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# Selective Inhibitors of $Ca^{2+}$-Binding Modulator of Phosphodiesterase Produce Vascular Relaxation and Inhibit Actin–Myosin Interaction

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SUMMARY

HIDAKA, HIROYOSHI, YAMAKI, TOKUO, TOTSUKA, TSUYOSHI & ASANO, MASAHISA (1979) Selective inhibitors of $Ca^{2+}$-binding modulator of phosphodiesterase produce vascular relaxation and inhibit actin-myosin interaction. *Mol. Pharmacol.* 15, 49–59.

To elucidate the role of $Ca^{2+}$-regulated activator protein (a protein modulator) of cyclic nucleotide phosphodiesterase in smooth muscle contraction, we determined the effects of various drugs that selectively inhibit a protein modulator-induced stimulation of cyclic nucleotide phosphodiesterase on contractile response of isolated rabbit aortic strip, and superprecipitation of bovine aorta smooth muscle actomyosin, considered to be an *in vitro* analogue of muscle contraction. Actomyosin preparation from bovine aorta smooth muscle contained approximately 200 units (2 $\mu$g) of protein modulator per mg actomyosin and superprecipitated by addition of ATP. Selective inhibitors of the stimulation of the phosphodiesterase by a protein modulator such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives, or psychotropic agents such as chlorpromazine, chlorprothixene, amitriptyline and desipramine produced relaxation of isolated aortic strips contracted by various agonists such as KCl, $CaCl_2$, norepinephrine, histamine, and prostaglandin $F_{2\alpha}$. The relaxation induced by these agents was not affected by treatment with adrenergic or cholinergic blocking agents such as propranolol and atropine, suggesting that such do not work through these receptors. These drugs not only reduced the extent of actomyosin superprecipitation but also prolonged the time required to attain the maximum superprecipitation, suggesting that they inhibit the superprecipitation of aorta smooth muscle actomyosin. The addition of various amounts of protein modulator prevented in dose-dependent fashion the inhibition by these compounds of actomyosin superprecipitation. The order of the potency of the drugs was much the same in assay systems such as inhibiting the stimulation of the phosphodiesterase by a protein modulator, superprecipitation of actomyosin and producing a relaxation of aortic strip. Other vasodilating drugs such as isoproterenol, sodium nitrite and fusaric acid had no effect on smooth muscle contraction process (superprecipitation of actomyosin). Papaverine, which is a potent inhibitor of cyclic nucleotide phosphodiesterase and not a selective inhibitor, served also as the control. However, even papaverine failed to inhibit the superprecipitation. Our results provide pharmacological evidence that a protein modulator participates in the regulation in smooth muscle contraction.

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## INTRODUCTION

Cheung (1) and Kakiuchi (2) reported that a protein activator (a protein modulator) of cyclic nucleotide phosphodiesterase exists in the mammalian brain. Both the enzyme activation by the protein modulator and the association between the enzymes and the modulator depend on the presence of $Ca^{2+}$ in the reaction media (3). This protein modulator is ubiquitously distributed in the animal kingdom (4). Several mammalian tissues and many invertebrates have been shown to contain high concentrations of the modulator, but very low amounts of $Ca^{2+}$-activatable phosphodiesterase (4–7). This observation led to the suggestion that the protein modulator may have functions other than its activation of phosphodiesterase. Recently several investigators (8, 9) have suggested that the regulation by $Ca^{2+}$ of the actin-myosin interaction in smooth muscle is thought to be made by a protein kinase and a phosphatase. In the presence of $Ca^{2+}$, the kinase phosphorylates the 20,000 dalton light chain of myosin and thereby allows the activation by action of the $Mg^{2+}$-ATPase activity. The kinase is not active in the absence of $Ca^{2+}$ and the phosphatase removes the phosphate groups from myosin molecule. Yagi and his associates (10) recently identified the activator protein of myosin light chain kinase as the $Ca^{2+}$-binding protein activator of the phosphodiesterase. More recently Dabrowska *et al.* (11) have reported that the protein modulator is a component of smooth muscle myosin kinase. Thus the regulatory proteins of smooth muscle are quite different from the troponin-tropomyosin system in skeletal muscle.

N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (12) and several psychotropic drugs such as chlorpromazine, chlorprothixene, amitriptyline and desipramine (13) were reported to be selective inhibitors of $Ca^{2+}$-activatable cyclic nucleotide phosphodiesterase.

In the present paper, we present evidence that these selective inhibitors produce a relaxation in vascular strips of rabbit aorta and inhibit bovine aorta smooth muscle actomyosin superprecipitation, an apparent *in vitro* analogue of muscle contraction. The essential role of a protein modulator in smooth muscle contraction is also discussed.

