0026-895X/88/060598-06-$02.00/0

MOLECULAR PHARMACOLOGY, 33:598-603

ML-9 Inhibits the Vascular Contraction via the Inhibition of Myosin Light Chain Phosphorylation

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Received June 3, 1987; Accepted March 15, 1988

SUMMARY

We investigated the effects of a newly synthesized compound, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9), a myosin light chain kinase (MLCK) inhibitor of superprecipitation of actomyosin, isometric tension development, and phosphorylation of the 20,000-Da myosin light chain (LC20) in vascular smooth muscle. Superprecipitation of actomyosin from bovine aorta was inhibited by the addition of ML-9 in a dose-dependent manner. In chemically skinned smooth muscles of the rabbit mesenteric artery, ML-9 inhibited the Ca^{2+}-independent contraction provoked by application of trypsin-treated MLCK. In the intact rabbit mesenteric artery, increases in LC20 phosphorylation reached a maximal value of 0.49 mol of P_i/mol of LC20 within 10 sec from a resting value of 0.15 mol of P_i/mol of LC20 and then declined to near the basal level during the maintained isometric force developed in response to 50 mM KCl. Preincubation with 10–30 μM ML-9 for 30 min significantly inhibited both the maximal rate and extent of KCl-induced contraction and the phosphorylation of LC20, in a dose-dependent manner. There was a linear relationship between the initial rate of tension development and the extent of LC20 phosphorylation at 10 sec after stimulation. ML-9 nonspecifically antagonized the contraction induced by various contractile agonists, such as $CaCl_2$, norepinephrine, serotonin, histamine, and angiotensin II. ML-9 dose dependently produced a shift to the right and down, in the dose-response curves, to all the agonists tested. These results suggest that ML-9 inhibits the actin-myosin interaction through the modulation of LC20 phosphorylation via the inhibition of MLCK activity. Thus, ML-9 may be a useful compound for investigating the physiologic role of myosin light chain phosphorylation by MLCK in living cells and tissues as well as *in vitro*.

Although the regulation of actin-myosin interactions in smooth muscle by Ca^{2+} is not fully understood, it has been generally accepted that the Ca^{2+}-dependent phosphorylation of the 20,000-Da light chain of myosin (LC20) is necessary for the contraction (1–3). The phosphorylation of myosin in smooth muscle is catalyzed by MLCK, the activity of which is absolutely dependent on the binding of a Ca^{2+}-CaM complex (2, 4). Because LC20 phosphorylation is important in the regulation of contractile activity in smooth muscle, physiologic or pharmacologic interventions might conceivably influence regulation of the extent of LC20 phosphorylation. These interventions might indirectly influence phosphorylation by altering the amount of Ca^{2+} or CaM available for activation of the MLCK or might also act directly by influencing the enzymatic properties of MLCK or phosphatase.

It is reported that the inhibition of contraction by CaM antagonists such as W-7 and fluphenazine was a result of inhibition of LC20 phosphorylation by inactivation of CaM (5–8). On the other hand, direct inhibition of MLCK activity may occur through cAMP-dependent phosphorylation of MLCK with a resultant decrease in the affinity of the enzyme for the Ca^{2+}-CaM complex (9–11). A drug that influences the phosphorylation of LC20 through the direct inhibition of MLCK activity has apparently not heretofore been developed.

Recently, we found that a newly synthesized compound, ML-9, is a direct and selective inhibitor of MLCK and that LC20 phosphorylation catalyzed by MLCK may be a key event in reactions related to the activation of human platelets with collagen (12).

We examined the effects of ML-9 on isometric tension development and LC20 phosphorylation in intact rabbit vascular smooth muscle, the objective being to characterize the pharmacologic properties of ML-9. The relationship between extent of inhibition of LC20 phosphorylation and inhibition of contractile activity by ML-9 was also investigated herein.

Materials and Methods

Experiments using bovine aorta smooth muscle actomyosin. Actomyosin was prepared from bovine aorta smooth muscle by a slight

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

ABBREVIATIONS: MLCK, myosin light chain kinase; CaM, calmodulin; KHS, Krebs-Henseleit solution; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; NE, norepinephrine; 5HT, serotonin; His, histamine; Ang II, angiotensin II; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine.

modification of the methods of a previous report (5). Superprecipitation of actomyosin (7.0 mg of protein/ml) was followed by an increase in A_{660} at 25° after addition of 2 mM Mg-ATP in the presence of 20 mM 3-(*N*-morpholino)propanesulfonic acid, 50 mM KCl, and 11 μM $CaCl_2$ at pH 7.0 in a total volume of 3 ml unless otherwise stated.

