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Selective Calmodulin Inhibition Toward Myosin Light Chain Kinase by a New Cerebral Circulation Improver, Ro 22-4839

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SUMMARY

Ro 22-4839, a new cerebral circulation improver, has shown to be a potent calmodulin antagonist toward myosin light chain kinase (MLCK). It inhibited *in vitro* activity of calmodulin-activated cyclic AMP phosphodiesterase isolated from either bovine heart or brain and ATP-induced superprecipitation of chicken gizzard actomyosin with respective IC_{50} values of 20 μM, 17 μM, and 2.0 μM. The inhibitory action of Ro 22-4839 on the contractile system of the smooth muscle was demonstrated directly by its inhibition of chicken gizzard MLCK. Ro 22-4839 was found to potently inhibit MLCK with an IC_{50} value of 3.1 μM but was unable to inhibit the activity of MLCK rendered Ca^{2+}/calmodulin independent by limited tryptic digestion. The inhibition of MLCK induced by Ro 22-4839 was completely overcome by addition of excess calmodulin. In contrast, Ro 22-4839 hardly inhibited calmodulin-activated Ca^{2+}, Mg^{2+}-ATPase from rat erythrocyte membrane or adenylate cyclase from rat brain. Use of hydrophobic fluorescence probes showed that Ro 22-4839 binds to the hydrophobic region of calmodulin like other calmodulin antagonists, trifluoperazine and W-7. However, the precise binding site of Ro 22-4839 to calmodulin is different from those of trifluoperazine and W-7, as suggested from differing IC_{50} values of these compounds against the probes. We conclude that Ro 22-4839 inhibits calmodulin-activated enzymes, most significantly of MLCK, highly specific to smooth muscle contractile systems by binding to the hydrophobic domain of the calmodulin and inducing its conformational change in the presence of calcium.

The versatile and indispensable function of Ca^{2+} as a mediator of biological information is now widely accepted. The calcium-binding protein calmodulin, originally discovered as a phosphodiesterase activator (1, 2), has been shown to be a primary Ca^{2+} acceptor protein and to regulate a variety of Ca^{2+}-dependent enzymes and processes (3, 4). In smooth muscle contraction, calmodulin also plays an important role in Ca^{2+} regulation (5). Ca^{2+}/calmodulin complex binds to and activates MLCK. Activation of MLCK results in the phosphorylation of the 20,000-Da myosin light chain and the stimulation of actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin (6, 7). In this context, agents which interfere with the interaction of Ca^{2+}/calmodulin complex with target enzyme(s) are expected to be of great pharmacological importance.

Ro 22-4839, 6,7-dimethoxy-1-(3,4-dimethoxybenzyl)-4{[4-(2-methoxyphenyl)-1-piperazinyl]methyl}isoquinoline, is a papaverine derivative found to show cerebral vasodilatory and vasospasmolytic actions as well as protective effects on erythrocyte shape change and learning deficiency in experimental animals (8–13). Inhibition of intracellular contractile elements of smooth muscle, i.e., calmodulin, by Ro 22-4839 was speculated from the fact that vasodilation induced by Ro 22-4839 was found to be both nonselective to various agonists and unaffected by extracellular Ca^{2+} concentration (9). W-7, a well known calmodulin antagonist, has also been reported to antagonize various constrictors (14). In the present study we investigated the *in vitro* calmodulin antagonistic action of Ro 22-4839 and characterized the mode of action. The conclusion drawn from the present work is that Ro 22-4839 is a calmodulin antagonist most significantly toward MLCK.

Materials and Methods

The source and purity of enzymes used in this study were as follows. Calmodulin-deficient cyclic AMP phosphodiesterase (bovine brain, specific activity 0.3 μmol/mg of protein; bovine heart, specific activity 9 μmol/mg of protein), cyclic AMP-dependent protein kinase (bovine heart, specific activity 1–2 nmol of phosphate/mg of protein), and calmodulin (bovine brain, more than 98% pure on sodium dodecyl sulfate-gel electrophoresis) were purchased from Sigma. MLCK and myosin light chain were purified from chicken gizzard. Adenylate cyclase and protein kinase C were partially purified from rat brain. Calmodulin-deficient membrane fraction enriched in Ca^{2+}, Mg^{2+}-ATPase was prepared from rat erythrocyte. Myosin B was prepared from chicken gizzard.

Calmodulin-activated phosphodiesterase assay. Calmodulin-activated cyclic AMP phosphodiesterase was assayed in a mixture of final volume 250 μl containing 40 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$,

ABBREVIATIONS: MLCK, myosin light chain kinase; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TFP, trifluoperazine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 9-AC, 9-anthroylcholine; NPN, *N*-phenyl-l-naphthylamine; TNS, 2-*p*-toluidinylnaphthylene-6-sulfonate.

