K-252a, a Novel Microbial Product, Inhibits Smooth Muscle Myosin Light Chain Kinase*

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Effects of K-252a, (8R*, 9S*, 11S*)-(−)-9-hydroxy-9-methoxy carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cy cloocta [ede]trindene-1-one, purified from the culture broth of Nocardiopsis sp., on the activity of myosin light chain kinase were investigated. 1) K-252a (1 × 10⁻⁸ M) affected three characteristic properties of chicken gizzard myosin-B, natural actomyosin, to a similar degree: the Ca²⁺-dependent activity of ATPase, superprecipitation, and the phosphorylation of the myosin light chain. 2) K-252a inhibited the activities of the purified myosin light chain kinase and a Ca²⁺-independent form of the enzyme which was constructed by cross-linking of myosin light chain kinase and calmodulin using glutaraldehyde. The degrees of inhibition by 3 × 10⁻⁴ M K-252a were 69 and 48% of the control activities with the purified enzyme and the cross-linked complex, respectively. Chlorpromazine (3 × 10⁻⁴ M), a calmodulin antagonist, inhibited the native enzyme, but not the cross-linked one. These results suggested that K-252a inhibited myosin light chain kinase by direct interaction with the enzyme, whereas chlorpromazine suppressed the enzyme activity by interacting with calmodulin. 3) The inhibition by K-252a of the cross-linked kinase was affected by the concentration of ATP, a phosphate donor. The concentration causing 50% inhibition was two orders magnitude lower in the presence of 100 μM ATP than in the presence of 2 mM ATP. 4) Kinetic analyses using [γ-³²P]ATP indicated that the inhibitory mode of K-252a was competitive with respect to ATP (Kᵢ = 20 μM). These results suggest that K-252a interacts at the ATP-binding domain of myosin light chain kinase. The direct action of the compound on the enzyme would explain the multivarious inhibition of myosin ATPase, superprecipitation, and of the contractile response of smooth muscle.

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Phosphorylation of the two 20-kDa light chains of myosin is recognized as a key regulatory process in smooth muscle contraction (for reviews see Refs. 1–3). The phosphorylation reaction is catalyzed by myosin light chain kinase. This enzyme shows high substrate specificity and is activated by Ca²⁺/calmodulin. Dephosphorylation of phosphorylated myosin light chain is catalyzed by myosin light chain phosphatase, which is Ca²⁺/calmodulin-independent. It is generally accepted that the phosphorylation of myosin is a prerequisite for the activation of myosin ATPase activity and actin-myosin interaction in various smooth muscle cells, i.e. the ATPase activity of the phosphorylated myosin is shown to be activated by actin, whereas in the dephosphorylated state, the activation by actin is not achieved. In addition, the activation of ATPase activity by actin correlated with the extent of myosin phosphorylation in reconstituted actomyosin (4). Actin-myosin interaction in natural actomyosin (called myosin-B) resulted in superprecipitation. The rate, but not the magnitude, of superprecipitation was also associated with the extent of myosin phosphorylation (5). Although the precise mechanism of this phenomenon has not been elucidated, both the superprecipitation and the ATPase activity of myosin-B have been used as in vitro models of contractile response.

The development of inhibitors of myosin light chain kinase may help to clarify the physiological function of the enzyme in cells and animals. Recently, K-252a has been isolated from the culture broth of Nocardiopsis sp. and found to inhibit phospholipid-sensitive, Ca²⁺-dependent protein kinase (protein kinase C) and calmodulin-dependent phosphodieserase (6–8). Further investigation revealed that the compound also inhibited cAMP- and cGMP-dependent protein kinases with comparable potencies and that its mode of inhibition for these three protein kinases was competitive with respect to ATP (9). Furthermore, K-252a has been found to inhibit the contraction caused by KCl, noradrenaline, and thromboxane A₂ in rabbit artery strips (10) and to inhibit the phosphorylation of the 20-kDa myosin light chain in intact platelets (11). From these findings, we have predicted that K-252a directly inhibits myosin light chain kinase. To demonstrate this hypothesis, we have monitored three activities of chicken gizzard myosin-B in the presence of K-252a: superprecipitation, the Ca²⁺-dependent ATPase activity, and the phosphorylation of the myosin light chain. In addition, we demonstrate the direct interaction of K-252a with myosin light chain kinase utilizing the purified enzyme and a Ca²⁺-independent form of the enzyme, prepared by cross-linking of the purified enzyme and calmodulin (12).

