MOLECULAR PHARMACOLOGY, 17, 66–72



# Calcium-Regulated Modulator Protein Interacting Agents Inhibit Smooth Muscle Calcium-Stimulated Protein Kinase and ATPase

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Received April 19, 1979; Accepted September 6, 1979

SUMMARY

HIDAKA, H., T. YAMAKI, M. NAKA, T. TANAKA, H. HAYASHI AND R. KOBAYASHI. Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. *Mol. Pharmacol.* 17: 66–72 (1980).

Reagents such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), chlorpromazine, prenylamine, and $N^2$-dansyl-L-arginine-4-t-butylpiperidine amide (No. 233) that interact with $Ca^{2+}$-regulated modulator protein (modulator protein, calmodulin) were found to inhibit dose dependently not only $Ca^{2+}$-dependent protein kinase (myosin light chain kinase), but also $Ca^{2+}$-dependent ATPase of chicken gizzard actomyosin. Inhibition of $Ca^{2+}$-dependent ATPase by these agents was prevented by the addition of modulator protein. These agents did not inhibit calcium-independent $Mg^{2+}$-ATPase of actomyosin. $Ca^{2+}$-dependent transfer of $^{32}P_i$ from [$\gamma$-$^{32}$P]ATP to the 20,000-dalton light chain of the gizzard myosin in the presence of $Mg^{2+}$ was also inhibited dose dependently by these agents. The concentrations of these agents producing 50% inhibition of the $Ca^{2+}$-dependent ATPase activity were found to be similar to concentrations producing 50% inhibition of myosin light chain phosphorylation, thereby suggesting that the inhibition of $Ca^{2+}$-dependent ATPase of actomyosin by these drugs is due to their inhibition of myosin light chain phosphorylation. W-7 bound to $Ca^{2+}$ modulator protein complex, but not to the modulator protein in the presence of EGTA. No. 233 and chlorpromazine inhibited the binding of W-7 to the $Ca^{2+}$–modulator complex, suggesting that No. 233 and chlorpromazine bind to modulator protein. The modulator protein has two classes of W-7 binding sites: three functional sites with a high affinity for W-7 ($K_{W-7}$ = 11 $\mu$M) and nine sites with a low affinity for the drug ($K_{W-7}$ = 200 $\mu$M). W-7 did not show a significant binding to actin, myosin, tropomyosin, and bovine serum albumin at the concentration of the drug capable of binding to modulator protein. Troponin C was the only protein other than modulator protein that bound W-7 significantly but the affinity ($K_{W-7}$ = 25 $\mu$M) of this protein for W-7 was lower than that of modulator protein. These results suggest that agents that interact with modulator protein produce relaxation of smooth muscle by inhibition of modulator protein-dependent myosin light chain phosphorylation thus suppressing the actin–myosin interaction and concomitant myosin ATPase activation.

## INTRODUCTION

Cyclic nucleotide phosphodiesterase inhibitor produces various pharmacological effects on tissues such as blood vessels, platelets, etc.; however, details of the mechanism of action are not well understood. Phosphodiesterase inhibitors, such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), chlorpromazine, chlorprothixene, and desipramine have been found to produce relaxation of isolated rabbit aortic strips contracted with agonists such as KCl, $CaCl_2$, norepinephrine, histamine, serotonin, and angiotensin (1–3). Prenylamine (4), chlorpromazine (4, 5), chlorprothixene (2), and desipramine (5) have been reported to inhibit selectively $Ca^{2+}$-regulated modulator protein (modulator protein, calmodulin)-induced stimulation of the phosphodiesterase. We have recently reported evidence that relaxation of vascular strips by these inhibitors is due to a direct effect on the smooth muscle actomyosin system (1, 2). Thus, these

This work was supported in part by Grant-In-Aid for Scientific Research (B) 248124 from the Ministry of Education, Science and Culture, Japan, and a grant from the Japanese Atherosclerosis Foundation.

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0026-895X/80/010066-07$02.00/0

studies suggest that modulator protein plays an important role in smooth muscle contraction. Other investigators (6, 7) suggested that regulation by $Ca^{2+}$ of the actin–myosin interaction in smooth muscle is mediated by a protein kinase and a phosphatase. Dabrowska *et al.* (8) have recently demonstrated that modulator protein is a component of smooth muscle myosin light chain kinase. We now report that several drugs known to be phosphodiesterase inhibitors also inhibit myosin light chain kinase ($Ca^{2+}$-modulator-dependent protein kinase). Our findings suggest that inhibition of myosin light chain kinase results in inhibition of $Ca^{2+}$-$Mg^{2+}$-ATPase of actomyosin. These inhibitors can serve as useful pharmacological tools for elucidating the biological significance of modulator protein-mediated reactions.