25 ng of calmodulin, 0.1 mM $CaCl_2$, 5 μM (0.2 μCi) cyclic [^{3}H]AMP, enzyme from either bovine heart (1 milliunit) or bovine brain (4 milliunits), and various concentrations of the test compounds. For the measurement of the basal activity, 1 mM EGTA was added instead of 0.1 mM $CaCl_2$ in the absence of calmodulin. The reaction was started by adding cyclic [^{3}H]AMP after preincubation for 5 min at 37°. The mixture was incubated for 30 min at 37° and the reaction was stopped by boiling for 3 min in a water bath. Then, a 10-μl portion was spotted onto Whatman No. 1 paper and developed in a solvent containing ethanol and 0.5 M ammonium acetate (5:2, v/v) along with unlabeled cyclic AMP, adenosine, and 5′-AMP. Separated spots were visualized by ultraviolet lamp and cut out for measuring radioactivity by a liquid scintillation counter.

Preparation and assay of adenylate cyclase. Adenylate cyclase was partially purified from rat brain by the method of Cheung *et al.* (15) using ECTEOLA-cellulose and assayed according to the method of Brostrom *et al.* (16). The standard reaction mixture of 250 μl contained 5 mM $MgCl_2$, 10 mM theophylline, 2 mM cyclic AMP, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.1 mM (40 μCi/mmol) [^{3}H]ATP, 25 ng of calmodulin, 50 mM glycylglycine buffer (pH 7.5), 5 mM phospho*enol*pyruvate, 20 mM KCl, and 10 μg of rabbit muscle pyruvate kinase. The incubation was carried out at 37° for 10 min and the reactions were terminated with 80 μl of 1 M HCl. Cyclic [^{3}H]AMP was isolated from the reaction mixture by the $Ba(OH)_2$-$ZnSO_4$ precipitation, followed by passage through a column (bed volume, 1 ml) of AG 50X resin and counted by a liquid scintillation counter.

Calmodulin-activated Ca^{2+}, Mg^{2+}-ATPase activity. Erythrocyte membranes were prepared from Wistar rats (250–300 g) according to the method Luthra *et al.* (17). The ATPase assay used was essentially the same as that described by Gopinath and Vincenzi (18). Reaction mixture in a final volume of 1.0 ml contained 2 mM ATP, and 18 mM concentration each of histidine-imidazole buffer, pH 7.1, 0.1 mM EGTA, 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain, 50 ng of calmodulin, 200 μg of erythrocyte ghost proteins, and compounds to be tested. Protein was determined by the method of Lowry *et al.* (19).

ATP-induced superprecipitation. Myosin B was prepared from chicken gizzard as described by Hidaka *et al.* (20). The superprecipitation was carried out at 37° and determined by measuring the increase in absorbance at 550 nm. Reaction mixture contained myosin B (0.2 mg of protein/ml), 42 mM KCl, 1.4 mM $CaCl_2$, 0.1 mM Mg-ATP, and 28 mM Tris-HCl (pH 7.0) in a total volume of 3 ml. Calmodulin was not added because sufficient amounts of calmodulin were present in the actomyosin preparation. The reaction was started by addition of Mg-ATP. The IC_{50} value was determined by log-probit analysis of the rate of superprecipitation ($\Delta A/t_{0.5}$).

Preparation of MLCK. The enzyme was purified by the method described by Guerriero *et al.* (21). Briefly, MLCK was extracted from the washed pellet of chicken gizzard by homogenization in 40 mM imidazole buffer (pH 7.5) containing 60 mM KCl, 25 mM $MgCl_2$, 5 mM EGTA, and 1 mM dithiothreitol. After centrifugation, the supernatant was fractionated with solid ammonium sulfate. The 40–60% saturation fraction was applied to a DE52 column (2.5 × 8 cm) and eluted with a linear NaCl gradient (0.04–0.30 M). The active peak of MLCK was pooled and further purified using a calmodulin-Sepharose affinity column.

Purification of myosin light chain. Myosin light chain was purified from chicken gizzard smooth muscle by the method of Adelstein and Klee (22). Freshly dissected gizzard muscle (300 g) was minced and washed. The washed pellet was resuspended and incubated for 20 min at 4° in 4 volumes of 40 mM Tris-HCl (pH 7.2), 40 mM KCl, 2 mM EGTA, 10 mM Mg-ATP, and 100 mg/liter streptomycin sulfate, followed by centrifugation. While monitoring the pH (7.2), 1 M $CaCl_2$ was slowly added to the supernatant to make it 20 mM with respect to $CaCl_2$, and the resulting precipitate was sedimented. The precipitate was resuspended in cold water (4°), and solid guanidine hydrochloride was added to make it 5 M with respect to guanidine. Myosin heavy chain was sedimented and removed by the addition of cold water and cold 95% ethanol by the procedure of Perrie and Perry (23). The supernatant fluid was concentrated with a rotary evaporator and guanidine hydrochloride was removed by gel filtration through a Sephadex G-25 column (2.5 cm × 75 cm). The eluate was lyophilized and applied to a DE52 column (2.5 cm × 7 cm). The light chain was eluted with a linear NaCl gradient (0.02–0.5 M). Purity of MLCK and myosin light chain was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MLCK assay. MLCK activity was measured in a reaction mixture (100 μl) containing 28 mM Tris-HCl (pH 7.0), 42 mM KCl, 1.4 mM $CaCl_2$, 6.25 ng of calmodulin, 0.1 mM [γ-^{32}P]ATP (50–200 cpm/pmol), 0.5 μg of enzyme, 0.2 mg of myosin light chain, and various concentrations of the test compounds. For studying the direct action of the compounds to MLCK, the enzyme was treated with trypsin to abolish its sensitivity to calmodulin according to the method of Tanaka *et al.* (24). Trypsin-treated MLCK activity was measured in a mixture (100 μl) as described above, except that 2 mM EGTA was included in the mixture instead of $CaCl_2$ and calmodulin. The reaction was carried out for 20 min at 30° and terminated by addition of 1 ml of 20% trichloroacetic acid containing 3 mg/ml bovine serum albumin. The acid-insoluble fraction was sedimented and washed five times with 1 ml of ice-cold 10% trichloroacetic acid. The washed pellet was dissolved in 1 ml of 1 N NaOH and radioactivity was measured by a liquid scintillation counter.

