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Modulation of actomyosin ATPase by thiotetromycin is mediated through conformational change of actin

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Abstract

Thiotetromycin isolated from the culture broth of *Streptomyces* sp. strain OM-674 slightly enhanced the superprecipitation and the ATPase activity of myosin B from skeletal muscle. The ATPase activity of troponin–tropomyosin-free myosin B was inhibited by thiotetromycin. The inhibitory effect of thiotetromycin was significantly attenuated by troponin–tropomyosin complex. The ATPase activity of actomyosin reconstituted from actin and myosin was inhibited by pretreatment of actin with thiotetromycin. Thiotetromycin induced a concentration-dependent decrease in the fluorescence intensity of actin and pyrenyl–F-actin. By using surface plasmon resonance (SPR), it was proved that thiotetromycin bound to actin. Thiotetromycin caused a concentration-dependent decrease in sedimentation of F-actin by hard centrifugation. This was a cross-correlation among the concentration-inhibition curves for thiotetromycin in the activity of actomyosin ATPase and the fluorescence intensity. These results suggest that thiotetromycin binds to actin to cause a conformational change, resulting in modulation of the interaction between actin and myosin, and in depolymerization of F-actin.

Keywords: Thiotetromycin; ATPase; Actin; Conformational change

1. Introduction

The force of muscle contraction is produced by the interaction between actin and myosin molecules in a process that involves cross-bridge cycling coupled with the hydrolysis of ATP (Inoue et al., 1979). ATP hydrolysis and the subsequent dissociation of the tightly bound products (ADP and Pi) produce an ordered series of allosteric changes in the conformation of myosin (Burton, 1992). Actomyosin is a precise machinery for the transduction of the chemical energy in ATP molecules into mechanical work. From the viewpoint of enzymology, myosin is an ATPase whose activity is stimulated by the interaction with actin. There is much evidence suggesting that the conformational changes of actin and myosin are tightly linked to cross-bridge cycling (Inoue et al., 1979). Although the binding sites involved in the interaction between myosin and actin molecules have been determined (Rayment et al., 1993), the role of these conformational changes and the interactions in muscle contraction remain to be elucidated. Therefore, novel tools will be useful for studying the molecular mechanism of contractile proteins including actin (Ohizumi, 1997).

Streptomycetes are in various aspects an interesting group of Gram-positive bacteria with the soil as their main habitat (Hensel et al., 1991). Thiotetromycin (Fig. 1), an antibiotic substance, was first obtained from the culture broth of *Streptomyces* sp. strain OM-674 (Omura et al., 1983). There have been no reports on the pharmacological and biochemical properties of thiotetromycin except its lipid metabolism inhibitory activity (Tomoda and Omura, 1993). The effect of thiotetromycin on actomyosin ATPase has not been reported yet. In this study, we present the first report indicating that thiotetromycin induces modulation of

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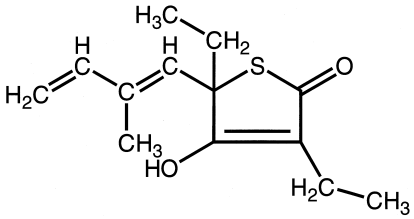

Fig. 1. Chemical structure of thiotetromycin.

actomyosin ATPase activity mediated through a conformational change in actin.

2. Materials and methods

2.1. Materials

Thiotetromycin was purified from the culture broth of *Streptomyces* sp. strain OM-674 as previously reported (Omura et al., 1983). Myosin B, myosin, actin, troponin and tropomyosin were prepared from rabbit skeletal muscle as described by Meng et al. (1995), Margossian and Lowey (1982), Spudich and Watt (1971), Kohama (1979) and Ebashi et al. (1971), respectively. Troponin–tropomyosin-free myosin B was prepared as described by Ebashi et al. (1968).

2.2. Superprecipitation assay

Superprecipitation was induced by adding 0.4 mM ATP in 0.3 mg/ml myosin B, 0.8 mM $CaCl_2$, 1 mM ethyleneglycol bistetraacetic acid (EGTA), 2 mM $MgCl_2$, 50 mM KCl and 20 mM Tris(hydroxymethyl)aminomethane (Tris)–HCl at pH 6.8 and 20°C, and the change in the absorbance at 660 nm was monitored.