## MATERIALS AND METHODS

*Experimental procedures.* The procedures used to isolate the various proteins were as follows. Chicken gizzard actomyosin and myosin were prepared according to the method of Sobieszek and Small (9). Briefly, chicken gizzards obtained up to 30 min after sacrifice were trimmed of the tough inner lining and fascia. The myofibrils were obtained by thorough homogenization of finely minced fresh gizzard followed by extraction with Triton X-100 and an extensive wash to produce a white residue. For the extraction of actomyosin, myofibrils were resuspended in an ice-cold medium of the following composition; 10 mM ATP (Na), 1 mM EDTA, 2 mM EGTA, 1 mM cysteine, 60 mM KCl, 40 mM imidazole (pH 7.1), and 100 mg/liter streptomycin. To this solution containing actomyosin extracted at the condition of low ionic strength as described above, 1 M $MgCl_2$ was slowly added to give a final concentration of 25 to 30 mM, and the actomyosin became opaque. The extract was then left at 4° for about 10 to 15 hr to form a compact gel. This gel was then centrifuged at 15,000*g* for 30 min and the pellet so obtained resuspended by gentle homogenization in a wash solution containing 60 mM KCl, 1 mM $MgCl_2$, 1 mM cysteine, 20 mM imidazole (pH 6.8), and 100 mg/liter streptomycin. The actomyosin was pelleted, resuspended, and stored in a buffer solution of 60 mM KCl, 1 mM cysteine, 40 mM imidazole (pH 7.0 at 25°) corresponding to that used for ATPase activity measurements. Experiments using this actomyosin were done within 2–3 days, as the calcium-sensitivity often decreased and disappeared in a preparation stored for 5–6 days. By centrifuging freshly extracted crude actomyosin for about 18 hr at 9,600 rpm (15,000*g*) we obtained a myosin fraction that contained little actin and tropomyosin impurities. This myosin was further precipitated by the slow addition of 1 M $MgCl_2$ with stirring to a final concentration of about 35 mM and was then collected by centrifugation. Chicken gizzard tropomyosin was prepared by the method of Ebashi *et al.* (10). The gizzard actin was purified according to the method of Spudich and Watt (11). Troponin was prepared by the method of Ebashi *et al.* (12) and troponin C was isolated by chromatography on DEAE-cellulose (13). Bovine brain modulator protein was purified according to the method of Teo *et al.* (14). Bovine aortic modulator protein was purified as described previously (2). Amino acid analysis of bovine aortic and brain modulator protein was kindly done by Dr. T. Kakuno, Division of Enzymology, Institute for Protein Research, Osaka University, Japan. The amino acid composition of bovine aortic modulator protein was similar to that of bovine brain modulator protein purified by the method of Teo *et al.* By comparison with modulator protein of the adrenal medulla (15), brain (16), heart (17), and gizzard (8), the bovine aortic modulator protein proved to be similar. The presence of tryptophan in the bovine aortic modulator protein was ruled out. We did not do experiments to identify trimethyllysine in the modulator protein molecule. Soluble and modulator protein-deficient cyclic nucleotide phosphodiesterase was prepared from human aortic smooth muscle as described previously (18).

*ATPase activity.* We measured ATPase activity of gizzard actomyosin using the method of Sobieszek and Small (9). ATPase activity measurements were carried out at 25° in a medium of 60 mM KCl, 250 μM ATP (Na), 1 mM cysteine, 40 mM imidazole (pH 7.0 at 25°), and either 2 mM EGTA plus 1.7 mM $MgCl_2$ or 0.1 mM $CaCl_2$ plus 1.7 mM $MgCl_2$. For assay at different concentrations of free $Ca^{2+}$, a Ca-EGTA/EGTA buffer system was used, taking the apparent dissociation constant for Ca-EGTA at pH 7.0 as 5 μM (19). The 3-ml assays were preequilibrated at 25° and the reaction initiated by adding ATP while mixing gently on a Vortex mixer. The reaction was terminated after 1 to 5 min by the addition of 2.0 ml 10% trichloroacetic acid. The preparations were filtered and inorganic phosphate was measured according to the method of Martin and Dotty (20).

*Phosphodiesterase activity.* Phosphodiesterase activity was measured by the method previously described (21). The reaction mixture contained 50 mM Tris–HCl, pH 8.0, 0.05 mM $CaCl_2$, 5 mM $MgCl_2$, 0.4 μM cyclic [$^3$H]-GMP (100,000 cpm), and the phosphodiesterase preparation in a total volume of 0.5 ml. 5′-[$^3$H]GMP formed by the phosphodiesterase was converted to [$^3$H]guanosine by the action of nucleotidase and the product isolated by cation exchange resin was counted in a liquid scintillation counter. Guanosine was confirmed by thin layer chromatography to be the only breakdown product from cyclic GMP in our assay (21). Recovery of guanosine was 95%.