Fluorescence measurements. Fluorescence measurements were made at room temperature as reported by Epstein *et al.* (25). Samples were prepared in a total volume of 3 ml containing 25 mM Tris-HCl (pH 7.0), calmodulin, 1.4 mM $CaCl_2$, 15 μM probe (TNS, NPN or 9-AC), and the compound to be tested. The mixture contained 400 μg, 100 μg, and 100 μg of calmodulin for measuring the fluorescence of TNS, NPN, and 9-AC, respectively. Excitation was at 365 nm for all probes, and emission intensity was measured from 400 to 500 nm.

Purification of protein kinase C. Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) was partially purified from rat brains as described by Inoue *et al.* (26). Protein kinase C was eluted by a linear NaCl gradient (0–0.4 M) on a DE52 column (2.5 × 7 cm).

Assays of protein kinase C and cyclic AMP-dependent protein kinase. Activities of protein kinase C and cyclic AMP-dependent protein kinase were assayed as described by Hidaka *et al.* (27).

Drugs and chemicals. The sources of materials used in this work were as follows: TFP, W-7, bovine brain calmodulin, histone H1 (type III-S), calmodulin-activated cyclic AMP phosphodiesterase from bovine heart and bovine brain, cyclic AMP-dependent protein kinase from bovine heart, TNS, and phosphatidylserine from Sigma; NPN from Wako Pure Chemicals Inc., Sephadex G-25 and calmodulin-Sepharose from Pharmacia Fine Chemicals; DE52 from Whatman; and [γ-^{32}P]ATP and cyclic [^{3}H]AMP from New England Nuclear. All other chemicals and materials were purchased from local commercial sources.

Results

Calmodulin-activated phosphodiesterase. Addition of 25 ng of calmodulin to the phosphodiesterase preparation increased the cyclic AMP hydrolyzing activity both in heart and brain 2- to 3-fold in the presence of 0.1 mM Ca^{2+}. Ro 22-4839 inhibited the activation of both enzymes in a concentration-dependent manner. The concentration of Ro 22-4839 which inhibited the activation of the enzyme by 50% (IC_{50}) was 20 μM in the heart, whereas corresponding IC_{50} values of W-7 and TFP were 39 μM and 10 μM, respectively (Table 1). Increasing Ca^{2+} concentration did not affect the inhibition induced by Ro 22-4839. These results indicate that Ro 22-4839 inhibits the activation of phosphodiesterase through direct binding to calmodulin, and not through inhibition of Ca^{2+}-calmodulin binding. Although inhibition of the bovine brain phosphodiesterase by W-7 (IC_{50} = 10.9 μM) and TFP (IC_{50} = 3.5 μM) was found

TABLE 1

IC_{50} values of Ro 22-4839, TFP, and W-7 on calmodulin-activated cyclic AMP phosphodiesterase from bovine heart and brain, calmodulin-activated adenylate cyclase from rat brain, and calmodulin-activated Ca^{2+}-ATPase from rat erythrocyte membrane

The activities of cyclic AMP phosphodiesterase, adenylate cyclase, and Ca^{2+}-ATPase were measured as described under Materials and Methods. IC_{50} values were the concentration of the compounds producing 50% inhibition of each enzyme activity in the presence of calmodulin. Each value represents the mean ± standard error of the means of three to eight separate experiments (indicated in parentheses), each assayed in duplicate.

Compounds	Phosphodiesterase		Adenylate cyclase	Ca^{2+},Mg^{2+}-ATPase
	Heart	Brain		
Ro 22-4839	20 ± 1.3 (8)	17 ± 0.76 (6)	>20 (30% inhibition at 20 μM) (3)	>30 (10% inhibition at 30 μM) (6)
TFP	10 ± 1.0 (5)	3.5 ± 0.35 (5)	16 ± 0.6 (3)	15 ± 2 (6)
W-7	39 ± 1.1 (6)	11 ± 2.2 (6)	47 ± 1.7 (3)	72 ± 7.3 (6)

to be stronger than that of bovine heart phosphodiesterase, Ro 22-4839 (IC_{50} = 16.7 μM) was equipotent for both enzymes.