Preparation of Ca^{2+}/CaM-independent MLCK. MLCK was purified from fresh chicken gizzard by the procedure described by Adelstein and Klee (4). The Ca^{2+} CaM-independent MLCK was prepared as follows: 0.6 mg/ml MLCK was treated for 20 min with trypsin (10 μg/ml) at 25° and this reaction was stopped by the addition of trypsin inhibitor (100 μg/ml). This trypsin-treated MLCK was active in the absence of Ca^{2+}/CaM and catalyzed Ca^{2+}-independent phosphorylation of the LC20 using isolated myosin light chains.

Preparation of vascular strips. Anesthesized male albino rabbits weighing about 2.5 kg were exsanguinated from the femoral arteries and the superior mesenteric artery (0.8–1.8 mm outside diameter) was quickly excised and placed in KHS. After removal of excess fat and adventitial connective tissue, the mesenteric artery was helically cut into strips of 1 × 10 mm (for tension recording) or 1.5 × 20 mm (for biochemical analysis).

Recording of mechanical responses. The vascular strips were vertically suspended in a 20-ml organ bath filled with KHS (for composition see below) the temperature of which was maintained at 37° (pH 7.4). A mixture of 95% O_2/5% CO_2 was constantly bubbled through the KHS in the organ bath.

The mechanical activity was recorded isometrically by means of a force-displacement transducer (TB-612T; Nihon Kohden Kogyo Co., Tokyo, Japan). An initial resting tension of 1 g was applied to the strips. Before initiation of the experiments, the strips were allowed to equilibrate for 1 hr in the KHS, during which time the bathing solutions were replaced every 15 min with fresh KHS. After the equilibration time of 1 hr, under resting tension, a submaximally effective concentration of KCl (50 mM) was administered twice. A cumulative dose-response curve for a contractile agonist was obtained by a stepwise increase in concentration of the agonist as soon as a steady response to the preceding dose had been obtained.

Effects of ML-9 on the dose-response curves for a contractile agonist were determined in the following manner. Two sequential dose-response curves for a contractile agonist were determined simultaneously on paired arterial strips with an interval of 60 min between each determination. Usually paired strips (from the same rabbit) were subjected to different treatments. To one strip, various concentrations of ML-9 were applied at the second trial; another strip was a control serving as an indicator of changes in tissue sensitivity during the course of the experiment.

For tension recording from chemically skinned muscles, the dissected mesenteric artery was carefully teased apart using jewelers' forceps along the longitudinal direction, and circular strips of 0.1 mm in width and 0.5 mm in length were prepared under a binocular microscope. The experimental procedures for the skinning of the tissue were much the same as those described by Itoh *et al.* (14) and Saitoh *et al.* (15). Saponin treatment was carried out by leaving the preparation for 20 min in relaxing solution containing 50 μg/ml saponin. The relaxing solution contained 130 mM KCl, 20 mM Tris-maleate, 5 mM $MgCl_2$, 5 mM ATP, and 4 mM EGTA at pH 6.8.

Biochemical analysis. The time course of LC20 phosphorylation was determined by freezing the strips at appropriate times during a contractile event. The strips were rapidly frozen by immersion into acetone precooled with dry ice and were then stored at −80° until biochemical analysis after measuring the weight.