*Phosphodiesterase activation assay.* Modulator protein, purified to homogeneity from bovine brain and aorta, was assayed by measuring the extent of stimulation of a fixed amount of modulator protein-deficient phosphodiesterase under standard conditions (18). One unit of modulator protein was equivalent to 10 ng protein. (One unit of modulator protein was defined as the amount necessary to produce 50% maximum activation of the modulator protein-deficient phosphodiesterase attainable under standard experimental conditions.) With a saturating concentration (150 ng) of modulator protein, the enzyme was stimulated approximately sixfold. This proved to be a consistent and reliable means of quantifying the activity of modulator protein.

*Phosphorylation procedures.* Phosphorylation of gizzard actomyosin was examined in the same reaction mixture used for measurement of ATPase activity except with larger amounts of the actomyosin (2–8 mg/ml) and

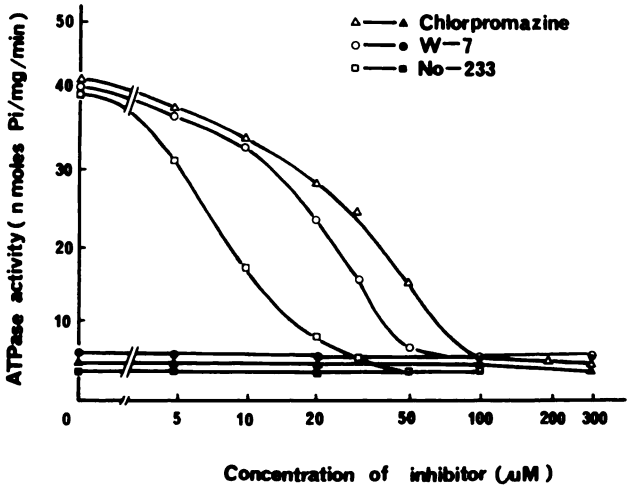


FIG. 1. *Effects of modulator protein interacting agents on gizzard actomyosin ATPase*

The reaction mixture (3.0 ml) contained: 40 mM imidazole pH 7.0, 1.7 mM $MgCl_2$, 1 mM cysteine, 60 mM KCl, 250 μM ATP, gizzard actomyosin (0.54 mg/ml), 100 μM $Ca^{2+}$ (open symbols) or 2 mM EGTA (closed symbols) and various concentrations of modulator protein interacting agents (○—●, W-7; △—▲, chlorpromazine; □—■, No. 233). The reaction was started by addition of ATP and carried out at 25°.

[γ-$^{32}$P]ATP (7.5 μCi/tube) in a total volume of 0.5 ml. The reaction was initiated by the addition of 250 μM ATP containing 7.5 μCi [γ-$^{32}$P]ATP and was terminated after 10 sec by the addition of 1 ml of ice-cold 10% trichloroacetic acid. Phosphorylation of the actomyosin was analyzed by two methods: (1) Following the addition of trichloroacetic acid, the reaction mixture was centrifuged at 2000 rpm for 5 min, the pellet was resuspended in ice-cold 5% trichloroacetic acid solution containing 2.5 mM ATP, and the centrifugation–resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 0.5 N NaOH and the radioactivity was measured with a liquid scintillation counter. (2) SDS (0.1 ml of a 10% solution) was added to the reaction mixture to terminate the reaction and appropriate amounts of the solution were subjected to tube gel electrophoresis. After electrophoresis, the gels were stained with Coomassie brilliant blue and the protein profile was recorded by densitometry or photography. The gel was then sliced at a width of 1 mm, each piece of gel was dissolved in 30% hydrogen peroxide solution by heating at 80° and counted by Cherenkov counting in a liquid scintillation counter.

*Sodium dodecyl sulfate-tube gel electrophoresis.* SDS–Tube gel electrophoresis was performed by the method of Weber and Osborn (22) using 10% polyacrylamide gels. Protein samples (25–100 μg) were applied to each gel. Coomassie brilliant blue was used for staining proteins. For determination of molecular weight by SDS–gel electrophoresis, soybean trypsin inhibitor, RNA polymerase, and bovine serum albumin were used as the standard proteins.

*Protein determination.* Protein was determined by the method of Lowry *et al.* (23) with purified modulator protein as a standard. This protein gave a single band upon SDS–gel electrophoresis.

*Drugs.* W-7 synthesized by the method of Hidaka *et al.* (3) was a gift from the Banyu Pharmaceutical Company Ltd. [$^3$H]W-7 (59.7 Ci/mol) was also donated by Banyu Pharmaceutical Company, Ltd. $N^2$-Dansyl-L-arginine-4-t-butylpiperidine amide (No. 233) was a gift from Professor Okamoto, Department of Physiology, Kobe University, and Mitsubishi Kasei Company, Ltd. Prenylamine was also gift from the Fujisawa Pharmaceutical Company, Ltd.