## METHODS

*Phosphodiesterase activity.* Soluble and protein modulator-deficient phosphodiesterase, the activity of which is dependent on $Ca^{2+}$ and a protein modulator, was partially purified from human aorta smooth muscle by DEAE-cellulose column chromatography (12). Phosphodiesterase activity was measured by the method previously described (14). The reaction mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.4 μM cyclic [$^3$H]GMP[3] (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 (14). Recovery of guanosine was 95%.

*A protein modulator activity.* Protein modulator was purified to homogeneity from bovine brain according to the method of Teo et al. (15) and from bovine aorta smooth muscle. This protein was purified from crude tropomyosin fraction (16) of bovine aortic smooth muscle by acid precipitation at pH 4.6, ammonium sulfate fractionation at the salt concentration from 35% to 65% saturation, DEAE-cellulose column and gel filtration on Sephadex G-100. A DEAE-cellulose column (1.5 × 20 cm) was equilibrated with 20 mM potassium phosphate buffer (pH 7.2). The dialyzed sample from ammonium sulfate fractionation was applied to the column, which was eluted with 200 ml of the same buffer. The column was then eluted with a linear gradient generated from 150 ml of the buffer and 150 ml of the buffer containing 0.7 M

[3] The abbreviations used are; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; cyclic GMP, guanosine cyclic 3′,5′-monophosphate; ATP, adenosine 5′-triphosphate.

KCl. The protein modulator was eluted between 0.25 M and 0.6 M KCl. The concentrated protein modulator solution from DEAE-cellulose column was applied to Sephadex G-100 column (1.5 × 85 cm) which was pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM magnesium acetate and 1 mM imidazole. The column was developed with the same buffer. Most of the protein was eluted prior to the activity peak of the modulator. The peak fractions of the modulator were collected and concentrated by ultrafiltration through a Sartorius membrane. This protein gave a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). One unit of a purified protein modulator was equivalent to 10 ng protein. One unit of a protein modulator was defined as the amount necessary to produce 50% maximum activation of the modulator-deficient phosphodiesterase attainable under standard experimental conditions (described above). With saturating concentration of the protein modulator, the enzyme is stimulated approximately 6-fold. This

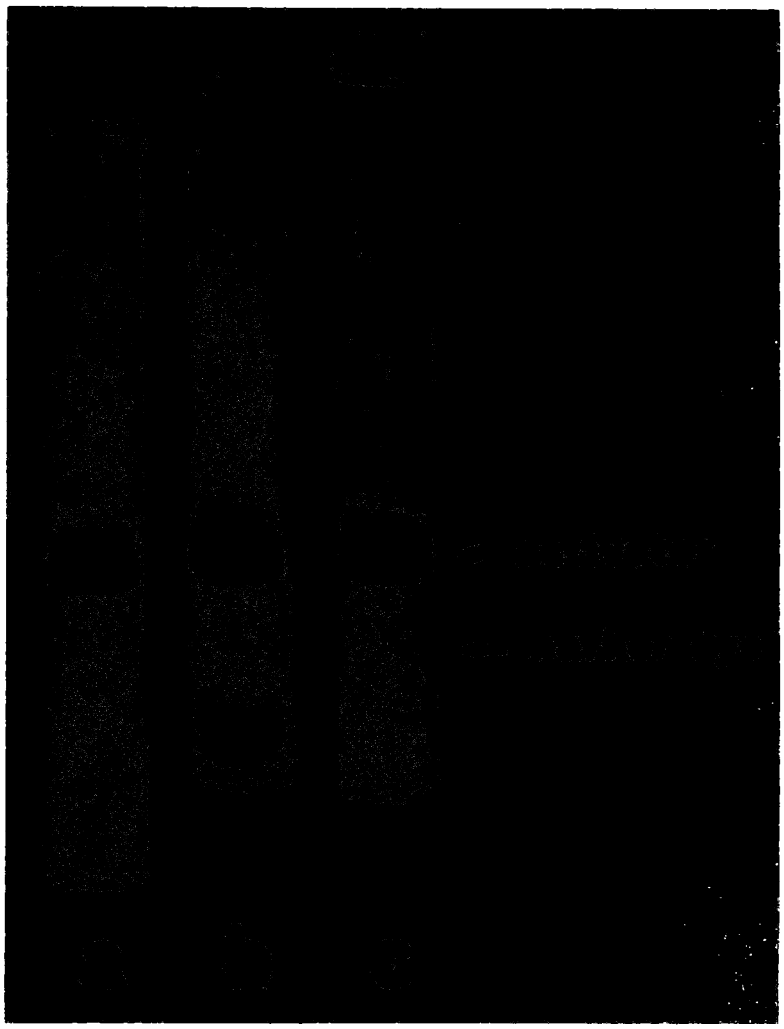


FIG. 1. *Disc polyacrylamide-gel electrophoresis of regulatory proteins from bovine tissues*

Disc electrophoresis was carried out using 10% polyacrylamide gel in 0.1 M phosphate buffer containing 0.1% SDS. a. protein modulator (30 μg) purified from bovine aorta tropomyosin fraction, b. protein modulator (25 μg protein) from bovine brain, c. mixture of aorta and brain protein modulator.

proved to be a consistent and reliable means of quantitating the activity of a protein modulator.