EXPERIMENTAL PROCEDURES

Materials

K-252a was isolated from the culture broth of Nocardiopsis sp. K-252 as described (6). Chlorpromazine was purchased from Sigma. DE52 was obtained from Whatman, Affi-Gel 15 from Bio-Rad, and phenyl-Sepharose CL-4B from Pharmacia LKB Biotechnologies Inc. [γ-³²P]ATP was purchased from Amersham Corp. All other reagents were of analytical grade.

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Preparation of Myosin-B

Myosin-B was prepared from chicken gizzard as described in another paper in detail.1

Preparation of Proteins

Calmodulin—Calmodulin was prepared from frozen pig brain, using modified procedures adapted from those reported by Yazawa et al. (18), Kakiuchi et al. (14), and Gopalakrishna and Anderson (15).

Phosphatase-free Myosin—Myosin was prepared from chicken gizzard according to the method of Ebashi (16) with some modifications (17). Myosin free from myosin light chain phosphatase was obtained after hydroxyapatite treatment of the above preparation of myosin as described (17).

Mixed Light Chains—Mixed light chains were prepared from the myosin preparation according to the method of Perrie and Perry (18).

Myosin Light Chain Kinase

Myosin light chain kinase was prepared by the method of Adelstein and Klee (19), with some modifications (17). In particular, ion exchange and gel filtration steps were omitted and calmodulin affinity column prepared from Affi-Gel 16 was used in place of calmodulin-Sepharose 4B.

Cross-linking of Myosin Light Chain Kinase and Calmodulin

Myosin light chain kinase and calmodulin were cross-linked covalently with glutaraldehyde as described by Nakamura and Nonomura (12). Briefly, myosin light chain kinase (0.33 mg) and calmodulin (0.22 mg) were mixed and dialyzed against 100 mM KCl, 10 mM Tris- HCl (pH 7.5), 0.1 mM CaCl2, 0.5 mM dithiothreitol, and 1 mM MgCl2. To the dialyzed solution (2 ml) was added 4 μl of 50% glutaraldehyde, and the mixture was incubated for 7 min at 0°C. After stopping the reaction by adding 100 μl of 1 M KSCN, the mixture was immediately dialyzed against 30 mM KCl, 20 mM Tris- HCl (pH 7.5), and 10 mM MgCl2. The dialyzed solution was used as cross-linked kinase.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (20). The concentrations of acrylamide determined by the method of Youngburg and Youngburg (21) with some modifications.

Phosphorylation of Myosin Light Chain

The reaction mixture used in the phosphorylation of myosin-B was the same as that used in the ATPase assays, except that the myosin-B concentration was 7.9 mg/ml and that the total volume was 0.5 ml. The reaction mixture used in the phosphorylation of the myosin light chain by purified myosin light chain kinase was as follows: 6.5 μg/ml myosin light chain kinase, 1.4 μg/ml calmodulin, 2 mg/ml phosphatase-free myosin, 2 mM ATP, 50 mM KCl, 10 mM imidazole- HCl (pH 6.85), 8 mM MgCl2, and either 0.1 mM CaCl2 or 1 mM EGTA. Calmodulin was omitted from the assays which used cross-linked myosin light chain kinase. After 5 min of incubation at 25°C, 0.1 ml of 20% trichloroacetic acid was added to stop the reaction. The acid precipitates were neutralized by solid Tris, solubilized by solid urea and 2-mercaptoethanol, and then subjected to urea-gel electrophoresis (22). The urea gel system was slightly modified.1 In the case of myosin-B, solid urea and 2-mercaptoethanol were added directly to the reaction mixture after 5 min of incubation. The degree of phosphorylation of the 20-kDa myosin light chain was determined by scanning the urea gels and measuring the area of peaks corresponding to the phosphorylated and unphosphorylated forms of the light chain using a Shimadzu Dual Beam Wave Length Chromatoscanner CS-930. For kinetic studies, 5-40 μM [γ-32P] ATP was added to a reaction mixture (0.25 ml) containing 25 mM Tris-HCl (pH 7.5), 4 mM MgCl2, 0.5 mM CaCl2, 1.4 μg/ml calmodulin, 108 μg/ml mixed light chains, 0.11 mg/ml myosin light chain kinase, and 0-50 mM K-252a. After preincubation for 5 min at 25°C without ATP, reaction was performed for 5 min at 25°C with ATP and terminated by adding half volume of 20% trichloroacetic acid. The acid precipitable materials were trapped on a nitrocellulose membrane filter (Toyo Roshi Co., Ltd.) and washed five times with 5% trichloroacetic acid. Radioactivity on the filter was counted using a Packard Tri-Carb Liquid Scintillation Spectrometer, model 4503.

