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Activity-Structure Relationship of Calmodulin Antagonists

Naphthalenesulfonamide Derivatives

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SUMMARY

The role of calmodulin in vascular response was investigated using two series of synthesized naphthalenesulfonamide derivatives. The actions of these compounds as calmodulin antagonists and vascular relaxants were shown to depend both on the chlorination of the naphthalene ring and on the length of the alkyl chain (C_5–C_{10}). A good correlation between potency in relaxation of these strips and affinity to calmodulin was obtained, thereby indicating that the mechanism of relaxation of these compounds is probably due to their effects on the Ca^{2+}-calmodulin-dependent process. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) produced relaxation of contracted vascular strips with various contractile agonists, whereas this derivative contracted the strip in the absence of contractile agonists, suggesting that this contractile response produced by W-7 is masked in the presence of a contractile agonist. To clarify the mechanism of W-7-elicited contraction of vascular strips in the absence of contractile agonists, the effect of W-7 was examined on the spontaneous efflux of [^{3}H]norepinephrine ([^{3}H]NE) which had been preloaded to the strip; W-7 was compared with *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), a chlorine-deficient derivative of W-7 which has a lower affinity for calmodulin than does W-7. Both W-7- and W-5-induced contractions were associated with increases in the [^{3}H]NE efflux and were inhibited by the addition of an *alpha*-adrenergic blocker, phentolamine. Other chlorine-deficient derivatives of W-7 which also had lower affinities for calmodulin increased the [^{3}H]NE efflux with vascular strip contraction, suggesting no correlation between the potency in increasing the [^{3}H]NE efflux and the affinity to calmodulin. Chlorine-deficient derivatives such as W-5 produced contraction in both the presence and absence of contractile agonists which were inhibited by phentolamine. Although W-7-induced relaxation of vascular strips was produced in the presence of Ca^{2+}, the increase in [^{3}H]NE efflux by W-7 and its derivatives was independent of Ca^{2+}, suggesting that this increase in the [^{3}H]NE efflux is not related to the Ca^{2+}-calmodulin system. Thereby, W-7, a calmodulin antagonist, has at least two kinds of pharmacological actions: one is calmodulin-related relaxation and another is calmodulin-independent contraction. These chlorine-deficient derivatives seems to serve as so-called control agents of the calmodulin antagonist W-7, and these pairs of structually related naphthalenesulfonamides are useful tools with which to elucidate the function of calmodulin.

INTRODUCTION

The important role of Ca^{2+} as an agent mediating excitation-response coupling is being given increasing attention (1). Biochemistry studies of Ca^{2+}-mediated cellular processes have increased following the recognition that calmodulin is a ubiquitous and extraordinarily versatile Ca^{2+}-binding protein (2). This substance mediates the control of a large number of enzymes by Ca^{2+} (3, 4). Calmodulin may also play an important role in the regulation of other processes, such as the assembly and disassembly of microtubules (5).

Like cyclic nucleotides, calmodulin is a universal regulator that is not tissue-specific or species-specific, and affects a large number of cellular functions.

Several calmodulin antagonists which bind to calmodulin in a calcium-dependent manner and inhibit selectively Ca^{2+}-calmodulin-induced activation of enzymes have been reported (6–8). However, the structure-activity relationship study of these calmodulin antagonists was

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not determined. We attempted to clarify the activity-structure relationship of calmodulin antagonists, naphthalenesulfonamide derivatives, that bind selectively to the Ca^{2+}-calmodulin complex; particular attention was given to the congeners that produce relaxation of isolated vascular strips contracted by various agonists. With the use of one of these calmodulin antagonists, W-7[2] and its derivatives, we recently demonstrated the role of calmodulin in platelet secretion, vascular contraction, and cell proliferation (7–12).

Recent studies have suggested that some effects of calcium on nerve function may be modulated by calmodulin (13, 14). In the present study, we investigated the effect of W-7 on sympathetic nerve terminals of isolated strips of aorta preloaded with [^{3}H]NE; evidence is presented that W-7 possesses bidirectional action on isolated vascular strip preparations.

METHODS

Preparation of phosphodiesterase and calmodulin. Calmodulin-deficient, Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was purified from bovine brain according to a method previously reported (15).

Calmodulin from bovine brain was purified to homogeneity according to a method previously described (9). Homogeneity was confirmed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Calmodulin activity was assessed according to its ability to increase the activity of calmodulin-deficient phosphodiesterase prepared from bovine brain. One unit of calmodulin is defined as the amount necessary to produce 50% maximal activation of calmodulin-deficient phosphodiesterase under standard conditions (16), and was equivalent to 10 ng of protein.

Binding studies. The equilibrium binding procedure was essentially as described by Hummel and Dreyer (17). The displacement of [^{3}H]W-7 from purified calmodulin in the presence of calcium ion by various concentrations of drugs under study was measured as previously reported (8). The buffer contained 0.5 μM [^{3}H]W-7, 20 mM Tris-HCl (pH 7.5), 20 mM imidazole, 3 mM magnesium acetate, and 100 μM $CaCl_2$ at 25°.

