

## Role of calmodulin in platelet aggregation. Structure-activity relationship of calmodulin antagonists.

M Nishikawa, H Hidaka

*J Clin Invest.* 1982;69(6):1348-1355. <https://doi.org/10.1172/JCI110574>.

### Research Article

Two series of derivatives of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), including a dechlorinated analog of W-7 (W-5) and various aminoalkyl chain analogs of W-7 (A-3, A-4, A-5, I-240, A-6) were synthesized and their structure-activity relationships with calmodulin antagonistic actions and their potencies in inhibiting human platelet aggregation *in vitro* were investigated. Their binding affinities to calmodulin in the presence of 100  $\mu\text{M}$   $\text{Ca}^{2+}$  were dependent both on the chlorination of the naphthalene ring and on the length of aminoalkyl chain. The ability of these derivatives to inhibit  $\text{Ca}^{2+}$ -dependent phosphorylation of 20,000-dalton myosin light chain from platelets correlated well with the magnitude of their binding affinity to calmodulin. W-7 (10-100  $\mu\text{M}$ ) inhibited in a dose-dependent manner platelet aggregation induced by collagen (2  $\mu\text{g}/\text{ml}$ ), ADP (5  $\mu\text{M}$ ), epinephrine (1  $\mu\text{g}/\text{ml}$ ), sodium arachidonate (0.83  $\text{mM}$ ), thrombin (0.125  $\text{U}/\text{ml}$ ), and A-23187 (10  $\mu\text{M}$ ). The  $\text{IC}_{50}$  value (concentration producing 50% inhibition of aggregation) of W-7 was lower in arachidonate- and collagen-induced aggregation than in ADP- or epinephrine-induced aggregation. A good correlation between the potency in inhibition of collagen-induced aggregation by W-7 and its derivatives and their affinities to calmodulin was obtained ( $r = 0.94$ ). Thus, the inhibitory mechanism of these compounds may be due to their effect on  $\text{Ca}^{2+}$ -calmodulin-dependent processes, such as 20,000-dalton myosin light chain phosphorylation. These data also support the hypothesis that the calmodulin-mediated system has an [...]

Find the latest version:

<https://jci.me/110574/pdf>



# Role of Calmodulin in Platelet Aggregation

## STRUCTURE-ACTIVITY RELATIONSHIP OF CALMODULIN ANTAGONISTS

MASAKATSU NISHIKAWA and HIROYOSHI HIDAKA, *Department of Pharmacology,  
Mie University School of Medicine, Edobashi, Tsu 514, Japan*

**ABSTRACT** Two series of derivatives of *N*-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide (W-7), including a dechlorinated analog of W-7 (W-5) and various aminoalkyl chain analogs of W-7 (A-3, A-4, A-5, I-240, A-6) were synthesized and their structure-activity relationships with calmodulin antagonistic actions and their potencies in inhibiting human platelet aggregation *in vitro* were investigated. Their binding affinities to calmodulin in the presence of 100  $\mu$ M  $\text{Ca}^{2+}$  were dependent both on the chlorination of the naphthalene ring and on the length of aminoalkyl chain. The ability of these derivatives to inhibit  $\text{Ca}^{2+}$ -dependent phosphorylation of 20,000-dalton myosin light chain from platelets correlated well with the magnitude of their binding affinity to calmodulin. W-7 (10–100  $\mu$ M) inhibited in a dose-dependent manner platelet aggregation induced by collagen (2  $\mu$ g/ml), ADP (5  $\mu$ M), epinephrine (1  $\mu$ g/ml), sodium arachidonate (0.83 mM), thrombin (0.125 U/ml), and A-23187 (10  $\mu$ M). The  $\text{IC}_{50}$  value (concentration producing 50% inhibition of aggregation) of W-7 was lower in arachidonate- and collagen-induced aggregation than in ADP- or epinephrine-induced aggregation. A good correlation between the potency in inhibition of collagen-induced aggregation by W-7 and its derivatives and their affinities to calmodulin was obtained ( $r = 0.94$ ). Thus, the inhibitory mechanism of these compounds may be due to their effect on  $\text{Ca}^{2+}$ -calmodulin-dependent processes, such as 20,000-dalton myosin light chain phosphorylation. These data also support the hypothesis that the calmodulin-mediated system has an important role in platelet function.