RESULTS

Inhibitory Effects of K-252a on Superprecipitation, ATPase, and Phosphorylation of Myosin Light Chain in Myosin-B

Fig. 1 shows the effects of K-252a and chlorpromazine on the superprecipitation of myosin-B. K-252a inhibited both the initial rate and the maximal turbidity in a dose-dependent manner (Fig. 1a). The concentration causing 50% inhibition (IC50) of the initial rate was between 1 X 10-5 and 1 X 10-4 M (Table I). Chlorpromazine, which is known to inhibit calmodulin, also inhibited superprecipitation of myosin-B although its effective concentration was more than 10 times higher than that of K-252a (Fig. 1b, Table I).

The specific activity of the myosin-B ATPase was 5.61 and 9.86 nmol Pi/min/mg in the absence and presence of Ca2+, respectively.1 K-252a inhibited the Ca2+-dependent ATPase activity with an IC50 value of ~1 X 10-6 M (Table I). Chlorpromazine also inhibited the Ca2+-dependent ATPase with an IC50 value between 1 x 10-4 and 1 x 10-5 M (Table I). In contrast, the Ca2+-independent activity was not inhibited by

1 Y. Nonomura and S. Matsui, manuscript in preparation.

2 The abbreviation used is: EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid.1

3 Since myosin content was estimated to be 30-50% of total myosin-B protein on an SDS-gel, the specific activity to myosin protein would be 2-3-fold higher than the value described above.
The extent of myosin light chain phosphorylation was determined by densitometry of urea gels. The initial rate of superprecipitation was measured by monitoring changes in absorbance at 660 nm as shown in Fig. 1. The ATPase activity was measured as described under “Experimental Procedures.” Controls in the presence and absence of Ca²⁺ were set at 0% and 100% inhibition, respectively.

### Table I

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* NT, not tested.

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* NT, not tested.

**Fig. 2. Effect of K-252a on the phosphorylation of myosin light chain in myosin-B.** Myosin-B (7.9 mg of protein/ml) was incubated for 0 or 5 min at 25 °C. The incubation mixture contained, in 0.2 ml of solution, 10 mM imidazole-HCl (pH 6.85), 8 mM MgCl₂, 0.2 mM ATP, and either 0.1 mM CaCl₂ (Ca⁺⁺, +) or 1 mM EGTA (Ca⁺⁺, −), and the indicated concentrations of K-252a. Reaction was stopped by adding half volume of 20% trichloroacetic acid and the acid precipitates were subjected to urea-gel electrophoresis. UP, unphosphorylated and P, phosphorylated 20-kDa myosin light chain. 17K, 17-kDa myosin light chain.

**Fig. 3. Effect of K-252a on the phosphorylation of myosin light chain by myosin light chain kinase.** Phosphatase-free myosin (20 μg) was phosphorylated by myosin light chain kinase (1.3 μg) for 0 or 5 min at 25 °C. The reaction mixture, in a final volume of 0.2 ml, contained 10 mM imidazole-HCl (pH 6.85), 8 mM MgCl₂, either 0.1 mM CaCl₂ and 1.4 μg/ml calmodulin (Ca⁺⁺/CaM, +) or 1 mM EGTA (Ca⁺⁺/CaM, −), 2 mM ATP, and the indicated concentrations of K-252a. Reaction was stopped by adding half volume of 20% trichloroacetic acid to the mixture, and the acid precipitates were treated and analyzed by urea-gel electrophoresis as described under “Experimental Procedures.” UP, unphosphorylated and P, phosphorylated 20-kDa myosin light chain; 17K, 17-kDa myosin light chain.

**Table I**

| Effects of K-252a and chlorpromazine on the phosphorylation of myosin light chain, superprecipitation, and the Ca²⁺-dependent activity of ATPase in myosin-B |

Ca⁺⁺. K-252a was found to inhibit the phosphorylation of the myosin light chain, with an IC₅₀ value between 1 x 10⁻⁶ and 3 x 10⁻⁷ M (Table II). Chlorpromazine also inhibited phosphorylation by 88% at 3 x 10⁻⁴ M (Table II).