Preparation of aortic strips and experimental procedures. Albino rabbits of either sex weighing 2.3–2.6 kg were killed by exsanguination of the common carotid arteries, and the thoracic aorta (3.0–4.0 mm outside diameter) between the heart and the diaphragm was quickly excised. After removal of excess fat and adventitial connective tissue, the aorta was helically cut at an angle of approximately 45° to the longitudinal axis, resulting in strips 2 mm wide and 25 mm long, according to the method of Lewis and Koessler (18) or that of Furchgott and Bhadrakom (19). The helical strips were fixed vertically between hooks in a water-jacketed (37° ± 0.5°) tissue bath containing 40 ml of modified Krebs-Henseleit solution. The composition of the bathing solution was as follows (in millimolar concentrations): 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 pH of the bathing solution was 7.5. The tissue bath solutions were maintained at 37° ± 0.5° 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 (SB-1T, Nihon Kohden Kogyo Company, Tokyo, Japan) by a silk thread. An initial resting tension of 2 g was applied to the aortic strips. Before the experiments were begun, preparations were allowed to equilibrate for 1 hr in the bathing solution. During the equilibration period, the solutions were replaced every 15 min.

After 1 hr of equilibration time under the resting tension, a submaximally effective concentration of KCl (40 mM) was administered two or three times until successive responses remained constant. Concentrated stock solutions of W-7 were added directly to the bathing solution in a volume of 0.4 ml to give the final concentrations desired.

The contractile effect of W-7 was also studied in aortic strips obtained from rabbits given reserpine in a dose of 1 mg/kg i.v. for 2 consecutive days. The rabbits were killed 16–20 hr after the second injection of reserpine.

Measurement of spontaneous [^{3}H]NE efflux by a superfusion technique. Helical strips of rabbit thoracic aorta were preincubated with 1×10^{-7} M [^{3}H]NE (specific activity 24.7 Ci/mmole; New England Nuclear Corporation, Boston, Mass.) in 1 ml of modified Krebs-Henseleit solution containing ascorbic acid, 100 mg/liter. After incubation for 60 min with [^{3}H]NE at 37°, aortic strips were mounted vertically and then superfused with modified Krebs-Henseleit solution at a flow rate of 1.5 ml/min, as described by Su and Bevan (20). The resting tension of 2 g was also applied to these strips, and the preparations were equilibrated for 120 min before the start of experiments. W-7 was added to the superfusing Krebs-Henseleit solution. The solution dripped from the strip directly into a collecting vial which was changed at 1-min intervals, and 10 ml of scintillation fluid (67 ml of toluene, 33 ml of Triton X-100, 400 mg of 2,5-diphenyloxazole, and 10 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene) were added to the vial. Tritium activity in the collected superfusate was determined by counting in a Beckman LS9000 liquid scintillation counter. Contractile tension developed by W-7 was recorded together with the spontaneous [^{3}H]NE efflux.

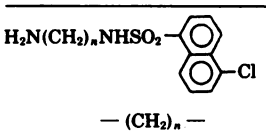
The tritiated norepinephrine content of the strip was measured as follows. After exposure to W-7, strips were superfused with Krebs-Henseleit solution for an additional 30 min in the absence of W-7. Strips were then removed from the bath, blotted with filter paper, weighed, solubilized in 1 ml of Protosol (tissue solubilizer, New England Nuclear Corporation), and kept overnight in an oven maintained at 50°. On the following day, 10 ml of toluene scintillation fluid (100 ml of toluene, 400 mg of 2,5-diphenyloxazole, and 10 mg 1,4-bis[2-(5-phenyloxazolyl)]benzene were added to the solubilized solution. Glacial acetic acid at a volume of 30 μl was then added to the vials to neutralize the solubilizer. The tritium content of the tissue was also determined.

[2] The abbreviations used are: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; NE, norepinephrine; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; W-9, *N*-(6-aminohexyl)-5-chloro-2-naphthanesulfonamide; W-6, *N*-(6-aminohexyl)-2-naphthanesulfonamide.

Drugs and chemicals. L-[^{3}H]NE (specific activity 24.7 Ci/mmole) was purchased from New England Nuclear Corporation. Other substances used included phentolamine mesylate (Regitine mesylate; CIBA Pharmaceutical Company, Summit, N. J.); atropine sulfate (Wako Pure Chemical Industries, Osaka, Japan); pyrilamine maleate (Sigma Chemical Company); methysergide dimaleate, hexamethonium bromide (Nakarai Chemicals, Kyoto, Japan); cocaine hydrochloride, tetrodotoxin (Sankyo Company, Tokyo, Japan); bretylium, tyramine hydrochloride (Merck & Company, Rathway, N. J.); and reserpine (Apoplon, Daiichi Pharmaceutical Company, Tokyo, Japan). W-7 and its analogues were synthesized by the method of Hidaka *et al.* (21).