*Relaxation of isolated rabbit aortic strip.* Albino rabbits of either sex weighing 1.9–2.6 kg were used. Thoracic aortae (2.5–5.0 mm outside diameter) were removed and cut helically at an angle of approximately 45° to the longitudinal axis into strips of 2.5 mm in width and 30 mm in length after removal of adventitial connective tissue (17). The helical strip was fixed vertically between hooks in a water-jacketed (37 ± 0.5°) tissue bath containing 40 ml of modified Krebs-Henseleit solution (18). The composition of the bathing solution was as follows (in mM); NaCl, 115.0; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgCl_2 \cdot 6H_2O$, 1.2; $NaHCO_3$, 25.0; $KH_2PO_4$, 1.2 and dextrose, 10.0. The tissue bath solutions were maintained at 37° ± 0.5° and bubbled with a mixture of 95% $O_2$ and 5% $CO_2$. The upper end of the strip was connected to the lever of a force-displacement transducer (SB-1T, Nihon Koden Kogyo Co., Tokyo, Japan) by silk thread. An initial resting tension of 1 g was applied to the aortic preparation. Cumulative dose-response curves for vascular relaxing responses of the agonists were obtained by increasing concentrations by a factor of about 3 while the previous dose remained in contact with the tissue (19). Each concentration was added only after the effect of the previous concentration had reached a maximum and remained constant. Concentrated stock solution (after adjusting pH 7.4) of drugs was added directly to the bathing solution in a volume of 0.4 ml to give the final concentration desired. Effects of various blocking agents such as propranolol and atropine on drug-induced relaxation were determined with these blocking agents prior to the addition of prostaglandin $F_{2\alpha}$. Whenever an $ED_{50}$ value was determined, responses to drugs were calculated as a percentage of the maximum relaxation obtained with the drugs. The $ED_{50}$ value was obtained from a plot of percent relaxation *versus* log concentration of the compounds.

*Superprecipitation of actomyosin (myosin B) from bovine aorta smooth muscle.* Calcium-sensitive actomyosin was prepared from bovine aortic smooth muscle tissue obtained from a local slaughter house. The smooth muscle was chopped with scissors, ground in a chilled meat grinder and blended with three volumes of solution containing 1 mM $NaHCO_3$ and 2 mM EDTA per gram tissue at low speed in a Waring blender in two 15-second operations. The material was kept in an ice bath for 30 min, centrifuged at 7,000 × *g* for 10 min, and the precipitate subjected to two additional suspension-precipitation cycles, following the same procedure.

This precipitate was dissolved in three volumes of Weber-Edsall solution (KCl, 0.6 M; $NaHCO_3$, 0.04 M; $Na_2CO_3$, 0.01 M) using a polytron PT-10 homogenizer. The supernatant solution which was obtained from this homogenate by centrifugation at 20,000 × *g* for 20 min was diluted with three volumes of distilled water. When salt concentration was brought to below 0.3 M by dilution with distilled water, actomyosin became insoluble. Actomyosin thus precipitated was collected by centrifugation and redissolved in 0.6 M of KCl solution. These treatments were repeated three times and the final 0.6 M KCl solution containing actomyosin was dialyzed against the same solution. The actomyosin preparation thus obtained from bovine aorta smooth muscle always contained approximately 200 units (2 μg) of protein modulator per mg actomyosin. When this actomyosin preparation was washed with 2 mM KCl solution containing 2 mM $NaHCO_3$ and 0.5 mM EGTA, this washed preparation lost protein modulator to some extent. The actomyosin preparation which was found to contain below a few units of modulator per mg actomyosin is referred as modulator-deficient actomyosin and was also used for superprecipitation study. Tropomyosin was purified from bovine aorta smooth muscle according to the method of Ebashi *et al.* (16). Superprecipitation of the actomyosin (0.3 mg protein/ml) was followed by an increase in $OD_{550}$ nm at 24° after addition of 250 μM of $Na_2ATP$ in the presence of 50 mM Tris-maleate, 60 mM KCl, 1.7 mM $MgCl_2$ and 100 μM $CaCl_2$ at pH 6.5 in a total volume of 3 ml unless otherwise stated. $T_{1/2}$ was defined as the time attaining half max-

imal increase in $OD_{550}$ nm. ATPase activity was assayed by measuring liberation of inorganic phosphate according to the method of Martin and Doty (20). The protein concentration was measured by the procedure of Gornall *et al.* (21). Concentrated stock solutions (after adjusting pH 6.5) of the drugs were added directly to the reaction mixture in a volume of 50 μl to give the final concentration desired, before the addition of ATP.

