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Modulation of Calmodulin Function and of Ca^{2+}-Induced Smooth Muscle Contraction by the Calmodulin Antagonist, HT-74

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SUMMARY

The relationship between the functions of calmodulin (CaM) and Ca^{2+}-induced smooth muscle contraction was investigated using a newly synthesized CaM antagonist, 3-(2-benzothiazolyl)-4,5-dimethoxy-*N*-[3-(4-phenylpiperidinyl)propyl]benzenesulfonamide (HT-74). We noted a selectivity of HT-74 for CaM, compared to other calcium-binding proteins and target enzymes of CaM. As HT-74 had no significant effect on the intensity of 8-anilino-1-naphthalene-sulfonic acid (ANS) fluorescence in the presence of the Ca^{2+}-CaM complex, the HT-74-binding sites may differ from those of naphthalenesulfonamides and phenothiazines which decrease ANS fluorescence. The Ca^{+2} binding to CaM was inhibited significantly by 1.0 μM HT-74, in sharp contrast to phenothiazines and naphthalenesulfonamides which increase the extent of the Ca^{2+} binding to CaM. Increasing CaM concentrations reversed the HT-74-induced inhibition of CaM-dependent enzymes such as myosin light chain kinase and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, with K_i values of 0.5 μM and 0.4 μM, respectively. In the presence of 0.3 μM HT-74, potassium-depolarized rabbit aortic strips pre-contracted with 0.3 mM $CaCl_2$ relaxed, and this relaxation was completely reversed by the addition of an excess amount of $CaCl_2$ (10 mM). This compound shifted the dose-response curve for $CaCl_2$ to the right, in a competitive manner. However, HT-74 inhibited the phenylephrine-induced contraction elicited in Ca^{2+}-free solution and the calcium ionophore A23187-induced contraction in the presence of calcium ion. Therefore, this agent affects intracellular actions of Ca^{2+} rather than membrane receptors or the influx of Ca^{2+}. HT-74 is a CaM antagonist which binds to CaM in a manner different from that heretofore reported. It inhibits Ca^{2+} binding to CaM and produces a competitive inhibition of Ca^{2+}-induced contractions of depolarized vascular smooth muscle.

Calcium ion is a major regulator of contractile protein interactions in smooth muscles. The release of Ca^{2+} into the cytoplasm is a primary event linking excitation to contraction. Contraction of smooth muscle depends on an increase in the concentration of sarcoplasmic free Ca^{2+} which activates the contractile elements. Ca^{2+} may bind to CaM, and the Ca^{2+}-CaM complex subsequently binds to and activates MLCK (1). Activation of this protein kinase results in phosphorylation of the light chain on myosin and the stimulation of actin-activated MgATPase activity of smooth muscle myosin. Biochemical, physiological, and pharmacological approaches all suggest that myosin light chain phosphorylation plays an important role in smooth muscle contraction (2).

Although mechanisms involved in activation of myosin phosphorylation require the binding of calcium ion to CaM (3), there is no apparent quantitative relationship between the Ca^{2+} binding to CaM and the regulation of smooth muscle contraction *in vivo*. We investigated the relationship between Ca^{2+} binding to CaM and smooth muscle contraction using a novel CaM antagonist, HT-74. We found that this compound binds to CaM in a manner different from that seen with naphthalenesulfonamides and phenothiazines. HT-74 has an inhibitory effect on the binding of Ca^{2+} to CaM and causes a competitive inhibition of Ca^{2+}-induced contractions of depolarized vascular smooth muscle.

Materials and Methods

Chemicals

Bovine brain CaM was purified by the method of Yazawa *et al.* (4). Myosin light chain kinase was purified from chicken gizzard by the method of Adelstein and Klee (5). Myosin light chain of chicken gizzard, used as a substrate for the kinase assay, was prepared according to the method of Perrie and Perry (6). The light chain was separated from CaM by DEAE-cellulose chromatography (7). CaM-deficient,

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ABBREVIATIONS: CaM, calmodulin; MLCK, myosin light chain kinase; HT-74, 3-(2-benzothiazolyl)-4,5-dimethoxy-N-[3-(4-phenylpiperidinyl)propyl] benzenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; ANS, 8-anilino-1-naphthalenesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; KBS, Krebs-bicarbonate solution; NE norepinephrine.

Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was purified as described previously (8). W-7 was synthesized by the method of Hidaka *et al.* (9). Calcium-45, cyclic [^{3}H]guanosine 3′,5′-monophosphate, and adenosine 5′-[γ-^{32}P]triphosphate were from Amersham International, Ltd. Dansyl-chloride and ANS were purchased from Wako Pure Chemical Industries, Ltd.

Synthesis of HT-74

3-(2-Benzothiazolyl)-4,5-dimethoxybenzenesulfonyl chloride [Fig. 1 (*1*)]. To a solution of chlorosulfonic acid (9 ml), 2-(2,3-dimethoxyphenyl)benzothiazole (3.0 g) was added portion-wise at −20° to −10°. After stirring for 2 hr, the reaction mixture was poured into ice-water. The resulting precipitates were collected by filtration, washed with distilled water, and dried to give 3-(2-benzothiazolyl)-4,5-dimethoxybenzenesulfonyl chloride (3.6 g), a pale yellow powder.

3-(2-Benzothiazolyl)-4,5-dimethoxy-*N*-(3-hydroxypropyl)-benzenesulfonamide [Fig. 1 (*2*)]. To a stirred suspension of 3-(2-benzothiazolyl)-4,5-dimethoxybenzenesulfonyl chloride (2.9 g) in dioxane (30 ml), a solution of 3-aminopropanol (1.5 g) in dioxane (10 ml) was added dropwise at room temperature. The reaction mixture was stirred for another hour and evaporated to dryness *in vacuo*. The residue was dissolved in ethyl acetate, and the mixture was washed with distilled water, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo*. The residue was recrystallized from aqueous ethanol to give 3-(2-benzothiazolyl)-4,5-dimethoxy-*N*-(3-hydroxypropyl)benzenesulfonamide (1.45 g) as white crystals, m.p. 184–186°. NMR [in d_6-dimethylsulfoxide-D_2O] (ppm): 1.4–1.8 (2H, multiplet), 2.85 (2H, triplet, j = 5 Hz), 3.38 (2H, triplet, j = 5 Hz), 3.83 (3H, singlet), 4.03 (3H, singlet), 7.3–7.7 (3H, multiplet), 8.0–8.2 (2H), 8.40 (1H, doublet, j = 2 Hz).Chemical analysis for $C_{18}H_{20}N_2O_5S_2$ was as follows, Calculated: C, 52.93; H, 4.93; N, 6.86. Found: C, 53.15; H, 4.86; N, 6.83.

3-(2-Benzothiazolyl)-*N*-(3-chloropropyl)-4,5-dimethoxybenzenesulfonamide [Fig. 1 (*3*)]. A mixture of 3-(2-benzothiazolyl)-4,5-dimethoxy-*N*-(3-hydroxypropyl)-benzene-sulfonamide (3.5 g), thionyl chloride (7 ml), and methylene chloride (100 ml) was refluxed for 5 hr. The reaction mixture was evaporated to dryness *in vacuo*. The residue was washed with water and recrystallized from $CHCl_3$-i-Pr_2O to give 3-(2-benzothiazolyl)-*N*-(3-chloropropyl)-4,5-dimethoxybenzenesulfonamide (3.6 g) as white crystals, m.p. 189–190°. NMR (in $CDCl_3$-d_6-dimethylsulfoxide) (ppm): 1.7–2.1 (2H, multiplet), 3.00 (2H, quartet, j = 4 Hz), 3.57 (2H, triplet, j = 4 Hz), 3.98 (3H, singlet), 4.08 (3H, singlet), 7.3–7.6 (3H, multiplet), 7.8–8.2 (2H), 8.55 (1H, doublet, j = 2 Hz). Chemical analysis for $C_{18}H_{19}ClN_2O_4S_2$ was: Calculated: C, 50.64; H, 4.49; N, 6.56. Found: C, 50.76; H, 4.62; N, 6.39.