TABLE 1

Effect of chlorinated naphthalenesulfonamide derivatives on [^{3}H]-W-7 binding to calmodulin, calmodulin-induced activation of phosphodiesterase, and isolated rabbit mesenteric arterial strips



— $(CH_2)_n$ —	Displacement of [^{3}H]W-7 from calmodulin, IC_{50}	Inhibition of PDE activation by calmodulin, IC_{50}	Vascular relaxation, ED_{50}
	μM	μM	μM
5	68	32	58
6	31	26	49
8	3.6	18	23
10	2.3	3.2	12

RESULTS

Chlorination of naphthalenesulfonamides and affinity to calmodulin. Two pairs of chlorinated and dechlorinated naphthalenesulfonamide derivatives were tested for their capacity to bind to calmodulin and to inhibit Ca^{2+}-calmodulin-dependent cyclic nucleotide phosphodiesterase, as shown in Fig. 1. We recently identified the calcium-dependent binding of W-7 to purified calmodulin from bovine brain (8). The capacity of chlorinated and dechlorinated naphthalenesulfonamide derivatives to compete for the specific binding of [^{3}H]W-7 to calmodulin is shown in Fig. 1. Chlorinated compounds bound avidly, whereas chloride-deficient analogues were approximately 9 times less potent. From the concentration of the unlabeled naphthalenesulfonamide derivatives necessary to compete for one-half the binding of 0.5 μM [^{3}H]W-7, one can evaluate the binding affinity for each of these compounds for purified calmodulin from bovine brain. Figure 1 summarizes the binding affinity of chlorinated and dechlorinated compounds to calmodulin and the potency of the inhibition of the compounds with regard to Ca^{2+}-calmodulin-induced activation of phosphodiesterase from bovine brain. In the inhibition of phosphodiesterase, the chlorinated group of these analogues was also potent, whereas the dechlorinated group was about 9-fold less potent. The chlorinated compounds bound more effectively to calmodulin and then inhibited cyclic nucleotide phosphodiesterase more potently than did the derivatives with hydrogen.

Hydrocarbon chain length of naphthalenesulfonamides and affinity to calmodulin. To assess which structural elements of the molecules are responsible for the affinity to calmodulin and the potency in phosphodiesterase inhibition, we studied the role of the length of the alkyl chain of naphthalenesulfonamide derivatives. As shown in Table 1, the capacity of the compounds preventing [^{3}H]W-7 from the specific binding to calmodulin increased with extension of the length of the hydrocarbon chain (C_5–C_{10}). Furthermore, the IC_{50} values related to the inhibition of the calmodulin-induced activation of Ca^{2+}-dependent phosphodiesterase decreased with increase in the length of the hydrocarbon chain. The degree to which these analogues inhibited the activation of phosphodiesterase was directly related to their ability to bind to calmodulin (Table 1).

Bidirectional action of W-7 on isolated vascular strips. The addition of W-7 at a concentration of 1×10^{-4} M caused a transient contraction followed by a persistent relaxation in K^+-contracted aortic strips of the rabbit (Fig. 2). When the response of aortic strips to W-7 was determined in the presence of 1×10^{-6} M phentolamine, the transient contraction produced by W-7 was abolished (Fig. 2). It is likely that the contractile response of the aortic strips to W-7 is the result of secretion of norepi-

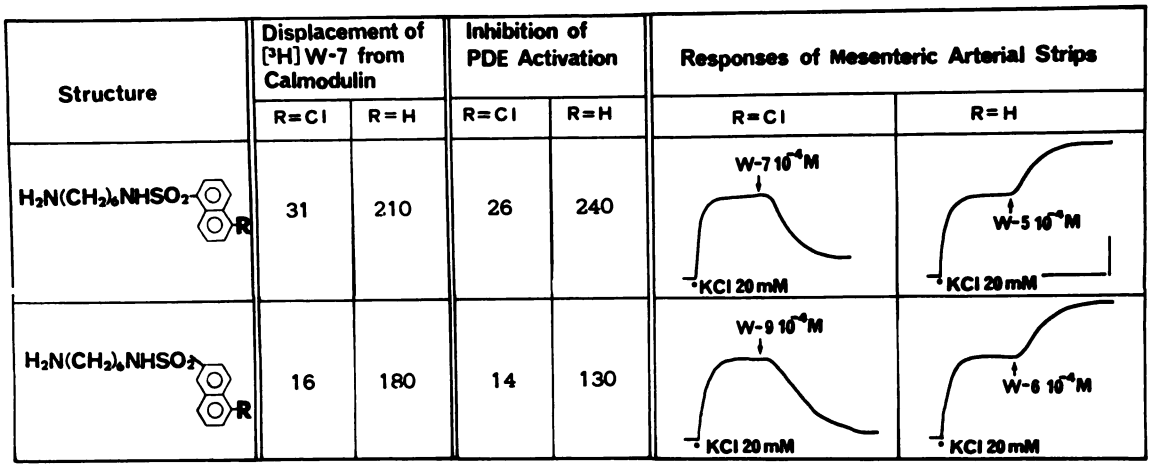


FIG. 1. *Effect of two pairs of naphthalenesulfonamide derivatives on [^{3}H]W-7 binding to calmodulin, calmodulin-induced activation of phosphodiesterase (PDE), and isolated rabbit mesenteric arterial strips*