## MATERIALS

We obtained bovine aorta smooth muscle tissue from aortae procured freshly from the slaughter house. Human aortae were obtained within four hours of autopsy from three Japanese patients, one of whom died of a heart attack (54-year-old male), one of stomach cancer (24-year-old female) and one with pneumonia (62-year-old male). N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), its derivatives (W-9, W-10) and fusaric acid were kindly provided by Banyu Pharmaceutical Co; chlorpromazine by Smith Kline & French Laboratories; chlorprothixene by Hoffman-La Roche; amitriptyline by Merck Sharp & Dohme; and desipramine by Merrel National Laboratories. 1-Norepinephrine bitartrate, histamine diphosphate and sodium nitrite were obtained from Wako; prostaglandin $F_{2\alpha}$ from Ono Pharmaceutical; papaverine and atropine sulfate from Katayama; dl-propranolol and isoproterenol hydrochloride from Sigma. All drugs were prepared daily and kept on ice during the course of the experiment.

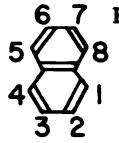
TABLE 1

*Effect of several pharmacological agents on activated and unactivated phosphodiesterase, isolated rabbit aortic strip and superprecipitation of actomyosin from bovine aorta*

Phosphodiesterase activity of a preparation partially purified from human aortic smooth muscle was measured in the presence and absence of 200 units of activator and various concentrations of the compounds under study, using 0.4 μM cyclic GMP as substrate. The addition of 200 units of a protein modulator produced about a 6-fold increase in phosphodiesterase activity. The addition of prostaglandin $F_{2\alpha}$ of .5 μM caused a sustained contraction in aortic strip and preparations thus contracted showed good relaxation in response to vasodilating drugs. The molar concentration of $ED_{50}$ of the compounds was obtained graphically from a dose-dependent relaxation curve of each compound. Superprecipitation of bovine aorta actomyosin after the addition of 250 μM ATP was determined by measuring the increase in optical density and expressed as time ($t_{1/2}$, min) attaining half maximal increase in $OD_{550}$ nm. Inhibition of superprecipitation by the compounds was calculated as follows:

$$[1 - (t_{1/2}\ \text{control}/t_{1/2}\ \text{compound})] \times 100.$$

| | Phosphodiesterase inhibition ($I_{50}$ μM) | | Vascular relaxation | Inhibition of superprecipitation |
|---|---|---|---|---|
| | activated | unactivated | $ED_{50}$ | $I_{50}$ |
| | | | μM | μM |
| N-(aminohexyl)-halogen-naphthalenesulfonamide | | | | |
| W-10 (5-Br, 2-R)[a] | 23 | 750 | 45 | 30 |
| W-9 (5-Cl, 2-R)[a] | 45 | 1300 | 72 | 46 |
| W-7 (5-Cl, 1-R)[a] | 67 | 2700 | 129 | 50 |
| Chlorprothixene | 36 | 50 | 30 | 16 |
| Chlorpromazine | 47 | 110 | 36 | 28 |
| Desipramine | 300 | 2000 | 59 | 78 |
| Amitriptyline | 250 | 1100 | 65 | 100 |
| Papaverine | 5.5 | 6 | 22 | no effect[b] |

[a] Structure:  R: $SO_2NH(CH_2)_6NH_2 \cdot HCl$.

[b] Papaverine did not affect superprecipitation of actomyosin up to 0.1 mM.

## RESULTS

*Inhibition of phosphodiesterase activity by various drugs in the presence and absence of a protein modulator.* Table 1 summarizes the effect of various drugs on the inhibition of phosphodiesterase in the presence and absence of a protein modulator, inhibition of superprecipitation of actomyosin and $ED_{50}$ values for relaxation of isolated aortic strips. The concentrations of drugs except papaverine producing 50% inhibition of phosphodiesterase activity in the presence of a protein modulator were less than those in the absence of a protein modulator (Table 1). The selective inhibition of the activation of phosphodiesterase by W-7 or psychotropic drugs has been already reported (12, 13). Increasing a protein modulator concentration in the presence of $Ca^{2+}$ is also reported to overcome W-7-induced (12) or psychotropic drug-induced (13) inhibition of phosphodiesterase activation. However, increasing calcium concentration affected neither the W-7-induced inhibition of phosphodiesterase activation (Fig. 2) nor the psychotropic drug-induced inhibition (13). Kinetic analysis of W-7-induced inhibition of activation of phosphodiesterase revealed that W-7 inhibits this activity in a competitive fashion with a protein modulator (Fig. 3). Psychotropic drugs also reportedly inhibit phosphodiesterase activity competitively with a protein modulator (13). Papaverine, which is a potent inhibitor of phosphodiesterase, did not affect the activation of phosphodiesterase by a protein modulator, and the concentration producing 50% inhibition of the phosphodiesterase was approximately 6 μM both in the presence and absence of protein modulator (Table 1).