HT-74. A mixture of 3-(2-benzothiazolyl)-*N*-(3-chlorpropyl)-4,5-dimethoxybenzenesulfonamide (2.2 g), 4-phenylpiperidine (0.9 g), triethylamine (1.1 ml), potassium iodide (1.1 g), and *N,N*-dimethylformamide (10 ml) was stirred at 100° for 1 hr. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with distilled water and dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was treated with alcoholic hydrogen chloride. The crude hydrochloride was recrystallized from ethanol to give 3-(2-benzothiazolyl)-4,5-dimethoxy-*N*-[3-(4-phenylpiperidinyl)propyl]benzenesulfonamide hydrocrhloride (1.1 g) as pale yellow crystals, m.p. 225–227°. Chemical analysis for $C_{29}H_{32}N_3O_4S_2$ HCl·½ H_2O was as follows, Calculated: C, 58.33; H, 5.91; N, 7.04. Found: C, 58.51; H, 5.80, N, 7.10.

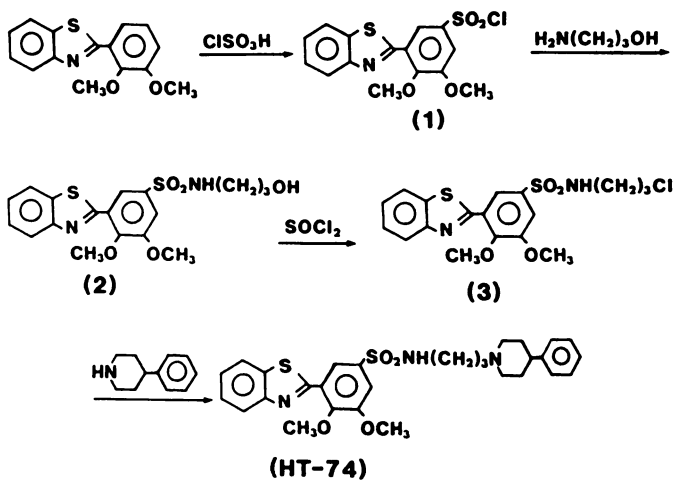

Fig. 1. Outline of chemical synthesis of HT-74.

Methods

As HT-74 at concentrations below 10 μM had no significant UV absorbance, we measured the change in intrinsic fluorescence of HT-74 to investigate the interaction between this compound and Ca^{2+}-binding proteins. Fluorescence measurements were made at 25° using an Aminco-Bowman spectrofluorometer, as reported previously (10). The fluorescence intensity of HT-74 was determined with excitation at 310 nm and emitted light monitored at 410 nm. Relative fluorescence values were uniformly corrected for dilution. Fluorometric titration of CaM with HT-74 or W-7 was performed by preparing samples containing 20 μM CaM and adding the indicated amounts of the compounds in 1 ml of 50 mM Tris-HCl (pH 7.6) and 0.1 mM $CaCl_2$ or 1 mM EGTA. The concentration of ANS was 40 μM. Fluorescence intensity was determined with excitation at 350 nm, and emission intensity was monitored at 500 nm.

The binding of Ca^{2+} to CaM was measured by the flow dialysis method (11), using a Teflon apparatus. Cellulose dialysis tubing served as the membrane separating the lower and upper chambers. The flow rate of solvent in the lower chamber was 170 ml/hr. The upper chamber contained 1.2 ml of 12 μM CaM. Nanopure II (Barnstead) was used to remove Ca^{2+} from all stock solutions. After the Nanopure II treatment, the calcium content of stock solution was less than 0.1 μM, as detected by atomic absorption spectrophotometer.

MLCK activity was assayed as described (12). Ca^{2+}-Dependent cyclic nucleotide phosphodiesterase was measured as reported (13).