**Inhibitory Effects of K-252a on Phosphorylation of Myosin Light Chain by Cross-linked Complex of Myosin Light Chain Kinase and Calmodulin—Myosin light chain kinase which has been cross-linked with calmodulin in the presence of Ca⁺⁺ retains its catalytic activity when Ca⁺⁺ is absent (12). We used the cross-linked enzyme to determine whether K-252a inhibits myosin light chain kinase independently of Ca⁺⁺. The results are presented in Fig. 4. Thirty percent of the myosin light chain was phosphorylated by the cross-linked enzyme after incubation with 2 mM ATP and 1 mM EGTA for 5 min at 25 °C. K-252a inhibited the phosphorylation (Fig. 4a), with an IC₅₀ value near 3 x 10⁻⁶ M (Table II). The inhibitory activity of K-252a on the cross-linked kinase was comparable to that on the native kinase. In contrast, chlorpromazine showed no inhibition of the cross-linked kinase, even at 3 x 10⁻⁴ M (Table II). The fact that chlorpromazine was able to inhibit only the native enzyme is consistent with its established role as a calmodulin antagonist. On the other hand, K-252a apparently inhibits phosphorylation by direct interaction with the kinase since both the native kinase and the cross-linked enzyme were affected to a similar degree.

### Inhibitory Effects of K-252a on Phosphorylation of Myosin Light Chain by Purified Myosin Light Chain Kinase—The observations on myosin-B suggest that K-252a inhibits myosin light chain kinase. In order to demonstrate the possibility more directly, we examined a reconstituted system consisting of myosin light chain kinase, myosin, and calmodulin as described under “Experimental Procedures.” Phosphatase-free myosin was used to dephosphorylate the phosphorylated myosin light chain. The results are illustrated in Fig. 3. The myosin light chain was completely phosphorylated after incubation for 5 min at 25 °C with purified myosin light chain kinase, calmodulin, and ATP in the presence of
K-252a, a Myosin Light Chain Kinase Inhibitor

**Figure 4.** Effect of K-252a on the phosphorylation of myosin light chain kinase and calmodulin. Phosphatase-free myosin (0.2 mg) was phosphorylated by the cross-linked kinase (5 µl) for 5 min at 25 °C. The reaction mixture, in a final volume of 0.2 ml, contained 10 mM imidazole-HCl (pH 6.85), 8 mM MgCl₂, either 0.1 mM CaCl₂ (Ca²⁺, +) or 1 mM EGTA (Ca²⁺, −), and the indicated concentrations of K-252a. Reaction was started by adding 2 mM ATP (a) or 100 µM ATP (b) and stopped by adding half volume of 20% trichloroacetic acid to the mixture. The acid precipitates were treated and analyzed by urea-gel electrophoresis as described under “Experimental Procedures.” UP, unphosphorylated and P, phosphorylated 20-kDa myosin light chain; 17K, 17-kDa myosin light chain.

**Figure 5.** Kinetic analysis of myosin light chain kinase inhibition by K-252a. Mixed light chains (27 µg) were phosphorylated by myosin light chain kinase (29 ng) in a reaction mixture (0.25 ml) containing 25 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 0.5 mM CaCl₂, 1.4 µg/ml calmodulin, 5-40 µM [γ-³²P]ATP and 0 (●), 12.5 (◆), 25 (▲), 50 nM (○) K-252a. Radioactivity incorporated into myosin light chain was measured as described under “Experimental Procedures.” From the secondary replots of the slope of the lines as a function of the concentration of K-252a, Kᵣ value was determined to be 20 nM.

Since K-252a inhibits various protein kinases, such as protein kinase C and cAMP- and cGMP-dependent protein kinases, by competing with ATP (9), we next examined the effect of ATP concentration on the inhibition of cross-linked myosin light chain kinase by K-252a. The extent of myosin light chain phosphorylation obtained at 100 µM ATP is similar to that found at 2 mM ATP (Fig. 4b). K-252a strongly inhibited the cross-linked kinase at 100 µM ATP (Fig. 4b) with an IC₅₀ value between 1 × 10⁻⁷ and 3 × 10⁻⁷ M. This range is about 30 times lower than that found with 2 mM ATP (Table II).

These data suggest that K-252a inhibits myosin light chain kinase by competing with ATP.

**Kinetic Study of Myosin Light Chain Kinase Inhibition by K-252a**—In order to determine the extent of phosphorylation at lower concentration of ATP, [γ-³²P]ATP was used in the assay (see “Experimental Procedures”). Under these conditions, the ATP level is lowered to 5-40 µM and the inhibitory potency of K-252a is markedly increased. Double-reciprocal plots of ATP concentration and reaction rate indicate that the inhibition is competitive with respect to ATP (Fig. 5). The secondary replots of the slope of the line as a function of the concentration of K-252a (Fig. 5, inset) show that the Kᵣ value is 20 nM. This value is comparable to the Kᵣ values for protein kinase C and cAMP- and cGMP-dependent protein kinases (9).