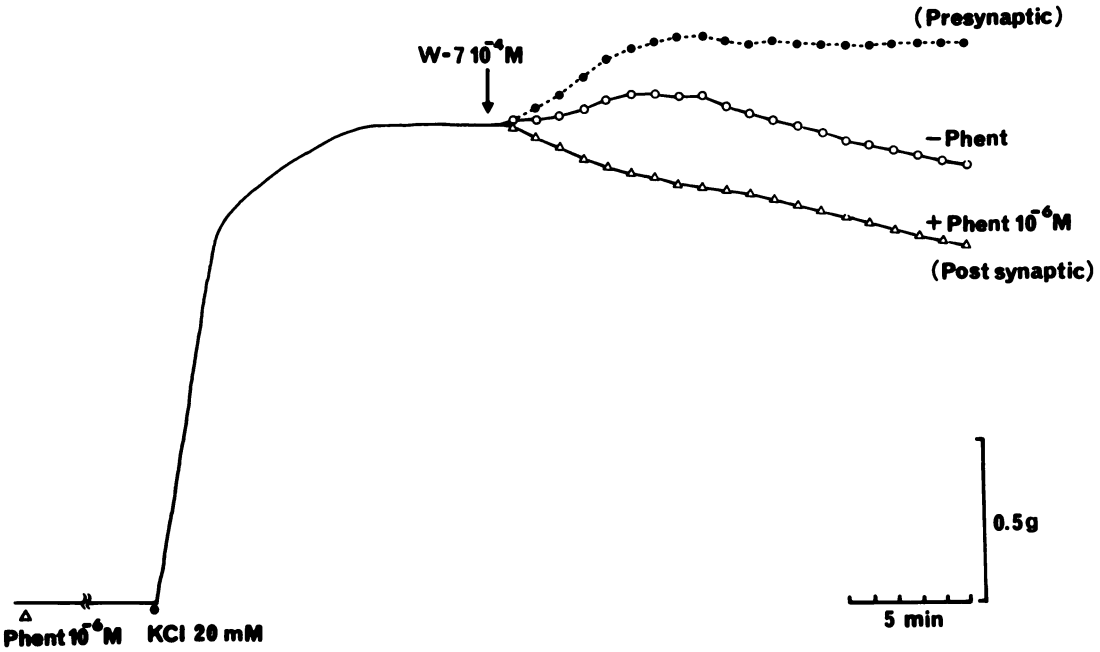


FIG. 2. *Responses of strips of rabbit thoracic aorta to W-7 in the absence or the presence of phentolamine (Phent)*

The aortic strips were contracted with KCl at a concentration of 20 mM. W-7 at a concentration of 1×10^{-4} M was added at the peak period of contraction (*arrow*). Phentolamine (1×10^{-6} M) was added 20 min before the addition of KCl. The response of aortic strips to W-7 in the absence of phentolamine (○——○) was significantly different from that in the presence of phentolamine (△——△); ●- - -●, "calculated" presynaptic excitatory response of aortic strip to W-7. For details see text.

nephrine from the sympathetic nerve terminals. From the effect of the treatment with 1×10^{-6} M phentolamine (*lower response* in Fig. 2) and from the control response, it is possible to calculate the presynaptic excitatory response to W-7. This is done simply by plotting responses to contractile tension in the absence of phentolamine plus reduced tension in the presence of phentolamine. The *broken line* in Fig. 2 shows the presynaptic effects of W-7. When W-7 was applied twice to the aortic strip with an interval of 60 min between each trial, the reproducibility of the contractile response was 85.0 ± 6.1% (N = 14). The presence of phentolamine antagonized in a dose-dependent manner the contractile response of the aortic strips to W-7 (Table 2), suggesting that the vascular contractile response to W-7 is mediated by the release of endogenous norepinephrine or by the direct stimulation of *alpha*-adrenergic receptors. The addition of atropine (1×10^{-6} M), pyrilamine (1×10^{-7} M), methysergide (3×10^{-7} M), and hexamethonium (1×10^{-5} M) did not alter the aortic contraction produced by W-7 (Table 2). These results indicate that W-7-induced aortic contraction is not mediated through muscarinic, histaminergic, serotonergic, and presynaptic nicotinic receptors.

The contractile response of the aortic strips to W-7 was not significantly potentiated by the addition of cocaine (5×10^{-6} M) and was not antagonized by the addition of either tetrodotoxin (1×10^{-7} M) or bretylium (1×10^{-5} M) (Table 2).

Preincubation of the aortic strips with 2 mM tyramine for 40 min prevented contractions induced by W-7 (Table 3). When the aortic strip was prepared from a rabbit given reserpine, 1 mg/kg, for 2 consecutive days, the contractile response to W-7 was weak as compared with findings in strips from non-dosed rabbits (Table 3).

NE release by W-7 from sympathetic nerve terminals of vascular strips. We examined the effect of W-7 on sympathetic nerve terminals using superfused aortic strips preloaded with [^{3}H]NE. The addition of W-7 at a concentration of 1×10^{-4} M contracted the superfused

TABLE 2

Effects of various blocking agents on W-7-induced contraction in rabbit aortic strips

Data are expressed as means ± standard error; N, number of preparations used.