Recording of mechanical activity. Male albino rabbits weighing 2.3–2.7 kg were killed by air embolism. The thoracic aorta and the superior mesenteric artery were rapidly removed and helically cut (at an angle of approximately 45° to the longitudinal axis) into strips of 2.5 × 30 mm, in the case of the aorta and 0.8 × 15 mm for the mesenteric artery, following removal of the adventitial connective tissue. The strips were fixed vertically between hooks in an organ bath containing 20 ml of KBS, of the following composition (mM): NaCl, 115; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; and dextrose, 10. The bathing solutions were maintained at 37° and bubbled with a mixture of 95% O_2-5% CO_2. The upper end of the strip was connected to the lever of a force-displacement transducer (TB-612T, Nihonkoden Kogyo Co., Tokyo, Japan) by a silk thread. An initial resting tension of 2 *g* or 1 *g* was applied to the aortic or mesenteric arterial strips, respectively. Before the experiments were commenced, preparations were allowed to equilibrate for 1 hr in KBS. After obtaining reproducible responses to 50 mM KCl, the effect of HT-74 on cumulative concentration-response curves for $CaCl_2$ were obtained in a Ca^{2+}-free, high K^+ KBS (in which KCl was increased to 80 mM and NaCl was reduced by an equimolar amount) by a stepwise increase in the concentration of $CaCl_2$, as soon as a steady response to the preceding dose had been obtained. The antagonistic effect of HT-74 on vascular contractile responses to other agonists in the KBS also was examined as follows: after precontraction was produced twice by NE, serotonin, or histamine, cumulative concentration-response curves to either agonist were obtained in the absence and in the presence of HT-74. Results shown in the figures were expressed as the mean values ± standard deviation. Effects of HT-74 on phenylephrine-induced contractions of aortic strips in Ca^{2+}-free solution and on calcium ionophore-induced contractions were investigated, as reported (14).

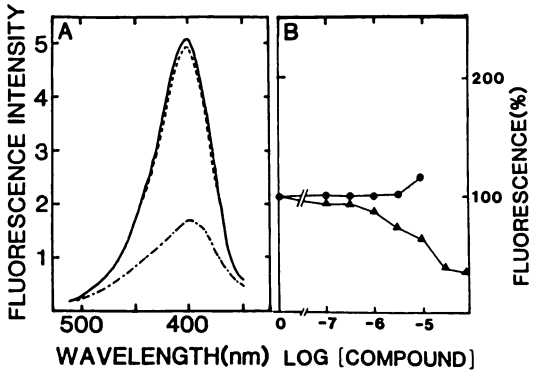

Fig. 2. Effect of calmodulin on HT-74 fluorescence (A) and effect of CaM antagonists on the fluorescence of ANS-CaM complex (B). A. Emission spectra of 2 μM HT-74 in 50 mM Tris-HCl (pH 7.6), without CaM (——) or with 0.5 μM CaM in the presence of 0.1 mM $CaCl_2$ (—— - ——) or 3 mM EGTA (- - -). Excitation was at 310 nm. B. The percentage of change in the fluorescence of ANS in the presence of Ca^{2+}-CaM complex as a function of HT-74 (●) and W-7 (▲). Excitation was at 350 nm and emission at 500 nm. Each cuvette contained 10 μM CaM, 40 μM ANS, and 50 mM Tris-HCl (pH 7.6).

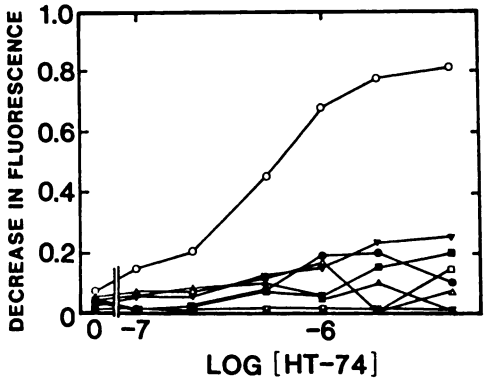

Fig. 3. Interaction of HT-74 with calmodulin and other calcium-binding proteins. Experiments were performed with each protein (2 μM) including CaM (○, ●), troponin C (△, ▲), parvalbumin (▽, ▼), or S-100 protein (□, ■) in 50 mM Tris-HCl (pH 7.5) and 0.1 mM $CaCl_2$ (*open symbols*) or 1 mM EGTA (*solid symbols*), as described under Materials and Methods. The decrease in fluorescence intensity of these samples was determined with excitation at 310 nm and emitted light monitored at 410 nm. Ca^{2+}-binding proteins were isolated as reported (28).