Calmodulin-activated adenylate cyclase. Rat brain adenylate cyclase that is partially purified is also activated by 25 ng of calmodulin about 10-fold. W-7 and TFP showed an inhibitory effect on this enzyme with IC_{50} values similar to those obtained in phosphodiesterase assay, whereas Ro 22-4839 had a relatively weak inhibitory activity (Table 1).

Calmodulin-activated Ca^{2+}, Mg^{2+}-ATPase. Calmodulin-deficient erythrocyte membrane yields Ca^{2+}, Mg^{2+}-ATPase that can be stimulated, on average, by 50 ng of calmodulin 2- to 3-fold above the basal activity. In contrast to the result of phosphodiesterase, Ro 22-4839, at concentrations approaching its solubility limit (30 μM), inhibited calmodulin-activated Ca^{2+}, Mg^{2+}-ATPase from rat erythrocyte ghost by only 10%, whereas W-7 and TFP showed inhibition with respective IC_{50} values of 72 μM and 15 μM (Table 1). The basal ATPase activity (in the absence of calmodulin) was not inhibited by these compounds.

Superprecipitation of chicken gizzard smooth muscle actomyosin. It is widely accepted that calmodulin plays an important role in the contraction of the smooth muscle. Taking the calmodulin-antagonistic action of Ro 22-4839 into consideration as to the vasodilating effect of this compound in isolated vessels (9), Ro 22-4839 is supposed to interact with some components involved in the contraction of smooth muscle. Therefore, we examined the effects of Ro 22-4839 on superprecipitation of myosin B which is well known as an *in vitro* model for the contraction of smooth muscle.

After the addition of ATP to the myosin B preparation from chicken gizzard, the increase in absorbance at 550 nm was observed, indicating that the contraction of actomyosin occurred (Fig. 1). Ro 22-4839 inhibited the superprecipitation in a concentration-dependent manner, with an IC_{50} value of 2.0 μM (Figs. 1 and 2). The potency of known calmodulin antagonists, W-7 and TFP, was relatively weak, with respective IC_{50} values of 220 and 38 μM. The key enzyme involved in the contraction of smooth muscle, MLCK, has been known both to have a calmodulin-binding domain and to be activated by calmodulin (28). This result indicates that Ro 22-4839 may inhibit MLCK through its inhibitory effect on calmodulin. This idea was confirmed by the experiments using the purified enzyme as described below.

Inhibition of MLCK. Purified MLCK from chicken gizzard was activated in the presence of Ca^{2+}/calmodulin and phosphorylated the myosin light chain. The activity in the absence of Ca^{2+}/calmodulin was within 10% of that in the presence of Ca^{2+}/calmodulin. Ro 22-4839 inhibited the activity of the enzyme in a concentration-dependent manner, with an IC_{50} value

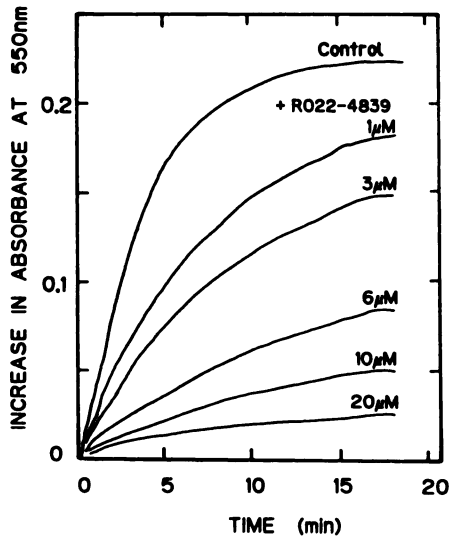

Fig. 1. Ro 22-4839 inhibits the ATP-induced superprecipitation of chicken gizzard actomyosin. Typical reaction of ATP-induced superprecipitation with or without Ro 22-4839 is shown. The superprecipitation was carried out using chicken gizzard myosin B prepared according to the method of Hidaka *et al.* (20). The reaction mixture contained myosin B (0.2 mg of protein/ml), 42 mM KCl, 1.4 mM $CaCl_2$, 0.1 mM Mg-ATP, and 28 mM Tris-HCl (pH 7.0) in a total volume of 3 ml. The reaction was started by addition of Mg-ATP at 37°. Ro 22-4839 was preincubated with myosin B for 10 min in the reaction mixture prior to the addition of Mg-ATP.

of 3.1 μM, whereas those of W-7 and TFP were 31 μM and 23 μM, respectively (Fig. 3). Fig. 4 shows the effect of the amount of calmodulin included in the assay. The inhibition of MLCK induced by Ro 22-4839 at 20 μM was completely overcome by adding a 30-fold excess amount of calmodulin in the assay.