Two-dimensional gel electrophoresis. The state of phosphorylation of the myosin light chains was determined on two-dimensional gel electrophoresis (16, 17). The frozen vascular smooth muscle strips (about 5 mg) were homogenized on ice in exactly 60 volumes (v/w) of a buffer containing 100 mM sodium pyrophosphate, 5 mM EGTA, 1 mM EDTA, 50 mM NaF, 10% glycerol, 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.1 mM leupeptin, final pH 8.8. The homogenate was then centrifuged at 7000 × *g* for 10 min. A measured volume of the supernatant fraction was removed, and proteins were precipitated from this fraction by addition of 100% trichloroacetic acid to a final concentration of 10%. Precipitated material was collected by centrifugation at 7000 × *g* for 10 min. The supernatant fraction was decanted, and the pellet was extracted three times with diethyl ether for 2 min, followed by air-drying at room temperature for 30 min to remove the residual ether. Precipitated proteins were then dissolved in 30 μl of a solution containing 8.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, and 2% Pharma-Lyte (pH 4–6:pH 3–7 = 4:1), and isoelectrofocused in the first dimension on 4% polyacrylamide gels containing 8 M urea, 2% Nonidet P-40, and 2% Pharma-Lyte (pH 4–6:pH 3–7 = 4:1). Subsequently, they were electrophoresed in the second dimension on 15% polyacrylamide gels in 0.1% sodium dodecyl sulfate and 0.38 M Tris·HCl, pH 8.8; the acrylamide was reduced to 4.5% in stacking gels. To visualize protein spots, polyacrylamide gels were stained in a solution containing 50% methanol, 10% acetic acid, and 0.1% Coomassie blue (R-250) followed by destaining in 50% methanol and 10% acetic acid and storage in 10% acetic acid. These conditions do not result in phosphorylation or dephosphorylation of myosin during homogenization and electrophoresis (18). The intensity of staining of the phosphorylated and unphosphorylated LC20 was quantitated by densitometric scanning using Transmittance/Refractance Scanning Densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA). Phosphorylation values were calculated by integration of the electrophoretic band corresponding to the phosphorylated LC20 as a percentage of the total integration of both the phosphorylated and nonphosphorylated LC20.

Statistics. Results shown in the text and figures were expressed as mean values ± standard deviation. The effects of ML-9 were compared with control responses (plus methanol only) sampled for each vascular strip. The results were statistically compared using Student's *t* test, paired *t* test, or analysis of variance. Statistical significance was assumed at $p < 0.05$. Correlation coefficients and regression lines were calculated by the method of least squares.

Electrolyte solutions. The KHS included (millimolar): NaCl, 115; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgCl_2 \cdot 6H_2O$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2, and dextrose, 10. For Ca^{2+}-induced contraction experiments, Ca^{2+}-free KHS ($CaCl_2 \cdot 2H_2O$ in the KHS was omitted and 0.1 mM EGTA was added) and high K^+, Ca^{2+}-free KHS (80 mM NaCl in the KHS was replaced with isotonic KCl and $CaCl_2 \cdot 2H_2O$ was omitted) were used.

Drug and chemicals. ML-9 was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were obtained from commercial suppliers in the highest grade available. ML-9 was soluble in 100% methanol; therefore, the stock solution was prepared and diluted to proper concentrations of the test solution.

Results

Inhibition by ML-9 of superprecipitation of bovine aorta actomyosin. When ATP was added to the reaction mixture containing bovine aorta actomyosin, superprecipitation of actomyosin occurred and this was determined by measuring the increase in absorbance at 660 nm. Addition of various concentrations of ML-9 produced inhibition of the superprecipitation in a dose-dependent fashion. However, the extent of inhibition of ML-9 was inversely proportional to the concentrations of Mg-ATP in the reaction mixture. Table 1 shows the percentage of inhibition of superprecipitation by 300 μM ML-9 in the presence of three concentrations of Mg-ATP. These results are consistent with the data that the inhibition of MLCK by ML-9 was competitive with respect to ATP (15).

Effect of ML-9 on Ca^{2+}-independent contractions. ML-9 was found to inhibit both Ca^{2+}/CaM-dependent and -independent smooth muscle MLCK with a similar concentration dependence and to suppress the Ca^{2+} (3 μM free Ca^{2+})-

TABLE 1

Effect of ML-9 on superprecipitation of actomyosin from bovine aorta smooth muscle

The extent of superprecipitation was expressed by the maximal change of light transmission expressed as a percentage, taking the difference between light transmission in the absence and presence of a given concentration of ATP as a value of 100%. Per cent inhibition of superprecipitation by ML-9 was calculated by dividing the per cent superprecipitation by that observed in the control run (same concentration of ATP and without ML-9), then multiplying by 100.