## INTRODUCTION

It is generally considered that  $\text{Ca}^{2+}$  plays a decisive role in regulating physiologic and biochemical pro-

cesses in platelets (1). It was reported (2, 3) that platelets contain relatively large quantities of calmodulin. In platelets, calmodulin probably mediates  $\text{Ca}^{2+}$  control of actin-myosin interaction through the phosphorylation of 20,000-dalton myosin light chain (MLC).<sup>1</sup> We have recently suggested (4) that a calmodulin-mediated system, such as  $\text{Ca}^{2+}$ -dependent MLC phosphorylation plays an important role in the phenomenon of platelet activation, using calmodulin antagonist *N*-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide (W-7). In the presence of calcium ions, calmodulin antagonists bind to calmodulin and inhibit selectively  $\text{Ca}^{2+}$ -calmodulin-dependent activation of enzymes (5, 6).

In the present work, using two series of derivatives of W-7, including a chlorine-deficient analog of W-7 (W-5) and aminoalkyl chain derivatives of W-7 (A-3, A-4, A-5, I-240, A-6), we attempted to clarify the relationship between their capacity to bind to calmodulin and their potency in inhibiting human platelet aggregation *in vitro*. We found that the affinity of these naphthalenesulfonamide derivatives for calmodulin was usually in parallel with the extent of inhibition of platelet aggregation.

## METHODS

**Preparation of calmodulin.** Calmodulin from bovine brain was purified to apparent homogeneity, according to the method described previously (7). The homogeneity was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, according to Weber and Osborn (8), using 0.1% SDS, 10% polyacrylamide, and 0.1 M sodium phosphate buffer (pH 7.2). Calmodulin levels were assessed

<sup>1</sup> *Abbreviations used in this paper:* DTT, dithiothreitol; GFP, gel filtered platelets;  $\text{I}_{50}$ , concentration of unlabeled compound producing 50% displacement of labeled W-7;  $\text{IC}_{50}$ , concentration producing 50% inhibition of aggregation; MLC, myosin light chain; PMSF, phenylmethylsulfonyl fluoride; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; W-7, *N*-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide.

Address correspondence to Dr. Hidaka.

Received for publication 24 August 1981 and in revised form 5 February 1982.

according to its ability to increase the activity of calmodulin-deficient,  $\text{Ca}^{2+}$ -dependent cyclic nucleotide phosphodiesterase prepared from bovine brain (7). 1 U of calmodulin was defined as the amount necessary to produce 50% maximum activation of the calmodulin-deficient phosphodiesterase, under standard conditions, and was equivalent to 10 ng protein.

**Binding studies.** The binding affinity to calmodulin was determined from the ability of the unlabeled compound to displace radiolabeled W-7 from calmodulin by the equilibrium-binding procedure. The equilibrium-binding procedure on a Sephadex G-50 gel filtration was essentially as described previously (6, 9). A column ( $0.9 \times 26.5$  cm) of Sephadex G-50 was equilibrated at  $25^\circ\text{C}$  with buffer containing  $0.5 \mu\text{M}$  [ $^3\text{H}$ ]W-7, 20 mM Tris  $\cdot$  HCl (pH 7.5), 20 mM imidazole, 3 mM magnesium acetate, and  $100 \mu\text{M}$   $\text{CaCl}_2$ . Purified calmodulin (180  $\mu\text{g}$ ) was used for each experiment. In studying the binding of nonradioactive compounds, we measured their ability to displace [ $^3\text{H}$ ]W-7 from calmodulin. The concentration of the unlabeled compounds that displaced 50% of labeled W-7 ( $\text{IC}_{50}$ ) is given for each compound.

**Preparation of platelet actomyosin.** All isolation procedures were performed at  $4^\circ\text{C}$ . Platelet actomyosin was extracted by the method of Hathaway and Adelstein (10), with minor modification. Human platelets were collected by sedimentation of platelet-rich plasma, at 2,000  $g$  for 20 min. The platelet pellet (5–10 g) was resuspended in 2 vol of 150 mM NaCl, 3 mM sodium citrate (pH 6.8), 2.5 mM dithiothreitol (DTT), and resedimented. The final platelet pellet was suspended in 2–3 vol of 20 mM Tris  $\cdot$  HCl (pH 7.5), 0.5 M KCl, 2.5 mM DTT, 5 mM  $\text{Na}_2\text{EDTA}$ , 1 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg/ml soybean trypsin inhibitor. The platelets were disrupted by the nitrogen decompression method with a Parr cell disruption bomb (Parr Instrument Co., Moline, IL). The homogenate was then centrifuged at 28,000  $g$  for 30 min to remove cellular debris. The resulting extract supernatant was dialyzed overnight against 20 vol of 20 mM Tris  $\cdot$  HCl (pH 7.5), 2.5 mM DTT, 5 mM EDTA, 0.4 mM PMSF. The actomyosin precipitate was then sedimented by centrifugation at 10,000  $g$  for 20 min. The supernatant was discarded and the actomyosin precipitate dissolved in 20 mM Tris  $\cdot$  HCl (pH 7.5), 0.5 M KCl, 2.5 mM DTT was the source of platelet actomyosin. The amount of protein migrating with the 20,000-dalton MLC was determined from absorbance scans of Coomassie Brilliant Blue-stained gels by comparing the peak areas of known quantities of the MLC of chicken gizzard with the peaks of 20,000-dalton MLC of the platelet actomyosin. Densitometry of the gels was carried out in a Beckman model 34 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at a wavelength of 560 nm.