2.3. ATPase assays

The reaction mixture for each ATPase was as follows (Ojima and Nishita, 1988; Kitada et al., 1989): 0.1 mg/ml myosin B or troponin–tropomyosin-free myosin B, 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 40 mM Tris–HCl (pH 6.8) for myosin B or troponin–tropomyosin-free myosin B ATPase; 0.1 mg/ml troponin–tropomyosin-free myosin B, 0.1 mg/ml troponin, 0.1 mg/ml tropomyosin, 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 40 mM Tris–HCl (pH 6.8) for the ATPase activity of myosin B reconstituted from troponin–tropomyosin-free myosin B, troponin and tropomyosin; various concentrations of actin and myosin, 2 mM ATP, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM Tris–HCl (pH 6.8) for the ATPase activity of actomyosin reconstituted from actin and myosin; 0.1 mg/ml myosin, 2 mM ATP, 500 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris–HCl (pH 6.8) for the Mg^{2+}–ATPase activity of myosin. The mixture was preincubated in the absence of thiotetromycin and ATP at 30°C for 5 min, followed by the addition of thiotetromycin and further preincubation. ATPase activity was determined from the amount of phosphate liberated, measured by the malachite green method described by Chan et al. (1986). In the control experiment, dimethyl sulfoxide was added instead of thiotetromycin. The final concentration of dimethyl sulfoxide was kept less than 0.5%.

2.4. Fluorescence enhancement of actin induced by thiotetromycin

The fluorescence intensity of intrinsic tryptophan residues of actin was recorded at 335 nm (excitation wavelength, 284 nm) with a Hitachi F-2000 fluorescence spectrophotometer. Thiotetromycin has no autofluorescence at the excitation and emission wavelengths used.

2.5. Fluorescence measurements of pyrenyl–actin

Actin purified from rabbit skeletal muscle was labeled at Cys-374 with *N*-(1-pyrenyl)iodoacetamide (Kouyama and Mihashi, 1981; Cooper et al., 1983). Pyrene-labeled actin was mixed with unlabeled actin to give a final concentration of 10% labeled actin. Mixed G-actin was polymerized for 3 h at 25°C in 50 mM KCl, 2 mM $MgCl_2$ and 2 mM Tris–HCl (pH 8.0). After thiotetromycin was added, the mixture was vortexed for 15 s and the fluorescence intensity excited at 365 nm and emitted at 407 nm was measured in a Hitachi F-2000 fluorescence spectrometer at 30°C and is given in the same relative values throughout. The total volume for assay was 1.0 ml.

2.6. Sedimentation experiment of actin

F-actin (0.05 mg/ml) in a solution containing 50 mM KCl, 2mM $MgCl_2$ and 2 mM Tris–HCl (pH 8.0) was centrifuged for 3 h at $100{,}000 \times g$. The protein concentration of the supernatant was measured (Lowry et al., 1951).

2.7. Surface plasmon resonance (SPR) measurement

The SPR670 biosensor (Nippon Laser and Electronics) has the potential to measure the interaction of biomolecules in real time. The binding event is monitored by using SPR detection (Karlsson et al., 1991; Yamamoto et al., 1997). The sensor chip SA surface was treated with 4,4-dithio dibutyric acid to immobilize protein. We detected the change of resonance angle after injection of 3×10^{-5} M thiotetromycin.