## RESULTS

*Inhibition of $Ca^{2+}$-dependent ATPase and phosphorylation of actomyosin.* The ATPase activity of chicken gizzard actomyosin was found to be dependent on calcium. Maximal stimulation of the ATPase activity (42 nM $P_i$/mg/min) was achieved in the presence of 10–100 μM $Ca^{2+}$. These data are in good agreement with the findings of Sobieszek and Small (9). Effects of W-7, No. 233, and chlorpromazine on the $Ca^{2+}$-dependent ATPase activity were examined in the presence of 100 μM $Ca^{2+}$ plus 1.7 mM $Mg^{2+}$ or 2 mM EGTA plus 1.7 mM $Mg^{2+}$. A typical experiment is shown in Fig. 1. These agents produced a concentration-dependent inhibition of $Ca^{2+}$-dependent ATPase but did not inhibit $Ca^{2+}$-independent ATPase. The concentrations of these agents producing 50% inhibition of $Ca^{2+}$-stimulated ATPase are summarized in Table 1. Inhibition by these agents of $Ca^{2+}$-dependent ATPase was prevented by addition of modulator protein in a dose-dependent fashion. A typical experiment is shown in Fig. 2. Inhibition of the ATPase by these drugs was not observed when the compounds were added to a reaction mixture that had been incubated for a few minutes, suggesting that all drugs tested had no effect on gizzard actomyosin ATPase in the phosphorylated form.

TABLE 1

*Effect of several compounds on modulator-stimulated phosphodiesterase, myosin light chain phosphorylation and $Ca^{2+}$-dependent ATPase*

Phosphodiesterase activity of a preparation purified from human aortic smooth muscle was measured in the presence of 200 units of modulator protein and 50 μM $CaCl_2$. $Ca^{2+}$-Dependent ATPase and $Ca^{2+}$-dependent phosphorylation of chicken gizzard smooth muscle actomyosin were measured in the presence of various concentrations of the compounds. The concentration of the compounds producing 50% inhibition of each enzyme activity was determined graphically. Dissociation constant for the compounds was obtained from kinetic studies as shown in Figs. 5 and 6. Values are means ± standard errors of three experiments.

| | Concentration ($I_{50}$, μM) of compounds producing 50% inhibition of | | | Apparent dissociation constant |
|---|---|---|---|---|
| | $Ca^{2+}$-$Mg^{2+}$-ATPase | Myosin light chain phosphorylation | Phosphodiesterase | |
| | | | | (μM) |
| W-7 | 27 ± 2 | 50 ± 3 | 67 ± 3 | 11[a] ± 1 |
| No. 233 | 11 ± 1 | 10 ± 1 | 17 ± 1 | 1.5[b] ± 0.3 |
| Chlorpromazine | 38 ± 2 | 50 ± 3 | 47 ± 2 | 2.4[b] ± 0.5 |
| Prenylamine | 34 ± 4 | 50 ± 3 | 18 ± 2 | 124[b] ± 20 |

[a] Value was obtained from Scatchard plots (Fig. 5).

[b] Value exhibits $K_i$ value of each compound for inhibition of binding of W-7 to modulator protein (see Fig. 6).

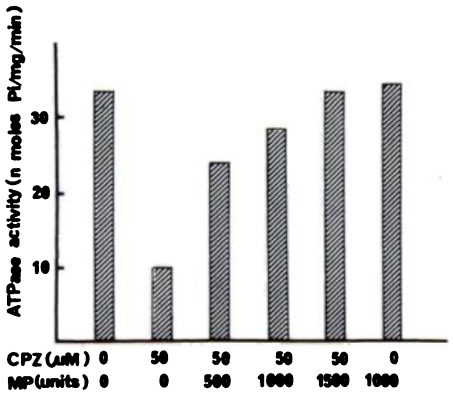


FIG. 2. *Recovery of chlorpromazine-induced inhibition of $Ca^{2+}$-dependent ATPase by addition of modulator protein*

The reaction mixture (3.0 ml) contained: 40 mM imidazole pH 7.0, 1.7 mM $MgCl_2$, 100 μM $Ca^{2+}$, 1 mM cysteine, 60 mM KCl, 250 μM ATP, gizzard actomyosin (0.54 mg/ml), 0 or 50 μM chlorpromazine, and various concentrations of purified bovine brain modulator protein. The reaction was started by addition of ATP and carried out at 25°.

These agents inhibited $^{32}P_i$ incorporation into the actomyosin from [γ-$^{32}$P]ATP at concentrations similar to those at which these agents produced inhibition of $Ca^{2+}$-stimulated ATPase (Table 1).