Conditions[a]	Concentration	N	Contraction by 1×10^{-4} M W-7[a]
	M		%
Control[b]		14	85.0 ± 6.1
+Phentolamine	2×10^{-7}	11	58.1 ± 5.7**
+Phentolamine	1×10^{-6}	9	36.3 ± 5.4**
+Atropine	1×10^{-6}	11	81.6 ± 6.9
+Pyrilamine	1×10^{-7}	8	88.2 ± 3.5
+Methysergide	3×10^{-7}	8	92.6 ± 3.8
+Hexamethonium	1×10^{-5}	9	87.4 ± 4.6
+Cocaine	5×10^{-6}	14	98.1 ± 7.0
+Tetrodotoxin	1×10^{-7}	8	79.6 ± 6.7
+Bretylium	1×10^{-5}	10	107.7 ± 4.5*

[a] A submaximally effective concentration of W-7 (1×10^{-4} M) was applied twice to the strips of rabbit thoracic aorta, and the first contraction by 1×10^{-4} M W-7 was taken as 100%. Phentolamine, atropine, pyrilamine, methysergide, hexamethonium, cocaine, tetrodotoxin, and bretylium were added 20 min before the second trial of W-7 (1×10^{-4} M).

[b] The second contraction by 1×10^{-4} M W-7 was 85.0 ± 6.1% with respect to the first contraction. The first contractile tension by 1×10^{-4} M W-7 was 993 ± 77 mg (N = 14).

* Significantly different from control ($p < 0.05$).

** Significantly different from control ($p < 0.001$).

TABLE 3

Effects of tyramine preincubation and reserpine pretreatment on W-7-induced contraction in rabbit aortic strips

Data are expressed as means ± standard error; *N*, number of preparations used.

Conditions	*N*	Tension developed by 1×10^{-4} M W-7[a]
		mg
Normal aorta	7	1147 ± 117
Tyramine pretreated aorta[b]	7	345 ± 100*
Reserpine pretreated aorta[c]	7	471 ± 70*

[a] Submaximally effective concentration of W-7 (1×10^{-4} M) was administered to the aortic strips under different conditions.

[b] Aortic strips were preincubated with 2 mM tyramine for 40 min and washed to remove tyramine from the medium. A strip thus prepared was used to examine the W-7 effect.

[c] Aortic strip obtained from a rabbit pretreated with 1 mg/kg reserpine for two consecutive days.

* Significantly different from normal aorta ($p < 0.001$).

aortic strip; and there was a significant increase in spontaneous efflux of [^{3}H]NE. A typical experiment is shown in Fig. 3A. The contractile tension developed by 1×10^{-4} M W-7 was 858 ± 79 mg ($N = 8$), and the spontaneous [^{3}H]NE efflux was increased by 1031 ± 95% ($N = 8$). The W-7-induced increase in the spontaneous tritium efflux was not affected by treatment with 1×10^{-6} M phentolamine (Fig. 3B). However, the contraction produced by W-7 was inhibited by treatment with phentolamine (Fig. 3). All such evidence suggests that W-7 contracts the aorta by effects on the sympathetic nerve terminals.

The relationship between the contractile tension developed by W-7 and the increase in the spontaneous [^{3}H]NE efflux is shown in Fig. 4. The addition of W-7 in concentrations ranging from 1×10^{-5} M to 3×10^{-4} M elicited a dose-dependent contraction and an increase in the spontaneous [^{3}H]NE efflux. The contraction and the change in the spontaneous [^{3}H]NE efflux caused by W-7 were closely correlated in these concentrations (Fig. 4).

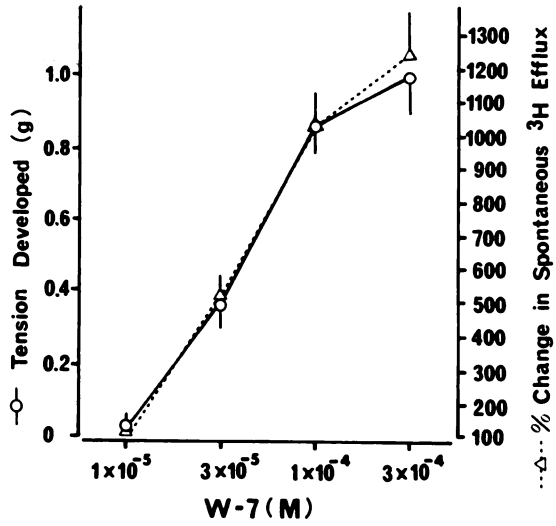


FIG. 4. *The relationship between the contractile tension developed by W-7* (○——○) *and the increase in spontaneous [^{3}H]norepinephrine efflux* (△- - -△)

Experimental conditions were the same as in Fig. 2. Data are expressed as means ± standard error of eight determinations for all concentrations of W-7.