ML-9	Mg-ATP	Inhibition
μM	mM	%
300	2	20.2 ± 10.8
300	0.4	37.2 ± 5.5
300	0.2	70.5 ± 7.7

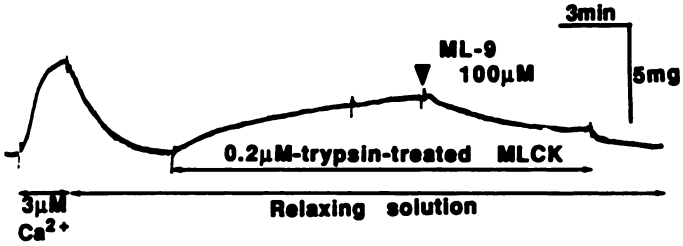

Fig. 1. Effect of ML-9 on Ca^{2+}-independent contraction. The contraction was evoked by application of 0.2 μM trypsin-treated MLCK and the time course of the relaxation was observed after the addition of 100 μM ML-9.

induced contraction of chemically skinned muscle fibers (15). We investigated the effect of ML-9 on Ca^{2+}-independent contraction of the skinned muscle fibers to exclude the possibility of a CaM antagonistic effect on vascular contraction. Ca^{2+}-independent MLCK preparation was achieved by limited proteolysis of the MLCK with trypsin (13). Incubation of the skinned fiber bundles with the Ca^{2+}-independent MLCK and ATP in the absence of Ca^{2+} elicited tension development.

ML-9 (100 μM) inhibited the trypsin-treated MLCK-induced contraction (Fig. 1). On the other hand, W-7, a CaM antagonist, (up to 100 μM) cannot relax the contraction (data not shown). These results suggest that ML-9 exerts its action at MLCK, but not CaM.

Inhibition by ML-9 of myosin light chain phosphorylation and mechanical response. Changes in phosphate content in the myosin molecule during contraction were studied by using intact vascular smooth muscle strips. We froze muscle strips at various times before and during force development, in response to 50 mM KCl. Two-dimensional peptide mapping of the phosphorylated LC20, after tryptic digestion, revealed that the site of phosphorylation in the LC20 of the myosin from KCl-contracted vascular strips was the same as the site phosphorylated by MLCK, using both purified myosin light chain and MLCK (data not shown).

Fig. 2 shows the time course of LC20 phosphorylation during tension development in intact vascular strips after stimulation by 50 mM KCl. In the rabbit mesenteric artery, muscles contracted with KCl generated an average of 1.5 ± 0.14 g of active tension. The contractile response reached a maximum at 2 min and was maintained up to at least 1 hr. Incubation of intact vascular strips in oxygenated KHS in the absence of passive tension was associated with low levels of LC20 phosphate content. Representative gel photographs are shown in Fig. 2. There was 0.15 ± 0.02 mol of P_i/mol of LC20 in the control vascular smooth muscle strips. In contrast, there was a near-maximal active isometric force development in response to stimulation with 50 mM KCl and in association with a rapid increase in LC20 phosphorylation. Temporally, the maximal extent of LC20 phosphate (0.49 ± 0.04 mol of P_i/mol of LC20) was obtained by 10 sec and preceded the attainment of isometric tension of 1.0 ± 0.1 g. The muscles stimulated in the presence of KCl showed a continued decrease in phosphate content from the maximal value to 0.25 ± 0.03 mol of P_i/mol of LC20 after 50 sec, a value all but unchanged after 120 sec. Pretreatment for 30 min with ML-9 inhibited the initial rate and maximal extent of LC20 phosphorylation during the first 2 min after the addition of 50 mM KCl. ML-9 also delayed the time course of the LC20 phosphorylation, in a dose-dependent manner.

We then examined the relationship between ML-9-induced inhibition of the extent of LC20 phosphorylation and isometric tension development. Because, in control muscles, the maximal LC20 phosphorylation was reached at 10 sec after stimulation, the correlation between phosphate content of the LC20 and initial rate of tension development measured 10 sec after initiation of contraction was examined. As shown in Fig. 3, the initial rate of isometric tension development correlated directly with the extent of phosphorylation of the LC20. In addition, there was a linear relationship between the LC20 phosphorylation and the isometric tension at 10 sec (data not shown).

ML-9 was also studied with regard to effects on the Ca^{2+}-induced contraction in depolarized vascular strips. The dose-response curves for $CaCl_2$ exhibited a parallel shift to the right after treatment with a lower concentration (3 μM) of ML-9, with no change in the maximal responses to $CaCl_2$ (Fig. 4a). At higher concentrations (10 and 30 μM) of ML-9, the dose-response curves shifed both rightward and downward, indicative of a noncompetitive antagonism.