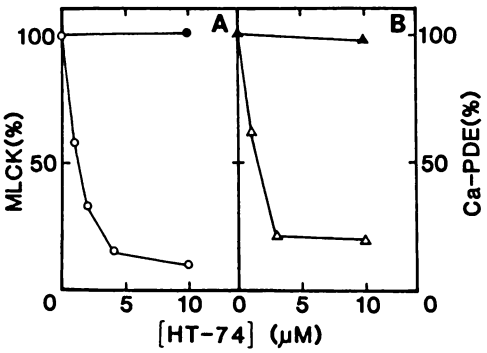

Fig. 4. Inhibition of myosin light chain kinase (A) and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (B) by HT-74. The effect of HT-74 on Ca^{2+}-CaM-dependent enzymes was assayed in the presence of 24 nM CaM and 0.1 mM $CaCl_2$ (○, △). The activity of trypsin-treated Ca^{2+}-CaM-dependent enzymes was determined in the presence of 1 mM EGTA and in the absence of Ca^{2+}-CaM complex (●, ▲).

TABLE 1

Effect of 0.3 μM HT-74 on the dose-response curve of rabbit mesenteric arterial strips in the presence of various agonists

Agonists	Control		HT-74 (0.3 μM)		
	N	−Log molar ED_{50}	*N*	% Maximum	−Log molar ED_{50}
$CaCl_2$	6	3.80 ± 0.16[a]	6	92.5 ± 4.3	3.20 ± 0.37[b]
Norepinephrine	6	7.35 ± 0.39	6	96.5 ± 1.3	6.80 ± 0.21[b]
Serotonin	6	7.00 ± 0.18	6	99.0 ± 0.5	6.30 ± 0.23[c]
Histamine	6	6.40 ± 0.34	6	99.5 ± 0.2	5.85 ± 0.18[c]

[a] Data are expressed as mean ± SD.
[b] Significantly different from control ($p < 0.05$).
[c] Significantly different from control ($p < 0.001$).

Results

Interaction between CaM and HT-74. As HT-74 proved to have fluorescent properties, we considered that it may be a useful probe for studying interactions with CaM. Fluorescence emission spectra of HT-74 in aqueous buffered solution with 0.5 μM CaM, in the presence or absence of calcium, are shown in Fig. 2A. The excitation wavelength was 310 nm. Binding of HT-74 to the purified CaM was monitored using a fluorospectrophotometer. In the presence of calcium ion, the fluorescence intensity of HT-74 decreased by 65% when purified CaM was added, and no significant decrease in the fluorescence was observed with CaM in the absence of Ca^{2+}. These results suggest that HT-74 interacts with CaM in a Ca^{2+}-dependent fashion. Ca^{2+} binding to CaM exposes hydrophobic regions that are capable of binding to a variety of hydrophobic fluorescent probe molecules such ANS (10, 15). At concentrations below 5 μM, HT-74 was effective in inhibiting CaM activity, but titration of the Ca^{2+}-CaM complex and ANS with this compound produced no significant change in the fluorescence intensity (Fig. 2B). With high concentrations of HT-74 (5–10 μM) there was a slight increase in fluorescence with ANS and the Ca^{2+}-CaM complex. This may be due to the intrinsic fluorescence intensity of HT-74 itself. Conversely, W-7, a well known CaM antagonist, suppressed the fluorescence of ANS with the Ca^{2+}-CaM complex at concentrations above 1 μM, a concentration similar to the one that inhibits CaM activity. These results suggest that HT-74 has distinctive binding sites which differ from those occupied by ANS and W-7. Fig. 3 shows the selective binding of HT-74 to CaM, compared with other Ca^{2+}-binding proteins such as troponin-C, parvalbumin, and S-100 protein. The Ca^{2+}-dependent interaction of HT-74 with CaM was determined by measuring changes in the fluorescence intensity, as a function of the concentrations of HT-74. Data derived from Fig. 3 indicate the apparent dissociation constant of 0.5 μM, with one binding site on CaM for HT-74. Moreover, we demonstrated the selectivity of this compound for CaM, compared to its target enzymes such as MLCK and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (Fig. 4). HT-74 inhibited, in a concentration-dependent manner, MLCK and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, in the presence of Ca^{2+}-CaM and 50% inhibition was attained at a concentration of 1.4 μM and 1.6 μM, respectively. In contrast, HT-74 in concentrations below 10 μM was not effective in inhibiting the catalytic fragment of these enzymes produced by trypsin treatment (Fig. 4) on the Ca^{2+}-dependent cyclic nucleotide phosphodiesterase in the absence of CaM. Because the purified MLCK had very low activity in the absence of Ca^{2+}-CaM, the effect of HT-74 on basal MLCK activity in the absence of Ca^{2+}-CaM could not