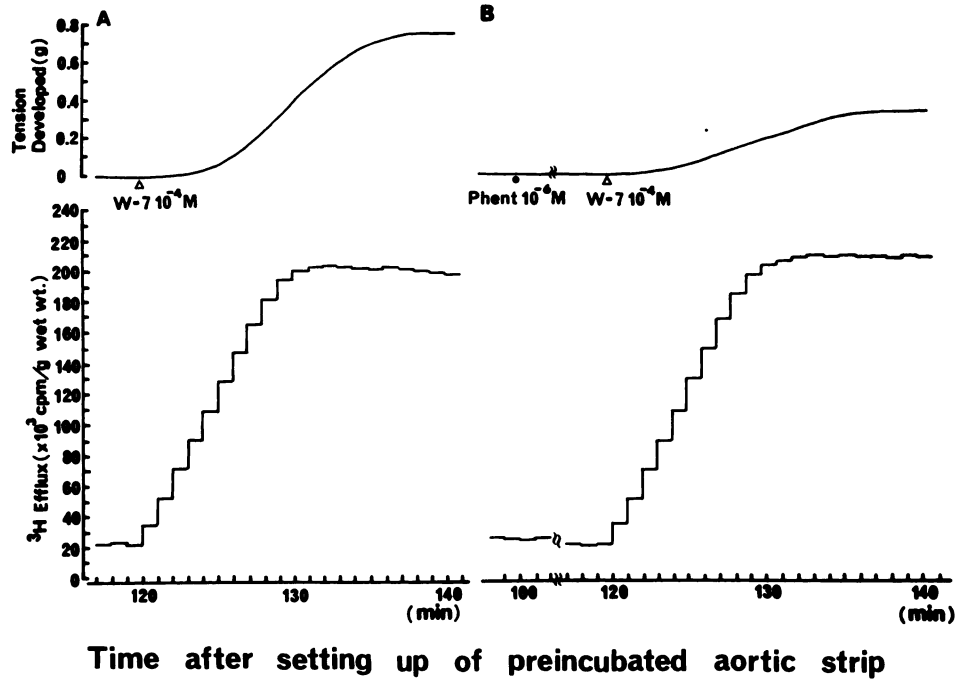


FIG. 3. *Effect of W-7 on spontaneous efflux of [^{3}H]norepinephrine in aortic strips of the rabbit*

The aortic strips were preincubated with [^{3}H]norepinephrine (1×10^{-7} M) for 60 min at 37°. After incubation with [^{3}H]norepinephrine, aortic strips were mounted vertically and then superfused at a flow rate of 1.5 ml/min, as described by Su and Bevan (20). The addition of W-7 (1×10^{-4} M) caused a contraction in the superfused aortic strip (A, *upper panel*) and an increase in spontaneous [^{3}H]norepinephrine efflux (A, *lower panel*). Phentolamine (*Phent*) significantly inhibited the contractile response of aortic strip to W-7 (B, *upper panel*). W-7-induced increase in spontaneous tritium efflux was not affected by phentolamine (B, *lower panel*). Phentolamine (1×10^{-6} M) was added 20 min before the addition of W-7.

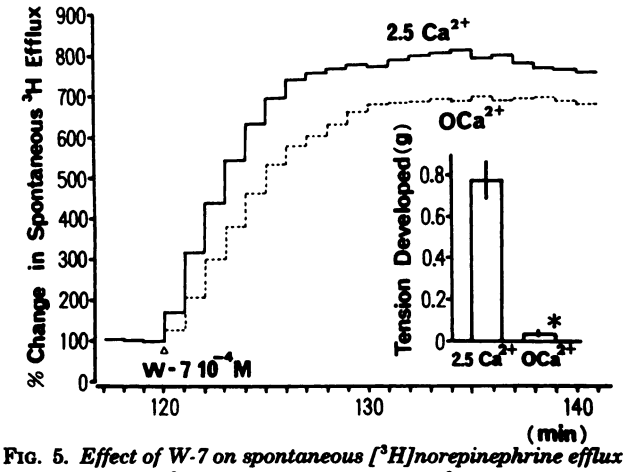


FIG. 5. *Effect of W-7 on spontaneous [^{3}H]norepinephrine efflux in the presence (2.5 Ca^{2+}, ——) or the absence (0Ca^{2+}, - - -) of calcium*

Experimental conditions were the same as in Fig. 2. W-7 elicited an increase in spontaneous [^{3}H]norepinephrine efflux in the presence of 10^{-4} M EGTA instead of Ca^{2+}-free solution. Contractile tensions developed by W-7 (10^{-4} M) in the presence or the absence of calcium are shown in the *inset*. Data are expressed as means ± standard error of eight determinations. *Significantly different from control (2.5 Ca^{2+}, $p < 0.001$).

Contribution of the Ca^{2+}-calmodulin mechanism to W-7-induced NE release. To determine whether the presynaptic excitatory effect of W-7 was produced through calmodulin, two different studies were performed.

First, the effect of W-7 on the spontaneous [^{3}H]NE efflux was examined in the presence and absence of calcium. Ca^{2+}-free solutions were prepared by omitting $CaCl_2$ from the superfusing Krebs-Henseleit solution. Additional experiments were conducted with Ca^{2+}-free solutions containing 0.1 mM EGTA made pH 7.4 with NaOH. Omission of Ca^{2+} from the superfusing solution did not prevent the W-7-induced increase in spontaneous [^{3}H]NE efflux (Fig. 5). This result strongly suggests that the release mechanism of W-7 is calcium-independent. When calcium was not added to the Krebs-Henseleit solution, the W-7-induced contraction did not occur (Fig. 5).

It is concluded that W-7 releases norepinephrine from sympathetic nerve terminals of the aortic strip by a mechanism differing from that of tyramine and electrical transmural stimulation.