To further examine the inhibitory effect of ML-9, the effects of this compound on the dose-response curves to each agonist were obtained from a single preparation, in the absence and presence of ML-9. NE, 5-HT, His, and Ang II were used as contractile agonists. Maximal contractile tension and ED_{50} values of each agonist were identical through two sequential trials (data not shown). The values in the first trial were taken as control and ML-9 was added at the second trial. As can be seen in Fig. 4, b–e, ML-9 produced a shift to the right and down in dose-response curves to all agonists tested, thereby indicating a noncompetitive antagonism. In this experiment, ML-9 exhibited a similar magnitude of antagonism among the dose-response curves for these agonists. The pD_2' values (negative logarithm of molar concentration of ML-9 producing a 50% reduction of the maximal response) were 4.56 ± 0.10, 4.55 ± 0.10, 4.71 ± 0.45, 5.06 ± 0.18, and 4.96 ± 0.39 (four experiments) for $CaCl_2$, NE, 5HT, His, and Ang II, respectively.

Discussion

We previously reported that a synthetic compound, ML-9, is a potent and selective inhibitor of MLCK (12, 15). From the kinetic analysis, ML-9 exhibited a selective inhibition toward MLCK with the apparent K_i value of 3.8 μM. The inhibitory action of ML-9 seems to be the result of direct effects on the active site of the enzyme (15). In addition, ML-9 also revealed that LC20 phosphorylation catalyzed by MLCK may be a key event in a series of reactions, in the case of activation of human platelets with collagen (12).

In this study, we demonstrated that a MLCK inhibitor,

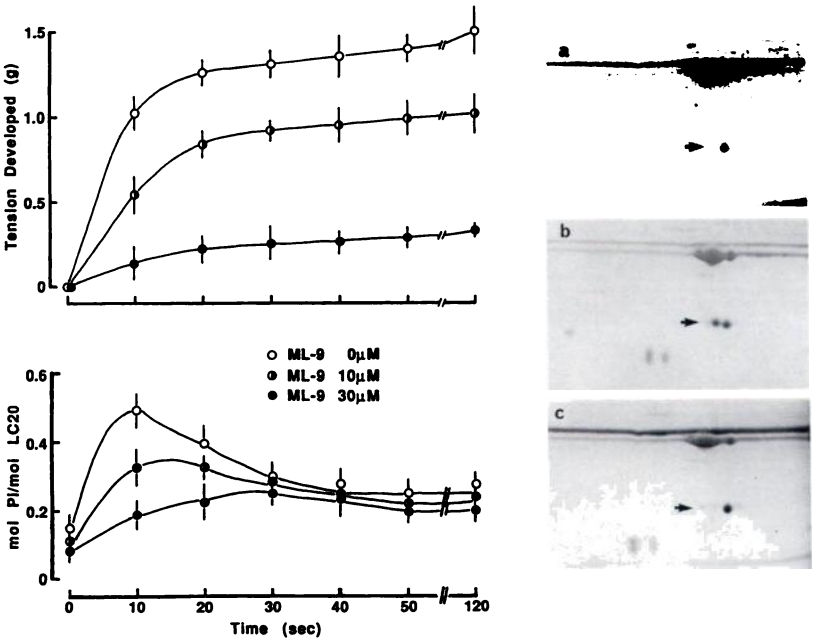

Fig. 2. Time course of force and LC20 phosphorylation in KCl-stimulated strips of vascular smooth muscle in the presence or absence of ML-9. Isometric tension recording and measurement of LC20 phosphorylation were independently performed. Vascular strips were frozen during rest or at indicated times between 10 sec and 2 min of stimulation. *Right panel* shows the two-dimensional gel electrophoresis of myosin light chain from representative smooth muscle samples (see Materials and Methods for details). a, Strip was frozen at rest; b and c, strips were frozen 10 sec after 50 mM KCl challenge in the absence or presence of 30 μM ML-9, respectively. The *arrow* indicates visible stained spots of LC20. LC20 phosphorylation transiently increased and then declined while the isometric tension rose monotonically to a plateau. Mean values are plotted ± standard deviation, three experiments.