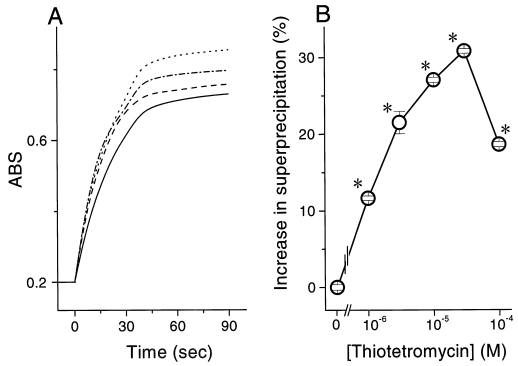

Fig. 2. Effect of thiotetromycin on superprecipitation of myosin B. (A) Typical recording traces of superprecipitation of myosin B in the presence of various concentrations (——: 0 μM, - - - - : 3 μM, ··· ··· ··· ··· ···: 10 μM, -·····-·····-·····: 30 μM) of thiotetromycin. Absorbance was measured at 660 nm. (B) Concentration-dependent effect of thiotetromycin on superprecipitation. The increase in superprecipitation is expressed as a percentage of the control activity (ABS_{660}, 0.6). The velocity of turbidity change was determined 30 s after application. Each point represents the mean ± S.E. from six experiments. * Significant difference from control.

2.8. Data analysis

Data are presented as the means ± S.E., and a statistically significant difference was determined by Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of thiotetromycin on superprecipitation of myosin B

The effect of thiotetromycin was examined on the superprecipitation of myosin B, an in vitro model of muscle protein contraction, by monitoring the turbidity change. After the addition of ATP, clearing occurred and then the turbidity increased. Thiotetromycin slightly enhanced the increase in turbidity without affecting clearing. As shown in Fig. 2, the maximum turbidity was increased by thiotetromycin. The initial velocity of the turbidity change increased with an increase in thiotetromycin concentration and reached a peak at 30 μM.

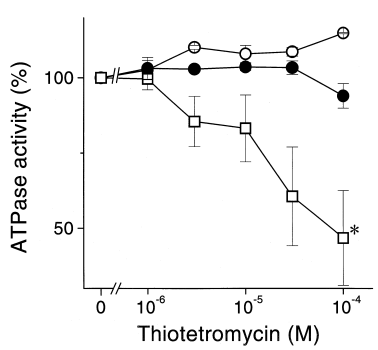

Fig. 3. Effects of various concentrations of thiotetromycin on the ATPase activity of myosin B. ○: Myosin B, ●: troponin–tropomyosin-free myosin B + troponin and tropomyosin, □: troponin–tropomyosin-free myosin B. The change is expressed as a percentage of the control activity. Each point represents the mean ± S.E. from three experiments. * Significant difference from control.

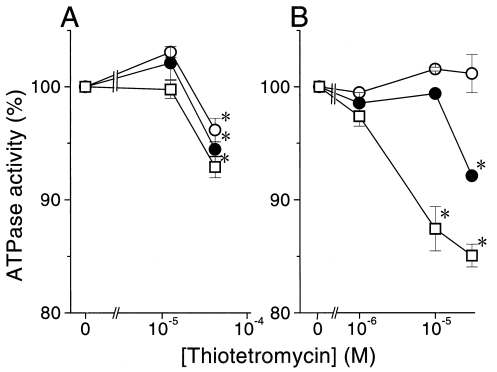

Fig. 4. Concentration-dependent effects of thiotetromycin on actomyosin ATPase activity. (A) Actomyosin ATPase activity with three different concentrations of myosin (○: 0.0025, ●: 0.005, □: 0.01 mg/ml) and 0.05 mg/ml actin. (B) Actomyosin ATPase activity with three different concentrations of actin (○: 0.1, ●: 0.15, □: 0.2 mg/ml) and 0.005 mg/ml myosin. The change was expressed as a percentage of the control activity. Each point represents the mean ± S.E. from three experiments. * Significant difference from control.

3.2. Effects of thiotetromycin on myosin B ATPase and other enzymes

The ATPase activity of myosin B was measured in the presence of various concentrations of thiotetromycin. As shown in Fig. 3, myosin B ATPase activity was slightly increased by thiotetromycin. But thiotetromycin caused a concentration-dependent decrease in troponin–tropomyosin-free myosin B ATPase activity. After the addition of troponin–tropomyosin complex to troponin–tropomyosin-free myosin B, thiotetromycin had no or little effect on the ATPase activity.