Second, the addition of W-5, which does not contain the chlorine group at position 5 of the naphthalene ring, at a concentration of 1×10^{-4} M caused a sustained contraction in K^+-contracted aortic strips (Fig. 6A). W-5 also elicited an increase in spontaneous [^{3}H]NE efflux (Fig. 6A). Contractile tension developed by 1×10^{-4} M W-5 was 977 ± 139 mg ($N = 5$), and the spontaneous [^{3}H]NE efflux was increased by 948 ± 145% ($N = 5$). Treatment of the aortic strip with 1×10^{-6} M phentolamine inhibited the W-5-induced contraction but did not alter the increase in spontaneous [^{3}H]NE efflux (Fig. 6B). As clearly shown in Fig. 6, W-5 did not relax the aortic

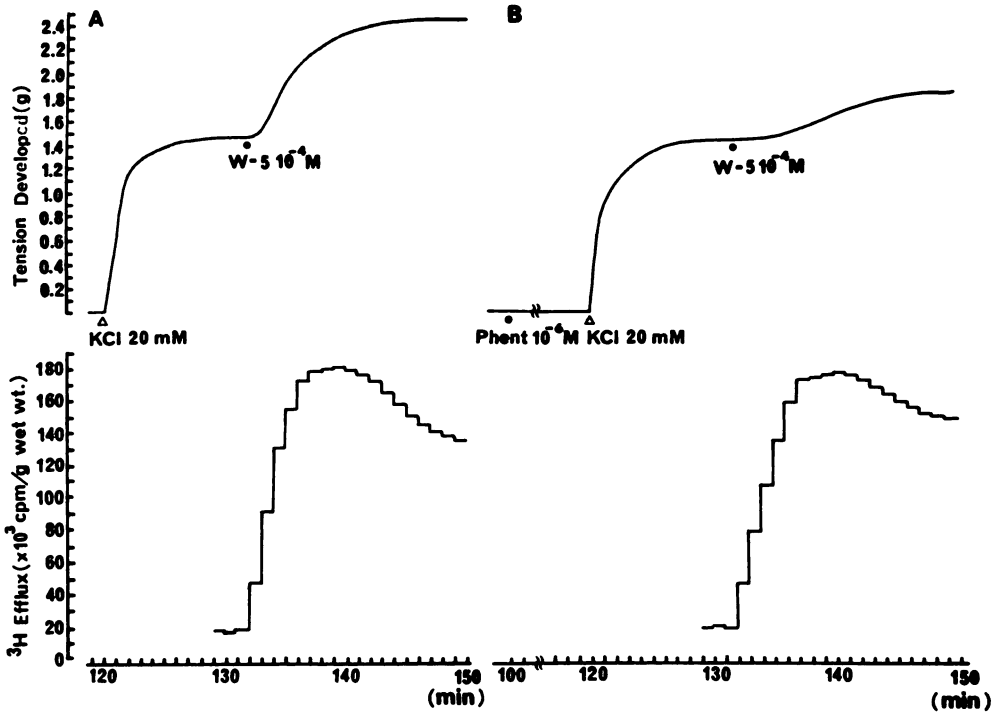


FIG. 6. *Effect of W-5 on spontaneous efflux of [^{3}H]norepinephrine in K^+-contracted aortic strips of the rabbit*

Experimental conditions were the same as in Fig. 2 with the exception of the K^+ contraction. The aortic strips were contracted with KCl (20 mM), and then W-5 (1×10^{-4} M) was added at the peak period of contraction. The addition of W-5 (1×10^{-4} M) produced a contraction in the superfused K^+-contracted aortic strip (A, *upper panel*) and an increase in spontaneous [^{3}H]norepinephrine efflux (A, *lower panel*). Phentolamine (*Phent*) significantly inhibited the contractile response of the aortic strip to W-5 (B, *upper panel*) but did not produce the relaxation observed in the response to W-7 (see Fig. 1). The W-5-induced increase in spontaneous ^{3}H efflux was not affected by phentolamine (B, *lower panel*). Phentolamine (1×10^{-6} M) was added 20 min before the addition of KCl.

strip. W-5 produced only contraction in the absence or the presence of contractile agonists. All of the evidence shown in Figs. 2–4 suggests that W-7 produces a dual action (contraction and relaxation) by affecting both presynaptic nerve terminals and postsynaptic effector cells, and that W-5 elicits only contraction through the activation of presynaptic nerve terminals.

As shown in Fig. 1, chlorinated naphthalenesulfonamide derivatives (W-7, W-9), which bind to calmodulin with a high affinity, caused a significant relaxation of isolated mesenteric arterial strips. In contrast, chlorine-deficient derivatives (W-5, W-6) of naphthalenesulfonamide produced contraction in K^+-contracted mesenteric arterial strips.

Moreover, the concentration of the derivative giving one-half of the maximal effect (ED_{50}) decreased with the length of the hydrocarbon chain (C_5–C_{10}), as shown in Table 2. These results also indicate that there is a good correlation between the ED_{50} values of relaxation and the affinity to calmodulin of each analogue.

DISCUSSION

Intracellular concentrations of free Ca^{2+} are important in governing the functional status of cells derived from the most primitive to the most complex organisms, as described by Kretsinger (1). Many investigations have supported the proposal that Ca^{2+} functions as a second messenger in biological systems, in a manner analogous to cyclic AMP. Consequently, the efforts of various workers have been directed to isolating Ca^{2+}-binding protein, including calmodulin, troponin-C, parvalbumin, and others. Calmodulin is now acknowledged to be a multifunctional Ca^{2+}-dependent regulator.