**Phosphorylation analyses of platelet actomyosin.** The phosphorylation of platelet actomyosin was determined according to the method previously described (4), and the assay mixture contained 20 mM Tris  $\cdot$  HCl (pH 7.5), 125 mM KCl, 10 mM  $\text{MgCl}_2$ , 0.5 mM [ $\gamma\text{-}^{32}\text{P}$ ]ATP (1  $\mu\text{Ci}/\text{tube}$ ), platelet actomyosin (1.5 mg/ml), various concentrations of the compounds, and  $100 \mu\text{M}$   $\text{CaCl}_2$  or 5 mM EGTA in a total volume of 0.2 ml. The reaction was performed at  $25^\circ\text{C}$  for 10 min by the addition of ATP and terminated by the addition of 20  $\mu\text{l}$  of 5% SDS containing 0.1 M 2-mercaptoethanol and 20% sucrose. The solution containing 60  $\mu\text{g}$  protein was applied to 0.1% SDS–10% polyacrylamide gel and electrophoresis was carried out. The gels were then stained with Coomassie Brilliant Blue, photographed, and sliced into 2-mm portions from which the radioactivity was eluted.  $^{32}\text{P}$  radio-

activity was analyzed by liquid scintillation spectrometry (Beckman Instruments, Inc.). The urea-glycerol (slab) gel electrophoresis was carried out by the method of Perrie et al. (11, 12), using 10% polyacrylamide, 40% (vol/vol) glycerol, and 25 mM Tris–150 mM glycine buffer, pH 8.6. Samples in 8 M urea containing 60  $\mu\text{g}$  of protein were applied to the gel.

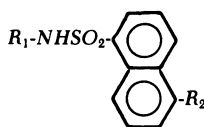
**Platelet aggregation studies.** Human blood was collected by venipuncture from healthy volunteers into acid citrate dextrose (volume preparation 1:6). Platelet-rich plasma (PRP) was prepared by centrifugation at 120  $g$  for 15 min. Platelet-poor plasma (PPP) was obtained by further centrifugation of blood at 1,500  $g$  for 10 min. Gel filtered platelets (GFP) were prepared by a modification of the method of Tangen et al. (14), using Sepharose 2-B, and were then suspended in Hepes buffer that contained a final concentration of 140 mM NaCl, 2.7 mM KCl, 1 mM  $\text{MgCl}_2$ , 0.1% dextrose, 0.35% bovine serum albumin, 3.75 mM  $\text{NaH}_2\text{PO}_4$ , 5 mM Hepes (pH 7.35). Platelet aggregation was studied by the turbidimetric method of Born (13), with a Rikadenki 4 Channel Aggregometer (RAM 41, Rikadenki Kogyo Co., Ltd., Japan) modified to provide continuous stirring and maintenance of constant temperature ( $37^\circ\text{C}$ ). The standard platelet reaction mixtures consisted of 0.27 ml of PRP or GFP suspension, 30  $\mu\text{l}$  of a drug solution (in saline), and the aggregating agent. In experiments involving platelet activation by thrombin, GFP suspension was used in place of PRP. All concentrations given refer to final concentrations. PRP was kept tightly capped at all times, and experiments were completed within 2–3 h of blood collection. Each experiment in which inhibition was obtained was followed by a control experiment which clearly demonstrated that the platelets in our conditions were still responsive to the stimulus in the absence of the inhibitor.