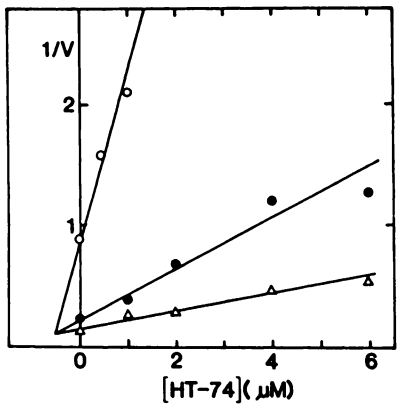

Fig. 5. Dixon plot of HT-74-induced inhibition of activation of MLCK. MLCK was assayed as described under Materials and Methods with CaM, 100 ng/ml (○), 2 μg/ml (●), or 20 μg/ml (△).

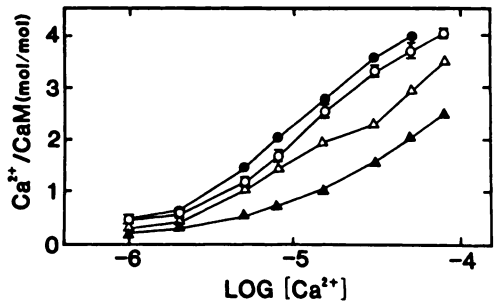

Fig. 6. Effect of HT-74 and W-7 on calcium ion binding to CaM. The Ca^{2+} bound to CaM, measured by the flow dialysis method in the absence (○) or presence of 0.5 μM HT-74 (△), 1.0 μM HT-74 (▲), or 10 μM W-7 (●), is shown as a function of the log of free molar Ca^{2+} concentration. The *points* represent three different experiments. *Vertical bars* of control experiments represent standard error.

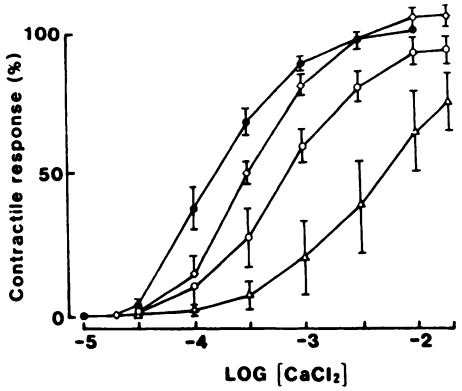

Fig. 7. Influence of HT-74 on the concentration-response curves for $CaCl_2$ in strips of the rabbit mesenteric artery. Each preparation was contracted in Ca^{2+}-free, 80 mM KCl solution. No (●), 0.1 μM (◌), 0.3 μM (○), or 1 μM (△) HT-74 was added 20 min before the addition of $CaCl_2$. In each preparation, the maximum tension developed in response to $CaCl_2$ in the control solution was taken as 100%. *Vertical bars* represent standard error. The number of preparations is 6 for each curve.

be measured accurately. Increasing the concentration of CaM reversed the HT-74-induced inhibition of MLCK and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, with K_i values of 0.5 μM (Fig. 5) and 0.4 μM, respectively, as determined by a Dixon plot (16). These results suggest that inhibition of these enzyme activities by HT-74 is due to its interaction with CaM.