Although it has been reported that calmodulin activates a number of enzymes and regulates the assembly and disassembly of microtubules (2–5) *in vitro*, the function of calmodulin has not been completely elucidated *in vivo*. In addition to the indirect immunofluorescence method of Means and Dedman (5), a pharmacological method using some calmodulin antagonists may be useful for investigating the role of calmodulin in cell function (8).

We have already reported that W-7 is a selective inhibitor of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase and that it produces vascular relaxation (7–10, 21). The mechanism involved in the action of W-7 is one of direct calcium-dependent binding to calmodulin (8). Therefore, W-7 was considered to produce relaxation of vascular smooth muscle by inhibiting calmodulin-dependent myosin light-chain phosphorylation (22), thus suppressing the actin-myosin interaction and concomitant myosin ATPase activation (22). Levin and Weiss (23) showed that Ca^{2+}-calmodulin-dependent phosphodiesterase was inhibited selectively by agents found clinically useful in treating psychoses but belonging to diverse structural classes, including phenothiazines, thioxanthenes, diphenylbutylamines, and others (23). Recently, they reported that trifluoperazine binds to calmodulin in a calcium-dependent manner (6). However, more recently Norman and his co-workers (24), using isomers, demonstrated that the interaction of these neuroleptic drugs with calmodulin may not be related to their clinical efficacy. In general, synthetic compounds have multiple functions such as have been demonstrated for psychotropic drugs (25, 26). This being the case, we attempted to clarify the structure-activity relationship of naphthalenesulfonamide derivatives in order to improve the pharmacological methodology. Our results suggest that the affinity to calmodulin and potency in relaxation of vascular strips depends on the chlorination of position 5 of the naphthalene ring and the length of the hydrocarbon chain (C_5–C_{10}). Furthermore, a good correlation between the potency in relaxation of vascular strips and affinity to calmodulin was obtained. Therefore, this mechanism of relaxation as related to these compounds is probably due to their effect on Ca^{2+}-calmodulin-dependent processes.

As clearly shown in this study, W-7 produced dual effects on isolated vascular strips. One effect was vascular relaxation through suppression of the actin-myosin interaction described above. Another effect was to increase the endogenous NE release by affecting the presynaptic sympathetic nerve terminals.

The W-7-induced NE release from presynaptic nerve terminals was confirmed by the following: (a) the addition of W-7 produced a significant contraction in isolated aortic strips; (b) this contraction was inhibited both by the addition of phentolamine, which blocks *alpha*-adrenergic receptors, and by pretreatment with reserpine, which depletes pooled NE in sympathetic nerve terminals; and (c) W-7 increased the spontaneous tritium efflux in [^{3}H]NE-preloaded aortic preparations. W-7 produced a release of NE in both the presence and the absence of calcium. Omission of Ca^{2+} from the superfusing solution and addition of 1×10^{-4} M EGTA to Ca^{2+}-free solution did not prevent the W-7-induced increase in spontaneous [^{3}H]NE efflux (Fig. 5). All of our findings clearly indicate that the presynaptic NE release mechanism of W-7 is calcium-independent, and in this respect is different from that of tyramine and electrical transmural stimulation. It has been reported that chlorpromazine and its derivatives stimulate neurosecretion in the absence of calcium ion (27, 28). Chlorpromazine is also well known to bind to Ca^{2+}-calmodulin complex with a high affinity, whereas chlorpromazine sulfoxide interacts weakly with calmodulin (29). However, the amount of norepinephrine liberated by chlorpromazine sulfoxide was similar to that seen with chlorpromazine (27).

W-5 has a structural formula similar to that of W-7, but it does not contain the chlorine group at position 5 of the naphthalene ring of W-7. W-5 also produced a significant contraction and an increase in spontaneous [^{3}H]NE release. However, this compound did not produce the aortic relaxation clearly demonstrated in the response to W-7. These results indicate that the affinity to calmodulin may not relate to the increase in [^{3}H]NE release by this compound. Although W-7 releases endogenous NE from presynaptic terminals, this compound does have a dual effect—contraction and relaxation. Finally, relaxation of the aortic strip by the addition of W-7 suggests that the postsynaptic inhibitory effect (relaxation) of the compound overcomes the presynaptic release of NE. Therefore, W-7 acts both at vascular smooth muscle actomyosin and at presynaptic terminals,

but the potency is significantly different. All of the naphthalenesulfonamide derivatives used which contain a chlorine group at the naphthalene ring produced vascular relaxation, and all of the derivatives used which contain no chlorine group at the naphthalene ring produced vascular contraction (Fig. 1). It is likely that the effect of non-chlorine naphthalenesulfonamide derivatives, or the release of NE, is not the result of an effect on the Ca^{2+}-calmodulin mechanism. We conclude that the chlorine group at the naphthalene ring is necessary to produce vascular relaxation and that only the W-7-induced relaxation was produced by the Ca^{2+}-calmodulin mechanism. These calmodulin antagonists are expected to be useful tools for investigating the function of calmodulin in various cellular processes.

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