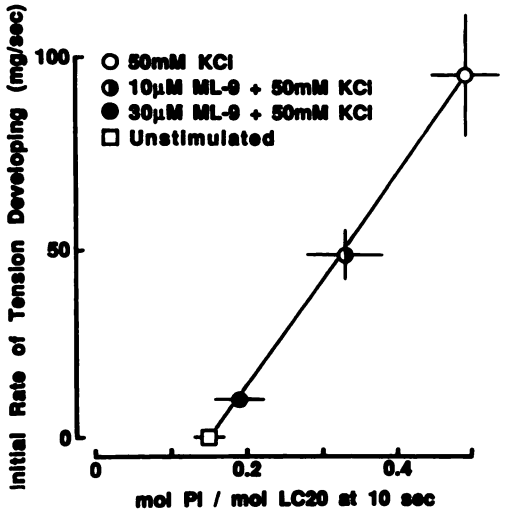

Fig. 3. Relationship between the extent of tension development and LC20 phosphorylation during 10 sec of stimulation with KCl. The mean ± standard deviation for each rate of tension development (mg/sec) and LC20 phosphorylation (mol of P_i/mol of LC20) from vascular strips stimulated by 50 mM KCl in the absence and presence of ML-9 is shown. ML-9 at 10 or 30 μM was added 30 min before the addition of KCl. These values were obtained from Fig. 2. The *solid line* represents the calculated line of regression ($r = 0.99$, $p < 0.01$).

ML-9, is able to inhibit the contractile response of intact vascular strips by affecting intracellular process(es) in smooth muscle contraction. First, superprecipitation of actomyosin from bovine aorta smooth muscle was inhibited by the addition of ML-9 in a dose-dependent fashion. Second, ML-9 inhibited the Ca^{2+}-independent contraction evoked by application of trypsin-treated MLCK in the chemically skinned fibers. These results may indicate that ML-9 suppresses the vascular contraction via the inhibition of MLCK activity *in vivo*.

The phosphorylation of the LC20 is thought to be a key regulatory step in smooth muscle contraction. In the present work, the transient phosphorylation of LC20 during isometric tension development was observed during the high K^+ stimulation of the rabbit mesenteric artery (Fig. 2). However, the time course of the LC20 phosphorylation was more rapid than that heretofore achieved by application of pharmacologic agonists (17, 19–25). The addition of 50 mM KCl resulted in a marked increase in the extent of LC20 phosphorylation from a resting value of 0.15 ± 0.02 to 0.49 ± 0.04 mol of P_i/mol of LC20, within 10 sec of incubation. Subsequently, there was a decline in the extent of phosphorylation to values of 0.30 ± 0.03 mol of P_i/mol of LC20 after 30 sec and 0.25 ± 0.03 after 50 sec (Fig. 2). ML-9 dose dependently inhibited the KCl-stimulated increases in LC20 phosphorylation and tension development. ML-9 also produced a delay of the time to attain maximal LC20 phosphorylation, the reason for which has yet to be elucidated.

Silver and Stull (8, 19) reported that the extent of LC20 phosphorylation during the initial period of contraction may be important in determining the steady state isometric tension and suggested that the maintained isometric force in smooth muscle may be dependent upon the maximal extent of LC20 phosphorylation obtained during an early temporal transient in the phosphorylation. Murphy and co-workers (17, 20, 21, 23, 24) found a good correlation between the degree of phosphorylation of the LC20 and the velocity of shortening but not the force output. In contrast, others (26, 27) reported a dissociation between LC20 phosphorylation and the active tension or maximal velocity of shortening. Our results show that there is a linear relationship between the initial rate of tension development and the extent of LC20 phosphorylation in the early phase (at 10 sec after stimulation) in the intact rabbit mesenteric artery (Fig. 3).