The effect of thiotetromycin on actomyosin ATPase was investigated at various concentrations of actin and

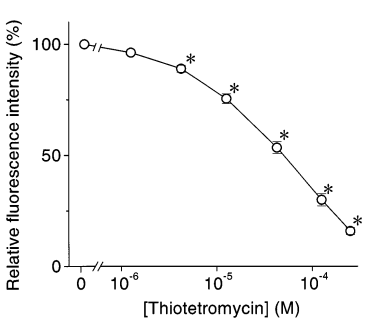

Fig. 5. Concentration-dependent effect of thiotetromycin on the fluorescence intensity of tryptophan in F-actin. The reaction mixture contained 0.05 mg/ml F-actin, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM HEPES–Tris (pH 6.8). The change was expressed as a percentage of the control activity. Each point represents the mean ± S.E. from three experiments. * Significant difference from control.

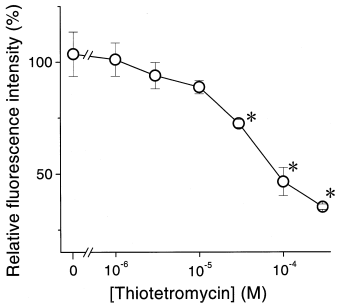

Fig. 6. Effects of different concentrations of thiotetromycin on fluorescence intensity of pyrenyl–F-actin. G-actin was polymerized for 3 h at 25°C in the presence of 50 mM KCl, 2 mM $MgCl_2$ and 2 mM Tris–HCl (pH 8.0), and then various concentrations of thiotetromycin were added. The change was expressed as a percentage of the control activity. Each point represents the mean ± S.E. from three experiments. * Significant difference from control.

myosin. As shown in Fig. 4B, in the presence of 0.2 mg/ml actin, thiotetromycin slightly decreased actomyosin ATPase activity in a concentration-dependent manner. The inhibitory effect of thiotetromycin was dependent on the concentration of actin (Fig. 4B), but not on the concentration of myosin (Fig. 4A). Thiotetromycin did not affect the Mg^{2+}–ATPase activity of myosin (data not shown). These results suggest that the effect of thiotetromycin on actomyosin ATPase activity is dependent on the concentration of actin, but not of myosin.

3.3. Effects of thiotetromycin on fluorescence intensity of tryptophan in actin

The fluorescence of intrinsic tryptophan has provided useful information on conformational changes of contractile protein molecules (Cooke, 1982). Addition of thiotetromycin to an actin solution caused a concentration-dependent decrease in the intensity of its fluorescence (Fig. 5), suggesting binding to actin.

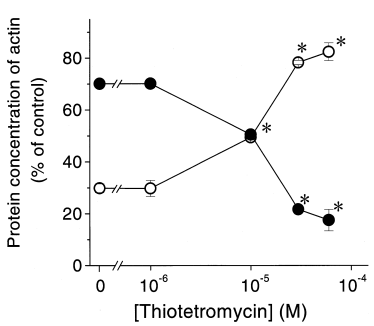

Fig. 7. Effect of thiotetromycin on sedimentation of F-actin. F-actin (0.1 mg/ml) in 50 mM KCl, 2 mM $MgCl_2$ and 2 mM Tris–HCl (pH 8.0) was centrifuged for 3 h at 100,000 × *g*. The protein concentration of the supernatant (○) and precipitate (●) after centrifugation was measured by the Lowry method. The change was expressed as a percentage of the control activity. Each point represents the mean ± S.E. from three experiments. * Significant difference from control.

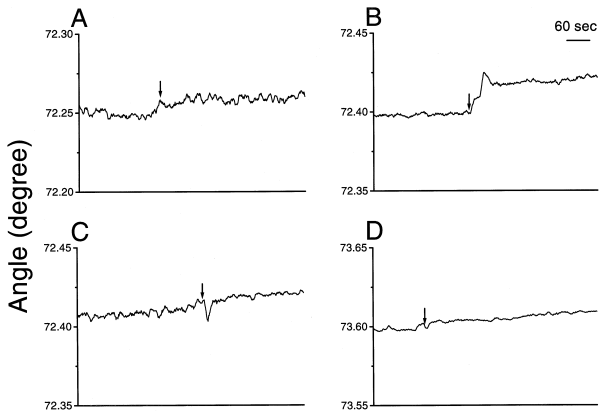

Fig. 8. Effect of thiotetromycin on contractile proteins in the SPR analysis. Typical recording traces of SPR signal of (A) myosin, (B) actin, (C) troponin and (D) tropomyosin after addition of 30 μM thiotetromycin. Thiotetromycin was added at the time indicated by the arrow. The data are representative of three experiments with similar results.