Actomyosin was analyzed by SDS–polyacrylamide gel electrophoresis after incubation of the actomyosin with [γ-$^{32}$P]ATP in the presence of $Ca^{2+}$. Only one protein band corresponding to 20,000 daltons was found to be phosphorylated. This phosphorylation was not observed when the actomyosin was incubated with [γ-$^{32}$P]ATP in the presence of EGTA. W-7, No. 233, prenylamine, and chlorpromazine produced concentration-dependent inhibition of $^{32}P_i$ incorporation into the 20,000-dalton component of actomyosin. A typical experiment using W-7 is shown in Fig. 3. The concentrations of these agents producing 50% inhibition of $^{32}P_i$ incorporation were estimated by plotting $^{32}P_i$ incorporation versus drug concentration and are summarized in Table 1.

*Interaction of W-7, No. 233, prenylamine, and chlorpromazine with modulator protein and other proteins.* The possible formation of the $Ca^{2+}$–modulator–agent complex has been investigated by the equilibrium binding technique of Hummel and Dreyer (24) on a Sephadex G-50 gel filtration column using tritium-labeled W-7. Figure 4 shows the elution profile for a typical binding experiment. The appearance of [$^3$H]W-7 peak and troughs in the profile is indicative of the binding of [$^3$H]W-7 to modulator protein (Fig. 4A) and to troponin C (Fig. 4B). The radioactivity peak coincides with the protein peak of the purified modulator protein. However, the radioactivity peak was not observed when EGTA instead of $Ca^{2+}$ was added (Fig. 4), suggesting that the binding of W-7 to modulator protein or troponin C is $Ca^{2+}$ dependent. For the calculation of the amount of bound W-7, only the data at peak regions have been used. The stoichiometry of the interaction between W-7 and $Ca^{2+}$–modulator protein or troponin C and the dissociation constant for the complex were determined from a Scatchard plot (Fig. 5) (25). The Scatchard plot with the lines by least-squares-analysis consists of two linear regions with different slopes (modulator protein). The results suggest that there are two types of W-7 binding sites on modulator protein that have different affinities. From the slopes, the dissociation constants of W-7 for the high and low affinity sites were calculated to be 11 and 200 μM, respectively. In kinetic studies (2) we found that the $K_i$ value of W-7 against modulator protein-induced stimulation of phosphodiesterase was 7.5 μM; thus, probably only the high affinity W-7 binding site is involved in the enzyme reaction. The stoichiometry of the interaction between W-7 and modulator protein may be calculated by the intercepts on the horizontal axis of the Scatchard plot extrapolated lines for the high and low affinity sites. This indicates that there are three high affinity W-7 binding sites and nine low affinity W-7 binding sites per mole of modulator protein. When gizzard actin, tropomyosin, myosin, and bovine serum albumin were examined for their binding to W-7, a significant radioactivity peak of W-7 was not observed. However, rabbit skeletal muscle troponin C was found to bind to W-7 significantly. The binding of W-7 to troponin C was weaker than the binding of W-7 to modulator protein (Fig. 4B). In contrast to modulator protein, there is one high affinity W-7 binding site per mol of troponin C. The dissociation constant of W-7 for this site is calculated to be 25 μM.

The effects of No. 233, chlorpromazine, and prenylamine on the binding of W-7 to modulator protein were studied in order to confirm that these agents directly interact with modulator protein. The stoichiometry of interaction between No. 233, chlorpromazine, or prenylamine and [$^3$H]W-7–modulator complex and the inhibitory constants of these agents for W-7-modulator complex were determined from a Dixon plot (Fig. 6) (26). No. 233 and chlorpromazine inhibited the binding of W-7 to modulator protein competitively and the $K_i$ values of these compounds against the binding of W-7 were 1.5 and 2.4 μM, respectively. These results suggest that the

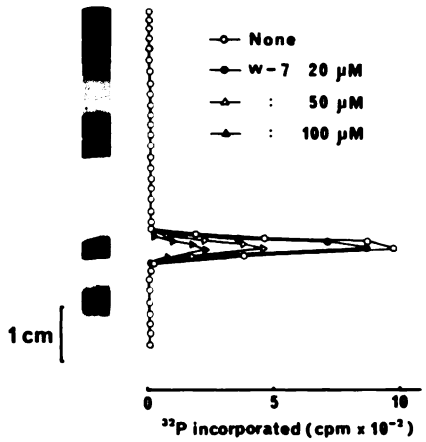


FIG. 3. *Effect of W-7 on $^{32}P$ incorporation into 20,000-dalton light chain of gizzard myosin*

The reaction mixture contained: 40 mM imidazole pH 7.0, 1.7 mM $MgCl_2$, 100 μM $Ca^{2+}$, 1 mM cysteine, 60 mM KCl, 250 μM ATP, various concentrations of W-7 (-○-, none; -●-, 20 μM; -△-, 50 μM; -▲-, 100 μM) and gizzard actomyosin (8 mg/ml). The same amount of phosphorylated actomyosin (100 μg) was applied on the gel. For further experimental details see MATERIALS AND METHODS.