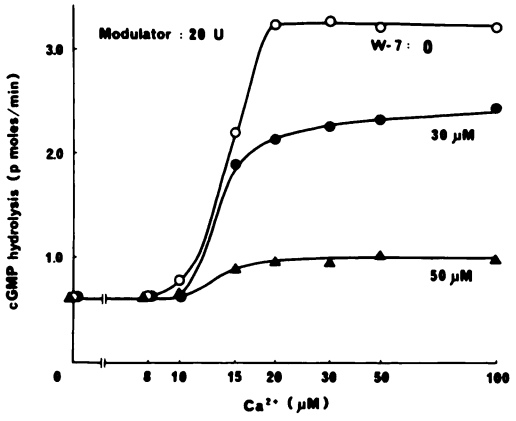



FIG. 2. *Effect of increasing calcium concentration on inhibition of activation of phosphodiesterase induced by W-7*

○ = W-7 (0); ● = W-7 (30 μM); ▲ = W-7 (50 μM). $Ca^{2+}$-dependent cyclic nucleotide phosphodiesterase was isolated by DEAE-cellulose column chromatography from human aorta smooth muscle and did not contain a protein modulator. Enzyme activity was measured in the presence of 20 units of a protein modulator. Each point is the mean of two determinations.

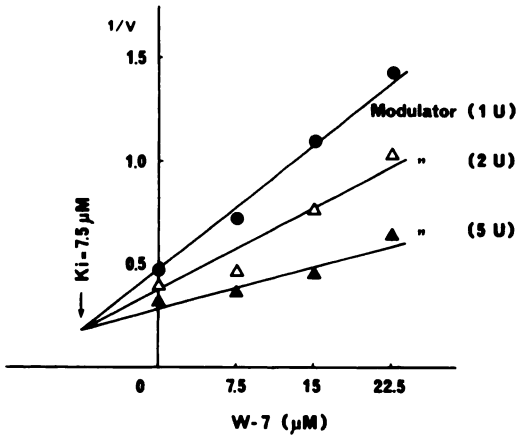



FIG. 3. *Kinetic analysis of W-7-induced inhibition of activation of phosphodiesterase was carried out by using Dixon plots (22)*

$Ca^{2+}$-dependent cyclic nucleotide phosphodiesterase was purified by DEAE-cellulose column chromatography. Enzyme activity was measured in the presence of 100 μM $CaCl_2$ and the substrate (cyclic GMP) concentration was 0.4 μM. Duplicate determinations were done in each experiment.

*Relaxation induced by various phosphodiesterase inhibitors in aortic strips.* The addition of prostaglandin $F_{2\alpha}$ in a concentration of .5 μM caused a sustained contraction in the aortic strip. Mean value of the contractile tension was 1039 ± 61 mg (N = 67). The preparation thus contracted showed good relaxation in response to vasodilating drugs. The addition of various drugs in concentrations ranging from 1 μM to 0.3 mM elicited dose-dependent relaxations. The molar concentration of $ED_{50}$ is shown in Table 1. The addition of W-9, W-10, chlorpromazine, chlorprothixene, amitriptyline and desipramine also produced a significant relaxation of thoracic aorta con-

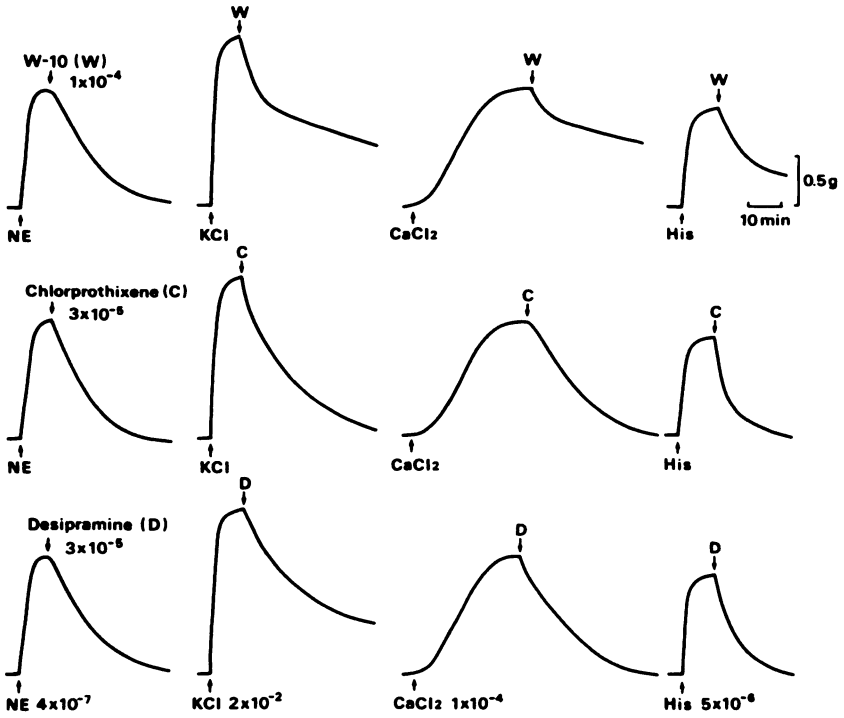



FIG. 4. *Responses of a strip of rabbit thoracic aorta to several selective inhibitors of $Ca^{2+}$-dependent cyclic nucleotide phosphodiesterase*