**Discussion**

The results of this study demonstrate that K-252a directly inhibits myosin light chain kinase. The following evidence confirmed the direct interaction of K-252a with the enzyme. First, K-252a inhibited superprecipitation, the Ca²⁺-dependent activity of ATPase, and myosin light chain phosphorylation of myosin-B to similar extents (Table I). These findings suggested that the inhibition of myosin light chain phosphorylation by K-252a resulted in the inhibition of superprecipitation and ATPase. Second, the activities of both myosin light chain kinase and the cross-linked enzyme are inhibited by similar concentration of K-252a (Table II). Chlorpromazine, which is known to be a calmodulin antagonist, inhibited the native kinase but not the cross-linked kinase (Table II). Chlorpromazine apparently interacts with calmodulin to inhibit myosin light chain kinase but fails to inhibit the cross-linked kinase, which is no longer dependent on Ca²⁺. On the other hand, the inhibition by K-252a of both the native and the cross-linked enzymes appears to be caused by the direct interaction of the compound with the catalytic domain of myosin light chain kinase. Third, K-252a inhibited the cross-linked kinase more potently in the presence of 100 µM ATP than in the presence of 2 mM ATP (Table II). This suggests that the inhibitory effect of K-252a on the activity of myosin light chain kinase is due to competition with ATP. Fourth, genetic analysis with the purified enzyme demonstrated that K-252a inhibited the activity of myosin light chain kinase competitively with respect to ATP (Fig. 5). These results indicate that K-252a inhibits myosin light chain kinase by direct interaction with its ATP-binding domain. In a previous paper (9), we reported that K-252a inhibited protein kinase C and cyclic nucleotide-dependent protein kinases competitively with respect to ATP. Since the ATP-binding sites of protein kinases show substantial homology with each other (25-27), K-252a may recognize some common structure occurring therein.

Chlorpromazine also inhibited both superprecipitation and Ca²⁺-dependent ATPase activity in myosin-B, but failed to inhibit the phosphorylation of the myosin light chain within the concentration range investigated (Table I). The lack of inhibition by chlorpromazine appears merely to be due to the high concentration of myosin-B used in the assay system; i.e. in comparison to the assay conditions for superprecipitation and myosin ATPase, about 20-fold higher concentration of myosin-B was used to measure phosphorylation (see “Experimental Procedures”). Consequently, chlorpromazine might be countered by high concentration of calmodulin, one of the constituents of myosin-B.

In spite of the extremely low Kᵣ value (20 nM) of K-252a for myosin light chain kinase, the concentrations of the compound required to inhibit light chain phosphorylation in my-
K-252a, a Myosin Light Chain Kinase Inhibitor

Osawa and Hida (28) have shown that K-252a is an inhibitor of the calmodulin-dependent activity of cyclic-nucleotide phosphodiesterase, with $K_i$ value of 1.5 $\mu M$, and not to inhibit its independent activity (29). Therefore, under conditions where the concentration of ATP is high, some interaction of the compound with calmodulin may contribute to the inhibition of myosin light chain kinase.

Although many compounds have been known to inhibit myosin light chain kinase by interacting with calmodulin (for reviews see Refs. 28–30), there are few inhibitors of the enzyme which have primary effects on the active site of the enzyme. Recently, Hida and his co-workers (31, 32) have found that naphthalenesulfonamides, such as A-3 and ML-9, inhibited myosin light chain kinase competitively with respect to ATP with $K_i$ values of 7.4 and 3.8 $\mu M$, respectively. Malencik et al. (33) demonstrated that 9-anthroylcholine competed with ATP for a common binding site of myosin light chain kinase with a $K_i$ value of 20 $\mu M$. Here we have found that K-252a is also an inhibitor acting directly on myosin light chain kinase, but with a $K_i$ value of 20 nM (Fig. 5).

The K-252a concentrations inhibiting superprecipitation and ATPase compare to those suppressing the contraction stimulated by KCl, noradrenaline, and thromboxane A$_2$ in rabbit artery strips (10). This strongly suggests that the inhibition of myosin light chain kinase by K-252a is responsible for its suppression of the contractile response.

In conclusion, K-252a, a microbial product, was found to be a potent and direct inhibitor of myosin light chain kinase with $K_i$ value of 20 nM. This compound is expected to be a good tool for studying the function of myosin light chain kinase.

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REFERENCES