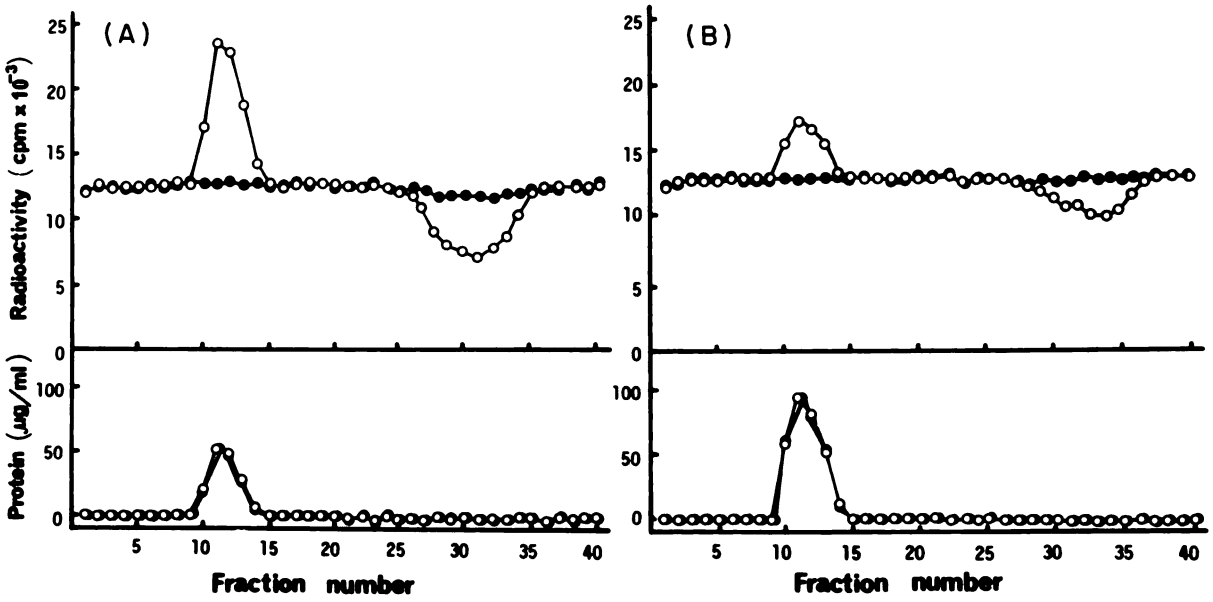


FIG. 4. *Elution profile for the measurement of W-7 binding by modulator protein or troponin C*

Sephadex G-50 (0.9 × 26.5 cm) was preequilibrated with the buffer containing 20 mM Tris–HCl, pH 7.5, 20 mM imidazole, 3 mM magnesium acetate, 0.5 μM [$^3$H]W-7, and 100 μM $Ca^{2+}$ (open circles) or 2 mM EGTA (closed circles) at 25°. Purified modulator protein (180 μg) (A) or troponin C (280 μg) (B) was used for each experiment. The gel filtration was carried out at 25° at a flow rate of 8.6 ml per hour and 0.86-ml fractions were collected. Samples (0.6 ml) of each fraction were analyzed for radioactivity.