The preparation was contracted with .4 μM norepinephrine (NE), 20 mM KCl, .1 mM $CaCl_2$ and 5 μM histamine (His). N-(6-aminohexyl)-5-bromo 2-naphthalenesulfonamide (W-10), chlorprothixene and desipramine at concentrations of .1 mM, 30 μM and 30 μM, respectively, were added at the peak period of contraction by each contractile agonist (↓).

tracted by norepinephrine (0.4 μM), histamine (5 μM), KCl (20 mM) and $CaCl_2$ (0.1 mM). Typical experiments of some of these drugs are demonstrated in Fig. 4. The addition of these agonists at the concentration indicated above caused a sustained contraction in the aortic strips at least for 20 min (norepinephrine and histamine) and 60–120 min (KCl, $CaCl_2$ and prostaglandin $F_{2\alpha}$) and contractile tensions developed by these agonists were as follows; 1125 ± 165 mg (norepinephrine, N = 7), 995 ± 145 mg (histamine, N = 7), 1690 ± 206 mg (KCl, N = 8) and 1130 ± 194 mg ($CaCl_2$, N = 6). Effects of various blocking agents on the drugs-induced relaxation in aortic strip contracted by prostaglandin $F_{2\alpha}$ were then examined. The relaxation of aortic strip produced by W-7, W-9, W-10, chlorpromazine, chlorprothixene, desipramine and amitriptyline was not affected by pretreatment with 5 μM propranolol and 10 μM atropine. Propranolol and atropine employed in this experiment antagonized responses which were produced by isoproterenol and acetylcholine, respectively. These results indicate that the drugs-induced relaxation was not mediated through receptors which could be blocked by propranolol and atropine.

*Effect of several pharmacological agents on superprecipitation of actomyosin from bovine aorta.* ATP-induced-superprecipitation phenomenon of actomyosin is considered to be an *in vitro* analogue of muscle contraction (23, 24, 25). We examined the effect of selective inhibitors of a protein modulator-induced stimulation of phosphodiesterase on superprecipitation of the actomyosin from bovine aorta smooth muscle. In contrast to skeletal muscle actomyosin, addition of ATP at concentrations of 10–20 μM increased the extent of superprecipitation (Δ $OD_{550}$ nm) of bovine aortic smooth muscle actomyosin but did not produce a marked change in $t_{1/2}$ (Fig. 5). A constant level of superprecipitation (Δ $OD_{550}$ nm)

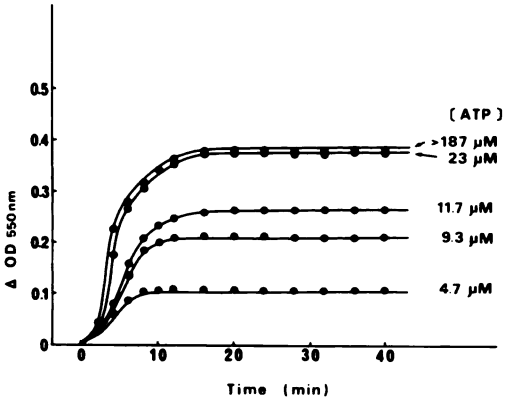



FIG. 5. *Effect of various concentrations of ATP on superprecipitation of the aorta smooth muscle actomyosin*

The reaction mixture consisted of actomyosin (0.3 mg/ml), KCl (60 mM), $MgCl_2$ (1.7 mM), $CaCl_2$ (100 μM) and Tris-maleate buffer (50 mM) pH 6.5 in a total volume of 3 ml. The reaction was started by addition of ATP and carried out at 24°. Extension of superprecipitation was determined by measuring the increase in $OD_{550}$ nm.

