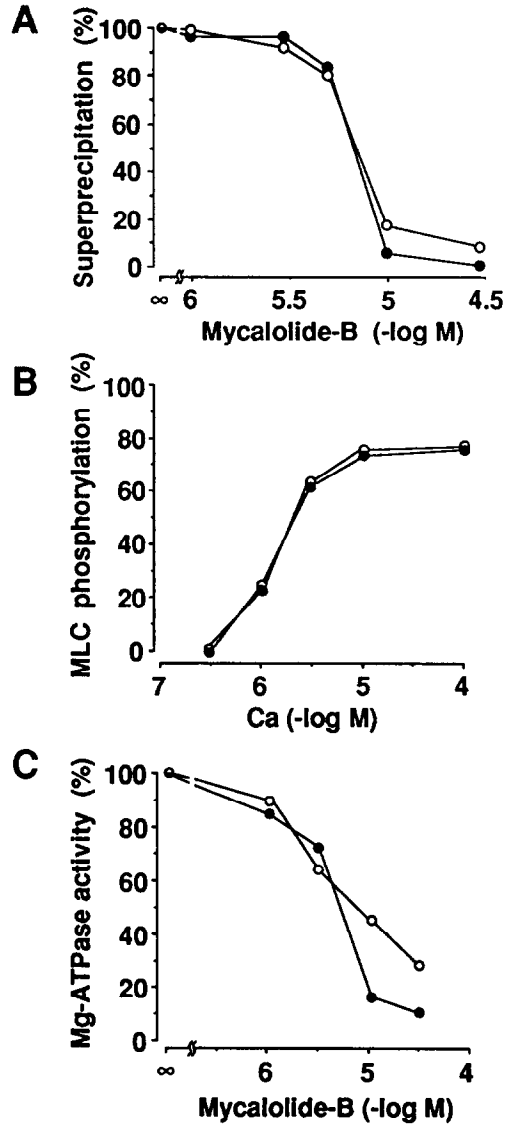

Fig. 4. Effect of mycalolide-B on superprecipitation (A), MLC phosphorylation (B) and Mg^{2+}-ATPase activity (C) of native actomyosin prepared from chicken gizzard. Preparations were treated with mycalolide-B for 120 min. Panel A: ○, 3 μM Ca^{2+}; ●, 10 μM Ca^{2+}. Turbidity of native actomyosin was continuously monitored and the maximum increase in the turbidity in the presence of 3 or 10 μM Ca^{2+} was normalized and plotted. Panel B: ○, control; ●, mycalolide-B 10 μM. Panel C: ○, Mg^{2+}-ATPase activity stimulated by 3 μM Ca^{2+} and 1 mM ATP; ●, Mg^{2+}-ATPase activity stimulated by 1 mM ATP without Ca^{2+} in the thiophosphorylated native actomyosin. Hydrolyzed inorganic phosphate (nmole P_i/mg protein) was measured at 30 min.

myosin, mycalolide-B (10 μM) also completely suppressed the Mg^{2+}-ATPase activity stimulated by 1 or 100 μM Ca^{2+} ($n = 4$ each).

Mycalolide-B (3–30 μM) did not change the (Ca^{2+}–Mg^{2+})-ATPase activity of erythrocyte membranes when assayed in the presence of 3 μM Ca^{2+} and 30 nM calmodulin (Control, 1.83 ± 0.09; 3 μM Ca^{2+}, 17.1 ± 0.14; 3 μM Ca^{2+} + 3 μM mycalolide-B, 17.0 ± 0.34; 3 μM Ca^{2+} + 10 μM mycalolide-B, 16.8 ± 0.23 nmol P_i/mg protein/min).

4. DISCUSSION

In vascular smooth muscle, it has been shown that high-K^+ depolarization and caffeine induce contractions by opening Ca^{2+} channels [17,18] and by releasing Ca^{2+} from the sarcoplasmic reticulum [19,20], respectively. Mycalolide-B inhibited these contractions with a similar time course at a similar concentration range. However, mycalolide-B did not change the stimulated $[Ca^{2+}]_i$. These results suggest that the relaxant effect of mycalolide-B is not due to change in Ca^{2+} movements.

In permeabilized smooth muscle, mycalolide-B inhibited the Ca^{2+}-induced contractions. Mycalolide-B also inhibited the superprecipitation and Mg^{2+}-ATPase activity of native actomyosin prepared from chicken gizzards. These results suggests that mycalolide-B directly inhibits contractile elements.

Contraction of smooth muscle is considered to be primarily regulated by the Ca^{2+}/calmodulin-dependent MLC phosphorylation [21,22]. The inhibitory effect of mycalolide-B, however, was not followed by the inhibition of MLC phosphorylation at any Ca^{2+} concentrations. Furthermore, mycalolide-B did not inhibit the calmodulin activity as measured by (Ca^{2+}–Mg^{2+})-ATPase of erythrocytes membrane. All of these observations suggest that mycalolide-B inhibits smooth muscle contractile element at the step(s) after the MLC phosphorylation.

In permeabilized smooth muscle pretreated with ATPγS and Ca^{2+}, ATP evokes sustained contraction in the absence of Ca^{2+} [9,10]. This contraction is due to the direct interaction between actin and thiophosphorylated myosin which is not dephosphorylated by protein phosphatases [9]. Mycalolide-B completely inhibited this contraction. Mycalolide-B also inhibited the Mg^{2+}-ATPase activity of thiophosphorylated native actomyosin prepared from chicken gizzards. These results suggest that mycalolide-B directly inhibits actin-myosin interaction.

We further examined whether mycalolide-B inhibits contractile elements of skeletal and cardiac muscles. Mycalolide-B completely inhibited both the Ca^{2+}-induced contraction in permeabilized skeletal muscle and Mg^{2+}-ATPase activity in native actomyosin prepared from skeletal muscle. Mycolalide-B also inhibited Ca^{2+}-induced contractions in permeabilized cardiac muscle. These results strongly suggest that mycalolide-B inhibits actomyosin not only in smooth muscle but also in skeletal and cardiac muscles. Further studies are now in progress to clarify the molecular mechanism of mycalolide-B on actin and myosin.

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