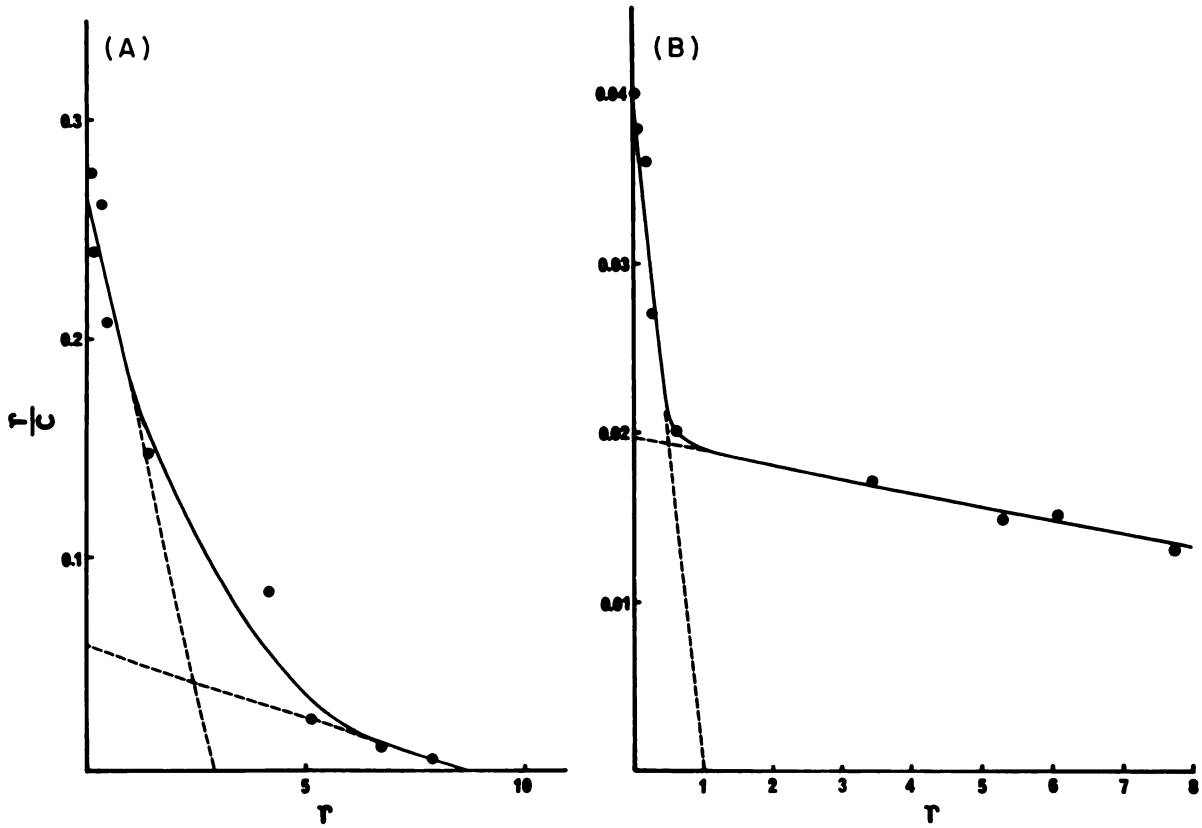


FIG. 5. *Scatchard plot for binding of W-7 by modulator protein (A) or troponin C (B)*

γ = The moles of W-7 bound per mole of modulator protein or troponin C and was calculated from the area under the peak region of the radioactivity profile. *C* = Concentration (μM) of W-7 in the buffer with which the modulator protein or troponin C is in equilibrium during the W-7 binding experiments.

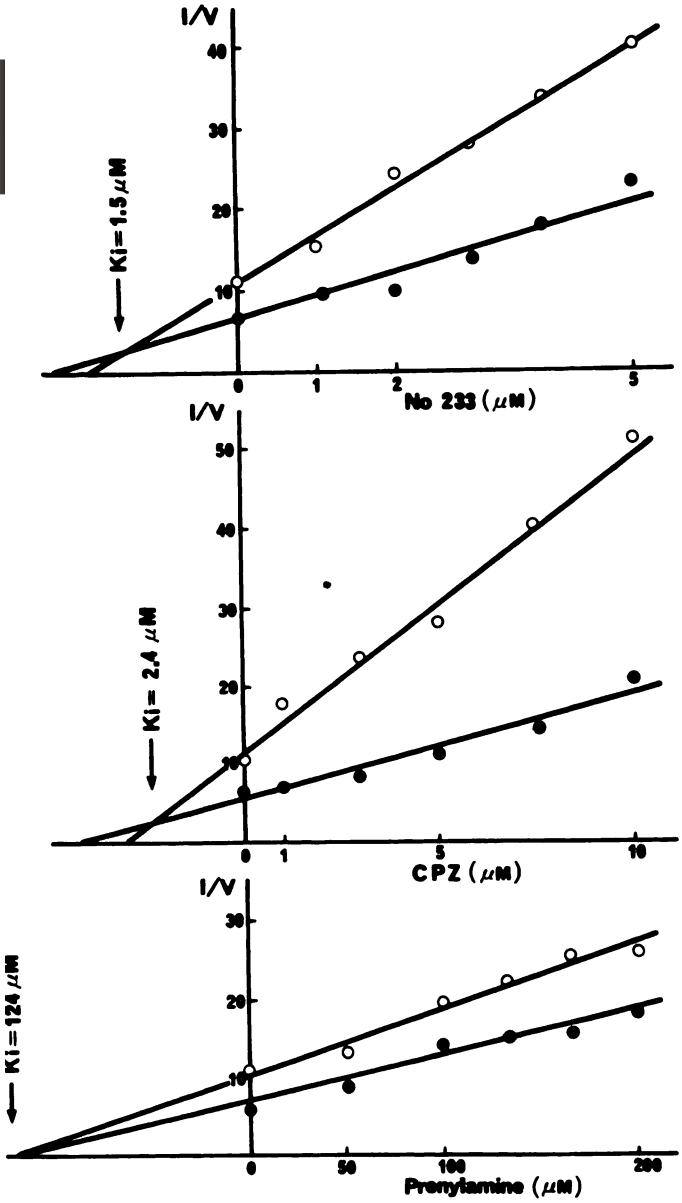


FIG. 6. *Kinetic analysis of chlorpromazine-, No. 233-, or prenylamine-induced inhibition of binding of W-7 to modulator protein*

The binding of W-7 (○, 0.25 μM; ●, 0.5 μM) to modulator protein was measured in the absence or presence of various concentrations of chlorpromazine, No. 233, or prenylamine. Velocity is expressed as moles of W-7 bound per mole of modulator protein.