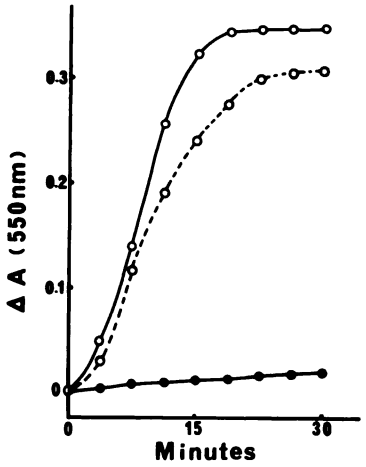



FIG. 6. *Superprecipitation of the modulator-rich actomyosin and the modulator-deficient actomyosin*

Superprecipitation of the modulator-rich actomyosin (0.3 mg/ml) (—○—), the modulator-deficient actomyosin (0.3 mg/ml) (—●—) and the modulator-deficient actomyosin + purified protein modulator (200 units, 2 μg) + purified tropomyosin (0.1 mg/ml) (- - -○- - -). The reaction mixture consisted of KCl (60 mM), $MgCl_2$ (1.7 mM), $CaCl_2$ (100 μM), Tris-meleate buffer (50 mM) pH 6.5 and ATP (250 μM).

was observed with more than 20 μM ATP. This actomyosin preparation was found to contain 200 units (2 μg protein equivalent) of protein modulator per mg actomyosin. However the actomyosin preparation which was found to lose a protein modulator by washing with 2 mM KCl solution containing 2 mM $NaHCO_3$ and 0.5 mM EGTA did not superprecipitate. The protein modulator content in such actomyosin was below 2 units per mg actomyosin. However this modulator-deficient actomyosin did superprecipitate by additions of 200 units of protein modulator and 0.3 mg of tropomyosin (Fig. 6). These results indicate that a protein modulator plays an essential role in the superprecipitation of actomyosin.

The effect of the compounds on superprecipitation of the modulator-rich actomyosin from bovine aortic smooth muscle was examined in the reaction mixture containing 50 mM Tris-maleate (pH 6.5), 100 μM $CaCl_2$, 1.7 mM $MgCl_2$, 250 μM ATP, and 0.3 mg/ml actomyosin. ATPase activity measured in this condition was 24 nmole/min/mg protein. Fig. 7 illustrates inhibition of superprecipitation of smooth muscle actomyosin by several vasodilating agents which inhibit selectively the stimulation of phosphodiesterase by a protein modulator. The figure shows that these drugs not only prolong the time required to attain maximum superprecipitation but also affect the extent of superprecipitation (maximum superprecipitation). When exogenous protein modulator was added, the inhibition of superprecipitation by these compounds was overcome. A typical experiment using chlorpromazine is shown in Fig. 8. Addition of various amounts of protein modulator prevented, in dose-dependent fashion, inhibition by chlorpromazine of superprecipitation (Fig. 8). The concentration of the compounds producing 50% inhibition of superprecipitation ($t_{1/2}$) is shown in Table 1. Papaverine, which was the most potent phosphodiesterase inhibitor among the compounds tested but did not inhibit selectively a protein modulator-induced stimulation of phosphodiesterase, had no effects on superprecipitation of smooth muscle actomyosin up to the concentration of 0.1 mM. Other vasodilators such as isoproterenol, sodium nitrite and fusaric acid did not affect the superprecipitation of the actomyosin up to the concentration of 0.1–1.0 mM. These reagents did not inhibit the