The pharmacologic regulation of smooth muscle contraction might occur through modulation of LC20 phosphorylation. Direct alteration of LC20 phosphorylation also occurs with the standard CaM antagonist W-7, which inhibits the activation of MLCK by CaM and suppresses vascular smooth muscle contraction (5–7), differing from Ca^{2+} channel blockers (28). The present data may be direct evidence that a synthesized compound that inhibits MLCK activity leads to the inhibition of

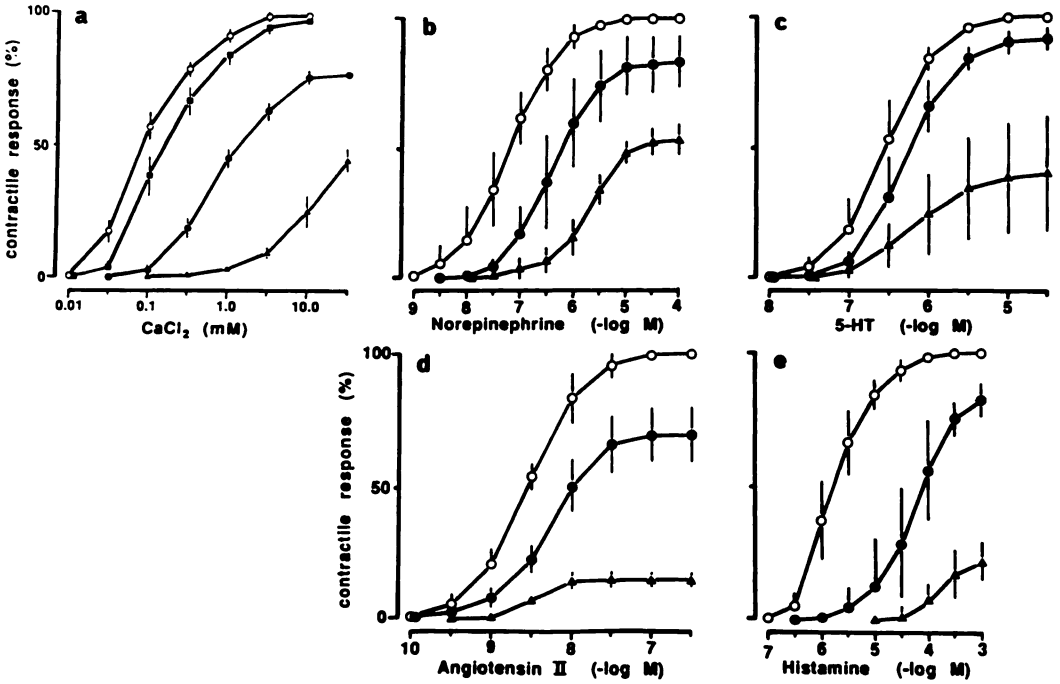

Fig. 4. Effect of ML-9 on the dose-response curves of rabbit mesenteric arterial strips for $CaCl_2$ (a), NE (b), 5-HT (c), Ang II (d), and His (e). ML-9 (10 or 30 μM) was added 30 min before the dose-response curve for each agonist and was present during the addition of each agonist. a, After recording the precontraction, the strips were exposed to Ca^{2+}-free KHS for 10 min. The preparations were then rinsed with high-K^+, Ca^{2+}-free KHS for 30 min, $CaCl_2$ was applied cumulatively to the organ bath, and the tension developed by 10 mM $CaCl_2$ was taken as 100%. The same procedure was carried out using various concentrations of ML-9 [0 (○), 3 (●), 10 (▲), and 30 (■) μM]. b–e, See Materials and Methods for details. Shown are the mean values and their standard deviations (five experiments) as *vertical bars*.

mechanical activity induced by KCl concomitant with a depressed LC20 phosphorylation in intact vascular smooth muscle.

ML-9 exhibited a noncompetitive antagonism against NE-, 5-HT-, Ang II-, His-, and $CaCl_2$-induced contractions, and the potency of antagonism was similar to these receptor agonists and $CaCl_2$ (Fig. 4). Thus, these compounds may well exert their actions at the intracellular or submembrane level and have an effect on basic and common events in smooth muscle contraction. It was reported that LC20 phosphorylation can be increased during stimulation with agonists, including NE, His, or 5-HT (16, 17, 25), as well as during K^+ depolarization in vascular smooth muscle. Although actual measurements of the extent of LC20 phosphorylation were not made with these agonists, the inhibition of contraction in rabbit mesenteric artery by ML-9 may be related to the inhibition of LC20 phosphorylation as is the case in KCl stimulation.

Thus, ML-9 should prove to be an effective compound for investigating the physiologic role of myosin light chain phosphorylation by MLCK in living cells and tissues as well as *in vitro*.

Acknowledgments

We thank M. Ohara of Kyushu University for critical reading of the manuscript.

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