W-7 binding sites of modulator protein are also responsible for binding of No. 233 or chlorpromazine. In contrast to No. 233 and chlorpromazine, prenylamine inhibited the binding of W-7 to modulator protein noncompetitively and the $K_i$ value of prenylamine against the binding of W-7 to modulator protein was 124 μM, suggesting that this drug might interact with modulator protein at sites different from W-7, No. 233, or chlorpromazine binding sites.

## DISCUSSION

Drugs interacting with modulator protein were found to produce relaxation of isolated vascular strips (1). Recently several investigators (8, 27) have suggested that modulator protein is involved in regulation by $Ca^{2+}$ of actin–myosin interaction in smooth muscle. The suggestion is based on the fact that myosin light chain kinase is composed of two components (catalytic component and modulator protein), both of which are essential for activity. In the presence of $Ca^{2+}$, the kinase phosphorylates the 20,000-dalton light chain of myosin and thereby allows the activation by the $Mg^{2+}$-ATPase activity. The present communication describes agents that interact with modulator protein and inhibit $Ca^{2+}$-stimulated protein kinase (myosin light chain kinase). The concentrations of these agents producing 50% inhibition of light chain phosphorylation are similar to concentrations required for 50% inhibition of the ATPase activity (Table 1), suggesting that the inhibition of light chain phosphorylation by these agents results in an inhibition of calcium-stimulated $Mg^{2+}$-ATPase of chicken gizzard actomyosin. The concentrations of these agents producing relaxation of aortic strips and the concentrations required for 50% inhibition of superprecipitation of bovine aorta smooth muscle actomyosin (2, 3) are much the same as the concentration of the drugs producing 50% inhibition of light chain kinase, as shown in Table 1. Our findings (1–3) suggest that modulator protein plays an important role in muscle contraction through a calcium-dependent phosphorylation of myosin light chain. However, these results do not exclude the possibility that these modulator interacting agents inhibit or affect other enzyme systems or proteins other than myosin light chain kinase. For example, W-7 was shown to bind to troponin C (Fig. 4B). Binding affinity of troponin C for W-7 was, however, lower than the affinity of modulator (Fig. 5). It is of interest that these inhibitors produce pharmacological actions such as hemolysis (28), inhibition of platelet aggregation[3] and vascular relaxation (2). Some of these pharmacological actions could be explained by their inhibition of modulator-dependent phosphorylation. It is likely that these modulator interacting agents also inhibit phosphorylation reactions catalyzed by other modulator-dependent protein kinase such as phosphorylase kinase (29) and glycogen synthase kinase (30). It should be noted that the agents tested in this experiment do not interact in a similar way with the modulator. Table 1 shows that prenylamine inhibited ATPase, light chain kinase, and $Ca^{2+}$-dependent phosphodiesterase to much the same extent as W-7, No. 233, or chlorpromazine but the apparent affinity of modulator for prenylamine was far less than that of other agents when the affinity was determined by measuring the ability of the drug to inhibit the binding of W-7 to modulator. Although this discrepancy cannot be explained unless direct binding of prenylamine to the modulator is determined, one possible explanation is that prenylamine binds to a site different from the W-7 binding site on modulator protein.

Pharmacological actions produced by these agents might be related not only to their inhibition of protein phosphorylation but also to their effects on cyclic nucleotides. It is known that $Ca^{2+}$, via its interaction with modulator protein, can regulate cyclic nucleotide metabolism. In the human aorta, $Ca^{2+}$-regulated cyclic nucleo-

[3] Unpublished observations.

tide phosphodiesterase hydrolyzes cyclic GMP preferentially (18). The use of modulator protein interacting agents, therefore, is one method for determining the importance of modulator-dependent reactions in biological reactions of various cells.

Although the modulator protein interacting agents have been shown to inhibit the *in vitro* reaction induced by enzymes requiring modulator protein, the utility of these agents for elucidation of the *in vivo* role of modulator protein should be restricted until the specificity of these drugs toward modulator protein has been clearly defined.

## ACKNOWLEDGMENTS

We thank M. Ohara, Kyoto University for critical reading of the manuscript, and Dr. T. Totsuka for helpful discussion.

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