3.4. Effects of thiotetromycin on fluorescence intensity of pyrenyl–F-actin

To analyze further the effect of thiotetromycin, actin was labeled with *N*-(1-pyrenyl)iodoacetamide as an indicator of conformational changes in actin molecules. As seen in Fig. 6, thiotetromycin decreased the fluorescence intensity of pyrenyl–F-actin.

3.5. Effects of thiotetromycin on sedimentation of actin

A sedimentation experiment was carried out to verify the above observations. F-actin was sedimentable by centrifugation for 3 h at 100,000 × *g* (Fig. 7). However, thiotetromycin decreased the amount of actin sedimented by centrifugation in a concentration-dependent manner. These observations confirm that actin was depolymerized in the presence of thiotetromycin.

3.6. Effects of thiotetromycin on contractile proteins determined by SPR measurement

In order to detect the conformational change in contractile proteins, SPR measurements were conducted. Thiotetromycin changed the SPR signal of actin (but not of myosin, troponin and tropomyosin), suggesting that thiotetromycin bound to actin (Fig. 8).

4. Discussion

It is generally accepted that the contraction of striated muscles is produced by the cyclical interaction of myosin molecules with ''regulated'' actin filaments consisting of actin, troponin and tropomyosin coupled to the breakdown

of ATP. A rise in intracellular Ca^{2+} concentration triggers muscle shortening by initiating the sliding of the myosin and actin filaments relative to each other. Ca^{2+} regulates this process by reversibly binding to troponin, which is bound to tropomyosin (Farah and Reinach, 1995; Gagne et al., 1997). The troponin–tropomyosin complex regulates the actin–myosin interaction by preventing the binding of the myosin cross-bridge to actin in the absence of Ca^{2+} (Tobacman and Adelstein, 1986). The superprecipitation of natural actomyosin has been used as an in vitro model of muscle protein contraction in skeletal muscle (Szent-Györgyi, 1951). Thiotetromycin slightly enhanced the superprecipitation and the ATPase activity of myosin B. However, the ATPase activity of troponin–tropomyosin-free myosin B and of actomyosin reconstituted from myosin and actin was decreased by thiotetromycin in a concentration-dependent manner, suggesting that the interaction of myosin and actin was inhibited by thiotetromycin. Furthermore, it is an important observation that this compound failed to cause a decrease in ATPase activity in the presence of a regulatory protein of muscle contraction such as the troponin–tropomyosin complex. It is probable that the thiotetromycin-induced decrease in actomyosin ATPase activity may be physiologically significant because of the sensitivity to troponin–tropomyosin complex.

The stimulatory effect of thiotetromycin was dependent on the concentration of actin, but not on the concentration of myosin. The Mg^{2+}–ATPase activity of myosin was not affected by thiotetromycin, thus eliminating the possible involvement of a direct action of myosin ATPase in the modulation of actomyosin ATPase. The SPR biosensor was used in the present study to measure the interaction between contractile protein and thiotetromycin (Sota and Hasegawa, 1998). Thiotetromycin changed the SPR signal of actin, but not that of myosin, troponin or tropomyosin, suggesting that there was selective binding to actin. The similarity among the concentration-inhibition curves for thiotetromycin in the intensity of tryptophan fluorescence of actin, the ATPase activity of reconstituted actomyosin and the intensity of pyrenyl–F-actin indicates a close relationship among the conformational change of actin, the interaction between actin and myosin, and the depolymerization of actin. Furthermore, in the sedimentation experiment, thiotetromycin decreased the amount of actin sedimented, providing further support for the depolymerization of actin. These results suggest that thiotetromycin binds to actin molecules to cause a conformational change of actin monomers in filaments, resulting in modulation of the interaction between actin and myosin. It is also suggested that thiotetromycin depolymerized F-actin by changing the conformation of the molecule.

Thiotetromycin may be a useful tool for studying the relationships between the structure and function of contractile proteins and the interaction among actin, myosin and troponin–tropomyosin complex.

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