Limited proteolysis of the enzyme with trypsin was reported to result in the loss of its Ca^{2+}/calmodulin sensitivity (24) and made it fully active even in the absence of Ca^{2+}/calmodulin because linkage between the catalytic domain and the calmodulin binding domain was highly susceptible to protease (29). Ro 22-4839, W-7, and TFP showed little or weak inhibition to MLCK treated with trypsin (30–50% inhibition at 20 μM for Ro 22-4839 and 10–20% at 300 μM for W-7 and TFP). These results indicate that Ro 22-4839 inhibits MLCK indirectly by acting on the calmodulin. As the MLCK inhibition caused by Ro 22-4839 correlated well with its results of actomyosin superprecipitation, the target of Ro 22-4839 in the inhibition of the contraction of smooth muscle may reside in its interaction with MLCK through Ca^{2+}/calmodulin.

The potency (IC_{50}) of calmodulin antagonism of the compounds tested varies from one assay system to another (Table

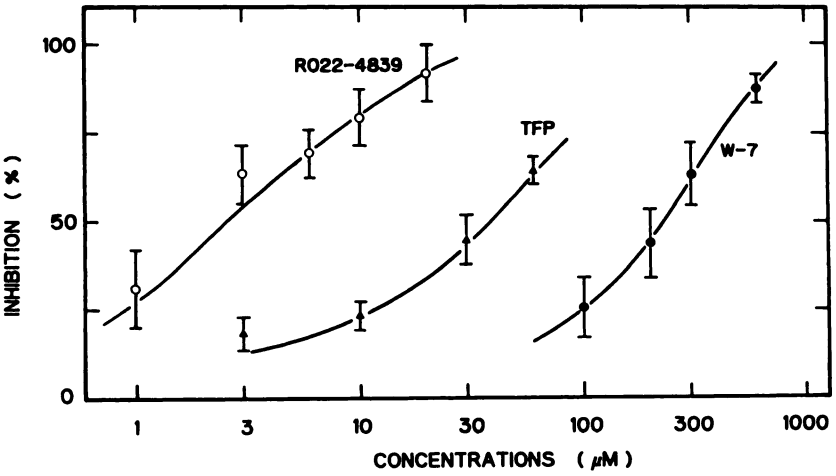

Fig. 2. Effects of Ro 22-4839, W-7, and TFP on ATP-induced superprecipitation of chicken gizzard actomyosin. The superprecipitation was performed as described in Fig. 1. Each point represents the mean ± standard error of three to four separate experiments. The IC_{50} value was determined by log-probit analysis of the rate of superprecipitation ($\Delta A/t_{0.5}$). ○, Ro 22-4839; ▲, TFP; ●, W-7.

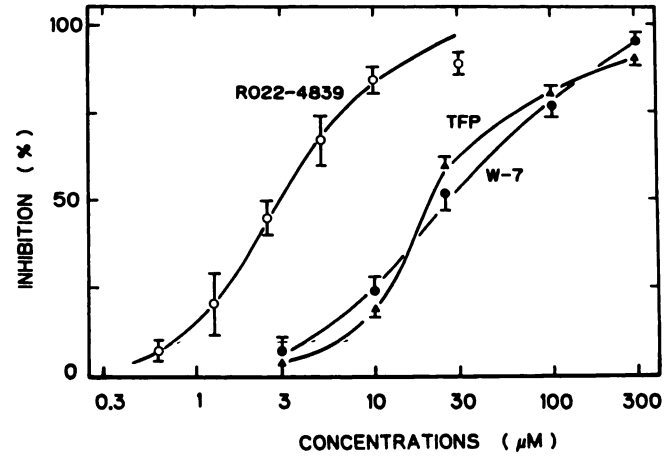

Fig. 3. Effects of Ro 22-4839, W-7, and TFP on Ca^{2+}/calmodulin-activated MLCK from chicken gizzard. The activity of MLCK was measured as described under Materials and Methods. Each point represents the mean ± standard error of three separate experiments each performed in duplicate. ○, Ro 22-4839; ▲, TFP; ●, W-7.

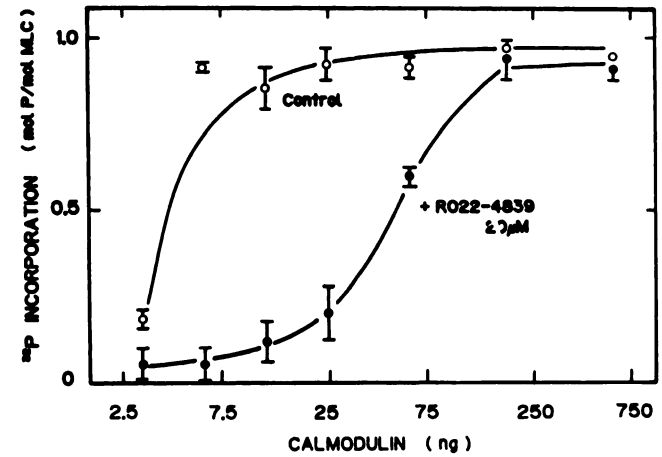

Fig. 4. Effects of increasing concentration of calmodulin on the inhibition of MLCK elicited by Ro 22-4839. The activity was measured as described under Materials and Methods [with (○) or without (●) 20 μM Ro 22-4839], except that the amount of calmodulin in the assay mixture was varied as indicated in the figure.