Effect of HT-74 on Ca^{2+} binding to CaM. Ca^{2+} binding

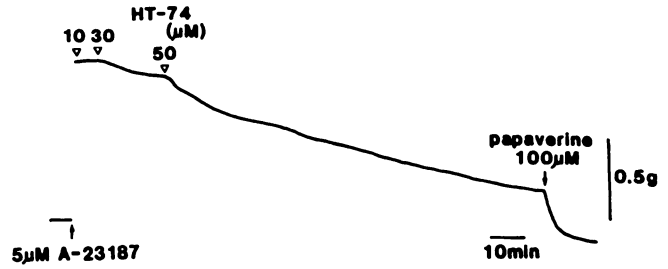

Fig. 8. Response of a strip of rabbit mesenteric artery to HT-74. The tonic contraction was produced by A23187 (5 μM). The *horizontal line* just on the *left* of the tracing indicates the initial level of tension before the addition of A23187. In the control experiment without HT-74, there was a sustained contraction, for at least 2 hr.

to CaM in the absence or presence of HT-74 or W-7 was investigated using the flow dialysis method (11), as described under Materials and Methods. CaM contains at least four calcium-binding sites (17). As shown in Fig. 6, HT-74 inhibited Ca^{2+} binding to CaM, whereas W-7 increased the affinity of Ca^{2+} binding to CaM. This result with W-7 is in agreement with data obtained by equilibrium dialysis (18) and NMR studies (19). MLCK and Ca^{2+}-dependent cyclic nucleotide phosphodiesterase activities, in the absence and presence of HT-74 and as a function of Ca^{2+} concentration, also were investigated. Increasing Ca^{2+} concentrations in the presence of 2 μM but not 24 nM CaM completely reversed the inhibition of the Ca^{2+}- CaM-dependent enzyme activities by 3 μM HT-74. These results indicate that HT-74 antagonizes calcium binding to CaM in an allosteric manner, that a direct displacement of ions may not be involved, and that a calcium-saturated CaM (4 mol of Ca/mol of protein) is not required.

Effect of HT-74 on Ca^{2+}-induced smooth muscle contraction. The addition of 20 mM KCl, a concentration that produces about 70% of the maximum contractile tension, caused a sustained contraction of the arterial strips. The addition of HT-74 in concentrations ranging from 10 nM to 3 μM elicited a concentration-dependent relaxation. The EC_{50} of HT-74 for the thoracic aorta was 0.54 μM.

To elucidate the mechanism of the HT-74-induced relaxation of the vascular strips, we examined the relaxing effect of HT-74 on vascular strips contracted by various contractile agonists. NE, serotonin, histamine, and $CaCl_2$ were the agonists used, and the EC_{50} values of each, in the absence or presence of 0.3 μM HT-74, are shown in Table 1. The EC_{50} values of the agonists increased significantly with the addition of 0.3 μM HT-74, but the maximum contractile tension elicited by various agonists was not reduced by HT-74. Moreover, the pharmacologically active concentrations of HT-74 were similar to those required for binding to CaM and inhibition of Ca^{2+}-, CaM-dependent enzyme activities and of Ca^{2+} binding to CaM. Cumulative concentration-response curves for $CaCl_2$ were obtained with concentrations ranging from 10 μM to 30 mM. HT-74, at concentrations of 0.1 μM and 1.0 μM, shifted the concentration-response curve rightward, in a competitive fashion (Fig. 7). Moreover, HT-74 significantly inhibited the phenylephrine-induced contraction elicited in Ca^{2+}-free solution (data not shown) and the calcium ionophore A23187-induced contraction in the presence of calcium ion (Fig. 8).