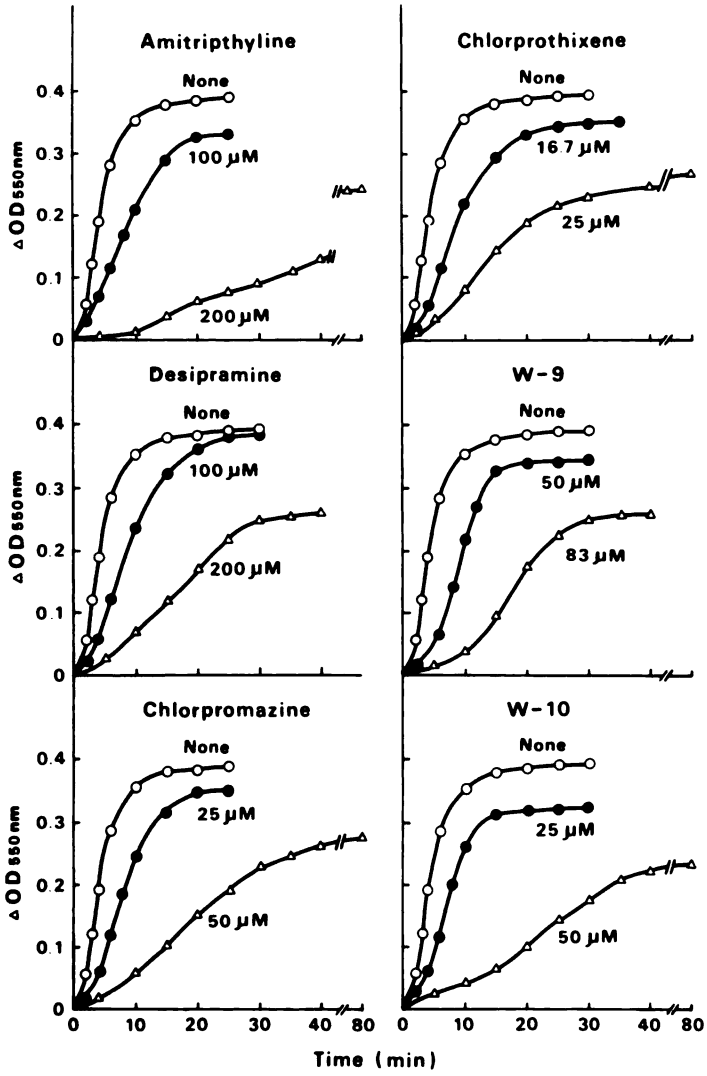



FIG. 7. *Effect of several selective inhibitors of $Ca^{2+}$-dependent cyclic nucleotide phosphodiesterase on superprecipitation of bovine aorta smooth muscle actomyosin*

The reaction mixture consisted of actomyosin (0.3 mg/ml), KCl (60 mM), $MgCl_2$ (1.7 mM), $CaCl_2$ (100 μM), Tris-maleate buffer (50 mM) pH 6.5, ATP (250 μM) and various concentrations of inhibitors in a total volume of 3 ml. The reaction was started by addition of ATP and carried out at 24°.

stimulation of the phosphodiesterase by a protein modulator.

## DISCUSSION

Collectively, these experiments represent the first report that isolated vascular strips were relaxed by several pharmacological agents affecting the smooth muscle contraction process (superprecipitation of actomyosin). However, well known vasodilators such as papaverine, isoproterenol, $NaNO_2$ and fusaric acid had no effect on the smooth muscle contraction process. All compounds which inhibit superprecipitation are selective inhibitors of a protein modulator-induced stimulation of phosphodiesterase. Psychotropic drugs, W-7, W-9, W-10, inhibited activated phosphodiesterase selectively at their lower concentrations, but they inhibited both activated and unactivated phosphodiesterase at their higher concentrations, suggesting that

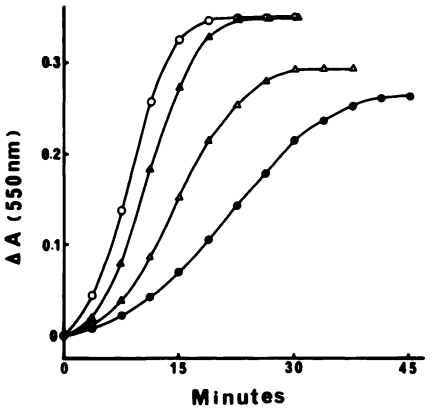



FIG. 8. *Prevention of chlorpromazine-induced inhibition of superprecipitation by activator protein*

The reaction mixture contained: 60 mM KCl, 1.67 mM $MgCl_2$, 100 μM $CaCl_2$, 50 mM Tris-maleate buffer (pH 6.5), 250 μM ATP, modulator-rich actomyosin (0.25 mg/ml) and none (control) (○), 40 μM chlorpromazine (●), 40 μM chlorpromazine + 3 μg purified protein modulator (△) or 40 μM chlorpromazine + 30 μg purified protein modulator (▲).

these reagents interact not only with a protein modulator but also the phosphodiesterase directly. As kinetic properties of activated phosphodiesterase differ from those of unactivated enzyme (26), the selective inhibition of activated phosphodiesterase by these reagents might be due to direct interaction of the reagents with activated phosphodiesterase ($Ca^{2+}$-modulator-phosphodiesterase complex) rather than due to the binding of the reagents to a protein modulator. However, direct interaction of the reagents with activated phosphodiesterase is not likely because these reagents inhibited the activated phosphodiesterase competitively with a protein modulator (Fig. 2). Moreover, it was found that $^3H$-labelled W-7 bound to a protein modulator in the presence of 100 μM $Ca^{2+}$ (unpublished observation). Levin and Weiss (27) recently reported that a protein modulator has the calcium dependent, high affinity binding site for phenothiazine antipsychotics. As the compounds tested all have similar properties in inhibiting the stimulation of phosphodiesterase by a protein modulator, the binding site for phenothiazine antipsychotics in a protein modulator may also be available for these compounds. If so, inhibition of superprecipitation by the compounds may result from the binding of the compounds to a protein modulator found in actomyosin from bovine aorta smooth muscle. Actually, recent works suggest that the regulation by $Ca^{2+}$ of the actin-myosin interaction in smooth muscle is due to the concerted action of a protein kinase (myosin kinase) and a phosphatase (8, 9), and a protein modulator is a component of smooth muscle myosin kinase (10, 11). Thus it is quite likely that a protein modulator plays an important role in muscle contraction. From our data, various vasodilators can be classified into at least three groups, those affecting actin myosin, those which affect membrane receptors and agents acting by other means such as papaverine. These selective inhibitors of a protein modulator can serve as an effective pharmacological tool for elucidating the regulatory mechanism in muscle contraction.

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