1, Figs. 2 and 3), suggesting that the mode of inhibition of these compounds to calmodulin was not identical.

Fluorescence measurement. To obtain information regarding binding characteristics of Ro 22-4839, W-7, and TFP to calmodulin, we used TNS, 9-AC, and NPN as hydrophobic fluorescence probes. In the presence of Ca^{2+}, the fluorescence intensity of hydrophobic fluorescence probes was greatly increased by calmodulin. Ro 22-4839 inhibited the fluorescence in a concentration-dependent manner (Fig. 5), with apparent IC_{50} values of 10 μM, 7 μM, and 5 μM for TNS, NPN, and 9-

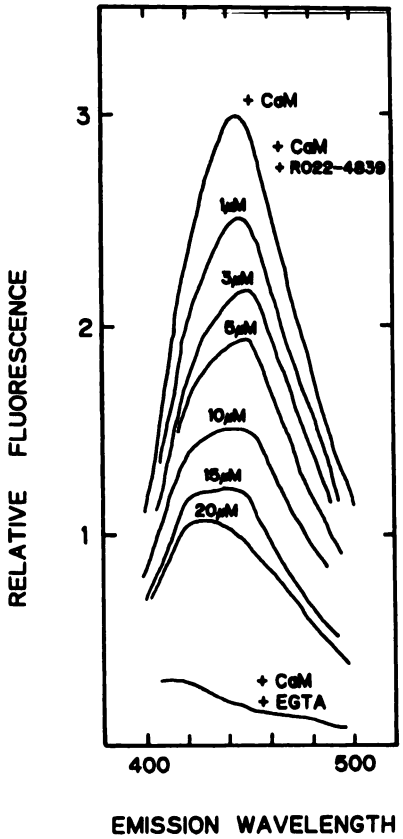

Fig. 5. Ro 22-4839 diminishes the fluorescence intensity of the hydrophobic fluorescence probe, TNS. Typical fluorescence measure with or without Ro 22-4839 is shown. Fluorescence measurements were made at room temperature as described (23). Samples were prepared in a total volume of 3 ml containing 25 mM Tris-HCl (pH 7.0), 400 μg of calmodulin (*CaM*), 1.4 mM $CaCl_2$, 15 μM TNS. Excitation was at 365 nm.

AC, respectively (Fig. 6). W-7 (IC_{50} = 14 μM for TNS, 4 μM for NPN, and 90 μM for 9-AC) and TFP (IC_{50} = 7.5 μM for TNS, 1.8 μM for NPN, and 2.5 μM for 9-AC) also inhibit the fluorescence of hydrophobic probes in a concentration-dependent manner (Fig. 6). These results indicate that hydrophobic domains of calmodulin become accessible to the fluorescence probes in the presence of Ca^{2+}, and that Ro 22-4839, W-7, and TFP seem to bind to these domains. However, the binding sites of these compounds to calmodulin are different from each other, as suggested by the differing IC_{50} values of calmodulin antagonists against the probes.

Effects on other protein kinases. Effects of Ro 22-4839 on other protein kinases were also investigated, including cyclic AMP-dependent protein kinase and protein kinase C. As shown in Table 2, Ro 22-4839 has little effect on both kinases, whereas W-7 and TFP exhibit weak but appreciable inhibition.

Discussion

In the present study, Ro 22-4839 was shown to inhibit various calmodulin-activated enzymes or processes including cyclic AMP phosphodiesterase from bovine heart and brain, ATP-induced superprecipitation, and MLCK with respective IC_{50} values of 20 μM, 17 μM, 2.0 μM, and 3.1 μM. In regard to calmodulin-activated phosphodiesterase from bovine heart, Ro 22-4839 was found to be twice as potent as W-7 and half as potent as TFP, both of which are well known calmodulin antagonists (14, 30). Of particular interest in the present study was the strong potency of Ro 22-4839 to inhibit the superprecipitation of actomyosin as well as the activity of MLCK from chicken gizzard smooth muscle, with respective IC_{50} values of 2.0 μM and 3.1 μM. The superprecipitation of actomyosin has been a well known *in vitro* model of the contraction of the smooth muscle, and one of the key enzymes of this process has been widely accepted as MLCK, which has a Ca^{2+}/calmodulin-binding domain (29) and is activated by calmodulin (28). The inhibitory action of Ro 22-4839, W-7, and TFP to MLCK treated with trypsin was found to be weak, indicating that the inhibition observed was due to its binding to calmodulin and did not appear to involve inhibition at the active site of the enzyme. This was confirmed by the experiment in which the inhibition elicited by Ro 22-4839 was completely overcome by an excess amount of calmodulin.