Discussion

The phosphorylation of myosin light chain of vertebratae smooth muscle and non-muscle cells by MLCK is thought to

play a major role in the regulation of contractile proteins (2). MLCK is totally dependent on the presence of calcium ion and CaM for activity (1). The enzyme is activated by the binding of 1 mol of CaM/mol of MLCK (2, 20). This Ca^{2+}-dependent reversible association and dissociation of CaM with MLCK is similar to the activation scheme proposed for other CaM-dependent enzyme systems such as Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (21).

$$Ca_n^{2+} + \text{CaM (inactive)} \rightleftharpoons Ca_n^{2+}\ \text{CaM (active)} \quad (1)$$

$$Ca_n^{2+}\ \text{CaM} + \text{MLCK (inactive)} \rightleftharpoons Ca_n^{2+}\ \text{CaM MLCK (active)} \quad (2)$$

According to this general scheme, activation first requires the formation of a Ca^{2+}-CaM complex (Eq. 1) that subsequently interacts with the inactive MLCK to form a catalytically active holoenzyme complex (Eq. 2). CaM antagonists such as W-7 and phenothiazine inhibit the formation of the catalytically active complex described as Eq. 2 (22). Conversely, the HT-74 we used in the present work may inhibit Ca^{2+} binding to CaM (Eq. 1).

It has been proposed that activation associated with an increase in cytoplasmic Ca^{2+} concentration is due to the Ca^{2+} binding to CaM, thus exposing the hydrophobic regions of Ca^{2+}-CaM complex (10, 15), and the ensuing interaction with MLCK leads to muscle contraction. These hydrophobic regions of CaM may be responsible for the binding of CaM antagonists such as naphthalenesulfonamides (23) as well as the binding of the enzymes. Naphthalenesulfonamide derivatives can displace the hydrophobic probe (such as ANS) from the hydrophobic surface of CaM (Fig. 2B) and increase the extent of the Ca^{2+} binding to CaM (Fig. 6). Similar findings were noted with trifluoperazine (18, 19). This class of CaM antagonists may block the process of Ca^{2+} information transduction from the Ca^{2+}-CaM complex of CaM-binding proteins such as MLCK. In contrast, HT-74 seems to be a selective inhibitor of the transduction of the Ca^{2+}-regulatory information system from calcium ion to CaM.

Although HT-74 shifted the concentration-response curve for $CaCl_2$ to the right, in a competitive manner, the possibility that HT-74 has pharmacological actions on the calcium channel can be excluded, for the following reasons. 1) HT-74 significantly antagonized the contractile response of vascular strips, almost to the same extent, with NE, serotonin, histamine, and $CaCl_2$. This antagonistic action is characteristic of CaM antagonists but not of calcium channel blockers. 2) As the apparent dissociation constant between CaM and HT-74 is similar to the pharmacological concentrations of HT-74, the vascular relaxing action may be due to an interaction with CaM. Not all calcium channel blockers interact with CaM at pharmacological concentrations, but at higher concentrations, some calcium channel blockers can bind to CaM (24, 25). 3) HT-74 inhibits the contraction produced by phenylephrine in the absence of extracellular Ca^{2+} and the calcium ionophore-induced contraction, thereby suggesting that this agent affects intracellular rather than extracellular actions of calcium ion. Part of the mechanism of action of HT-74 does not involve inhibition of the influx of Ca^{2+}, and the contractile processes after receptor activation or membrane depolarization are inhibited. Therefore, HT-74 may antagonize Ca^{2+} action on smooth muscle through an inhibitory effect on Ca^{2+} binding to CaM. Although the inhibition of MLCK by HT-74 could not be overcome completely by excess calcium ion, different domains of CaM may be responsible for the activation of other enzymes (26), and CaM-binding proteins such as caldesmon (27) may be responsible for regulation of myosin activity.

The exact binding sites of HT-74 on CaM remain to be elucidated. The observation that HT-74 binds in the presence of calcium ion, with a high affinity, indicates that it does not bind to the Ca^{2+}-binding loops in competition with calcium ion. The molecular pharmacological properties of HT-74 described in this paper suggest that this newly synthesized compound is a novel and unique CaM antagonist which differs completely from all heretofore available compounds. Investigations of calcium ion information systems related to smooth muscle contraction should be greatly facilitated with use of HT-74.

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