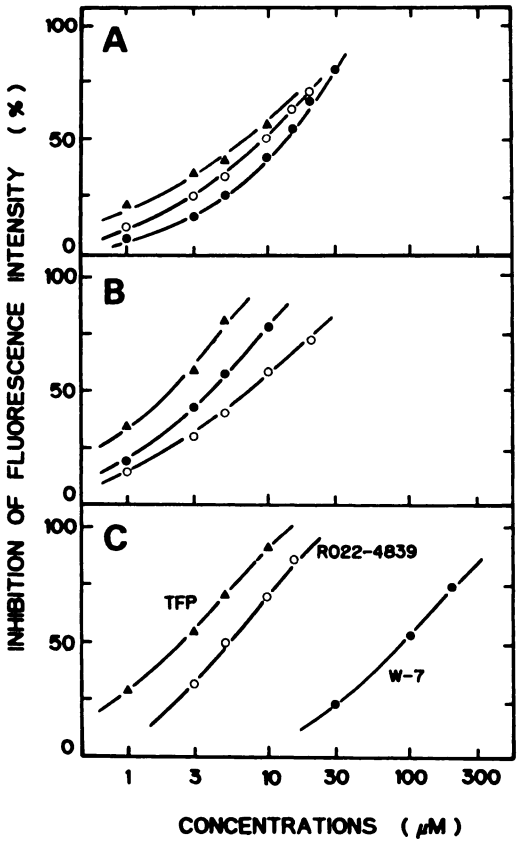

Fig. 6. Effects of calmodulin antagonists on fluorescence produced by hydrophobic fluorescence probes. Fluorescence measurements were performed as described in the legend of Fig. 5. The amounts of calmodulin in the reaction mixture for measuring the fluorescence of TNS (A), NPN (B), and 9-AC (C) were 400 μg, 100 μg, and 100 μg, respectively. Each point represents the mean of two separate experiments. ○, Ro 22-4839; ▲, TFP; ●, W-7.

TABLE 2

Effects of Ro 22-4839, TFP, and W-7 on protein kinases

The activities of cyclic AMP-dependent protein kinase and protein kinase C were measured as described in Materials and Methods. Each value represents the mean ± standard error of the mean of three to five separate experiments (indicated in parentheses), each performed in duplicate.

Compounds	IC_{50}[a]	
	A-Kinase[b]	Protein kinase C[c]
	μM	
Ro 22-4839	>20 (3)	>20 (3)
TFP	32 ± 4.2 (5)	80 ± 10 (4)
W-7	130 ± 12 (4)	200 ± 20 (4)

[a] The concentrations of the compounds producing 50% inhibition of each enzyme activity.
[b] Cyclic AMP-dependent protein kinase from bovine heart.
[c] Ca^{2+}-activated, phospholipid-dependent protein kinase from rat brain.

There is also a possibility that Ro 22-4839 inhibits superprecipitation by acting on a site other than MLCK, because the actomyosin preparation is heterogeneous. Recent biochemical and physiological studies have indicated that, whereas myosin phosphorylation-dephosphorylation is the primary Ca^{2+}-mediated regulatory process in smooth muscle, other Ca^{2+}-dependent control mechanisms may exist which modulate the actin-myosin interaction and thereby the contractile state of the muscle. Therefore, there is a possibility that Ro 22-4839 acts on this state. However, we feel that the main target of Ro 22-4839 is calmodulin, which stimulates MLCK, because the MLCK inhibition caused by Ro 22-4839 correlated well with its results of actomyosin superprecipitation.

Ro 22-4839 has been shown to dilate isolated vascular vessels constricted by various agonists including K^+, Ca^{2+}, prostaglandin $F_{2\alpha}$, and incubated blood, and its IC_{50} values have been almost comparable to that in MLCK inhibition (9). The inhibition of MLCK by Ro 22-4839 through Ca^{2+}/calmodulin could explain these results because Ro 22-4839 could induce vasodilation by affecting more basic events common to these constrictors. W-7 has also been reported to show antagonism to various vasoconstrictors such as KCl, $CaCl_2$, norepinephrine, serotonin, histamine, and angiotensin II (14, 20).

The relatively weak potency of Ro 22-4839 to inhibit calmodulin-activated Ca^{2+},Mg^{2+}-ATPase of erythrocytes could explain the result that Ro 22-4839 did not affect the ATP content of erythrocytes (10). The ATP content of erythrocytes was reduced from 983 to 161 μmol/liter of packed cells by Ca^{2+} loading, which was due to ATP consumption by membrane

Ca^{2+}-ATPase, whereas Ro 22-4839 at 10 and 30 μM did not influence the ATP reduction. Additionally, Ro 22-4839 has been reported not to inhibit the slow, inward current of isolated guinea pig papillary muscle in contrast to calcium antagonists (9). These observations and the ineffectiveness of Ro 22-4839 on Ca^{2+} uptake and Ca^{2+} release from isolated canine sarcoplasmic reticulum (31) suggest that Ro 22-4839 has little effect on Ca^{2+} flux in plasma membrane or Ca^{2+} uptake and release from sarcoplasmic reticulum. Recent studies (32–34) have shown that agonist-induced activation of protein kinase C could induce smooth muscle contraction. However, Ro 22-4839 does not inhibit protein kinase C as well as cyclic AMP-dependent protein kinase at concentrations up to 20 μM. The solubility limit of Ro 22-4839 may not seriously interfere with these interpretations because the effects of Ro 22-4839 on protein kinases were clearly different from those of W-7 or TFP at the same concentration. These observations further support the idea that Ro 22-4839 would exhibit its vasodilating action through the Ca^{2+}/calmodulin pathway.

The binding of Ro 22-4839 to calmodulin was characterized by using fluorescence probes (Fig. 5). It has been proposed that the binding of Ca^{2+} to calmodulin induces structural change to expose the hydrophobic region (35, 36) to which calmodulin antagonists bind and thereby inhibit the interaction of calmodulin with target proteins or enzymes (37). Fluorescence of hydrophobic probes was intensified by its binding to the hydrophobic region of calmodulin (36). Our results with fluorescence probes suggest that Ro 22-4839 interacts directly with the hydrophobic region of calmodulin as W-7 and TFP (Figs. 5 and 6). However, the precise mode of action of these three compounds may be different from each other, as suggested by the differing IC_{50} values of the compounds against the probes.

At present it is uncertain how calmodulin antagonists interfere with the interaction of calmodulin with target enzymes. Our data showed that Ro 22-4839 inhibited calmodulin-activated events in the order MLCK = superprecipitation > phosphodiesterase > adenylate cyclase >> Ca^{2+},Mg^{2+}-ATPase, whereas W-7 inhibited in the order MLCK > phosphodiesterase = adenylate cyclase > Ca^{2+},Mg^{2+}-ATPase > superprecipitation, and TFP in the order phosphodiesterase > adenylate cyclase > Ca^{2+},Mg^{2+}-ATPase > MLCK superprecipitation, indicating that conformational changes induced by various calmodulin antagonists are not the same and that some compounds show a great deal of selectivity toward calmodulin-regulated enzymes. Similarly, Hidaka *et al.* (38) have shown that the inhibition spectrum of prenylamine is different from that of W-7 using phosphodiesterase, Ca^{2+},Mg^{2+}-ATPase, and MLCK.

We must consider the possibility that the observed differences in IC_{50} are due to differences in the amount of available free inhibitor rather than a difference in affinity of the inhibitor for calmodulin. However, we conclude that our results are valid because two heterogenous preparations, Ca^{2+},Mg^{2+}-ATPase and chicken gizzard actomyosin, showed markedly different reactivity to the inhibitors tested. Indeed, Ro 22-4839 showed a potent inhibition against superprecipitation, a crude preparation.

W-7 and TFP were found to inhibit the phosphodiesterase preferably from bovine brain rather than from heart, but not Ro 22-4839 (Table 1). Similar results have been reported by Kubo *et al.* (39), that flunarizine inhibits the phosphodiesterase of bovine brain, but not of heart. These different effects of various calmodulin antagonists also support the idea that the binding characteristic of one calmodulin antagonist to a certain enzyme is not always the same as others. Recently, Mills *et al.* (40) have reported that allosteric interactions among the drug/protein sites on calmodulin might provide a mechanism for selectivity, directing calmodulin to specific target proteins. In this context, it is speculated that Ro 22-4839 induces some conformational change in Ca^{2+}/calmodulin complex which greatly affects the affinity of Ca^{2+}/calmodulin to MLCK with lesser effects on other calmodulin-related enzymes.

Erythrocyte deformability in which calmodulin also plays an important role as a modulator of cytoskeleton may be of critical importance for the capillary flow dynamics. In recent studies, it has been shown that Ro 22-4839 inhibits erythrocyte shape changes and filterability decreases *in vitro* (11). Thus, it seems reasonable to speculate that Ro 22-4839 is acting on the cytoskeleton of erythrocytes as a calmodulin antagonist (41).

In conclusion, Ro 22-4839 inhibits calmodulin-activated enzymes, most significantly of MLCK, highly specific to vascular smooth muscle contractile systems. The mechanism of blood flow regulation has recently begun to be better understood. Contractability/elasticity of blood flow caused by high deformability of erythrocyte membrane are thought to be important in blood flow regulation. These factors are in turn controlled by the calcium messenger system, namely, the calcium-calmodulin branch, which responds to intracellular calcium levels. Ro 22-4839 was discovered to be an intracellular calcium antagonist which selectively inhibits calmodulin and which mainly affects the blood vessels and erythrocytes. Therefore, Ro 22-4839 is expected to show clinical efficacy in the treatment of sequelae of strokes, prevention of recurrence, and amelioration of the various symptoms accompanied by cerebral arteriosclerosis. Ro 22-4839 is now under clinical trials in Japan.

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