**Serotonin release.** Serotonin release was measured by the following procedure. Platelet aggregation was monitored in a Rikadenki 4 Channel Aggregometer (RAM 41). 100 parts of PRP were first incubated for 30 min at  $37^\circ\text{C}$  with one part of [ $^{14}\text{C}$ ]serotonin creatinine sulfate (30–50 mCi/mmol) diluted in 70% ethanol (10  $\mu\text{Ci}/\text{ml}$ ). Under these conditions, >85% of the radioactivity was taken up into the platelets. 1  $\mu\text{M}$  imipramine was added to PRP to prevent the reuptake of secreted [ $^{14}\text{C}$ ]serotonin. PRP (0.72 ml) thus treated was mixed with 80  $\mu\text{l}$  of saline (control) or the compound (various concentrations) and was preincubated at  $37^\circ\text{C}$  with stirring for 3 min in the aggregometer cuvette; then 80  $\mu\text{l}$  of collagen was added. Aggregation was monitored for an additional 5 min, and 0.2 ml of 0.5% glutaraldehyde was subsequently added and mixed to stop the reaction. Samples were centrifuged at 2,500  $g$  for 20 min. The radioactivity of the supernatant (0.8 ml) was determined by liquid scintillation counting. [ $^{14}\text{C}$ ]Serotonin released from platelets was expressed as a percentage of total uptake.

Aggregation responses were quantified both as the rate (slope) and maximum extent of aggregation. The rate (slope) of aggregation was determined graphically in chart centimeter, by drawing a line through the steepest portion of the curve following the addition of the aggregating agents. The maximum extent of aggregation was calculated by the maximum change in light transmission expressed as a percentage, taking the difference between light transmission for PRP and PPP as a value of 100%. Percent inhibition of aggregation by a test compound was calculated by dividing the percent aggregation by that observed in the control run, then multiplying by 100.

**Materials.** W-7, [ $^3\text{H}$ ]W-7 (59.7 Ci/ml), and its analogs (W-5, A-3, A-4, A-5, I-240, A-6) were synthesized by the

TABLE I  
Structures of Naphthalenesulfonamide Derivatives and  
Displacement of Labeled [<sup>3</sup>H]W-7 from Calmodulin  
by These Compounds



Compound	R <sub>1</sub>	R <sub>2</sub>	I <sub>50</sub>
			μM
A-3	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>2</sub> -	Cl	230
A-4	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>3</sub> -	Cl	140
A-5	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>4</sub> -	Cl	100
I-240	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>5</sub> -	Cl	68
W-7	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>6</sub> -	Cl	31
A-6	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>8</sub> -	Cl	3.5
W-5	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>6</sub> -	H	210

Equilibrium binding procedure in the presence of calcium was performed as described in Methods. The I<sub>50</sub> is given for each compound. All binding experiments were run in triplicate.

method of Hidaka et al. (15). Chemical structures of these compounds are shown in Table I. Synthesis of some derivatives was made under the assistance of Banyu Pharmaceutical Company, Ltd. and Nippon Chemiphar Company, Ltd. Adenosine 5'-[γ-<sup>32</sup>P]triphosphate (3,000 Ci/mmol) and [<sup>14</sup>C]serotonin creatinine sulfate (30–50 mCi/mmol) were obtained from the Radiochemical Center, Amersham Corp., Buckinghamshire. The following reagents were used in this investigation: *l*-epinephrine (Sankyo Pharmacological, Tokyo), ADP, arachidonic acid (99% pure) (Sigma Chemical Co., St. Louis, MO), collagen (collagen reagent Horm, Hormon-Chemie, München). Collagen was diluted further with SKF Horm buffer to the desired concentration immediately before use. Sodium arachidonate was prepared from arachidonic acid by the method of Kinlough-Rathbone et al. (16). Thrombin (bovine, Parke-Davis Division of Warner-Lambert Inc., Detroit, MI) was stored as a 500 U/ml solution in 50% glycerol at –20°C until use and was diluted in saline to a desired concentration immediately before use. Ionophore A-23187 (Calbiochem-Boehringer Corp., American Hoechst Corp., San Diego, CA) was dissolved in dimethylsulfoxide.

## RESULTS

**Interaction of naphthalenesulfonamide derivatives with calmodulin.** Various naphthalenesulfonamide derivatives were tested for their capacity to bind to calmodulin in the presence of 100 μM Ca<sup>2+</sup>. We have recently identified the calcium-dependent binding of W-7 to purified calmodulin from bovine brain (6). Table I shows the concentration of these compounds required to displace 50% of 0.5 μM [<sup>3</sup>H]W-7 from calmodulin (I<sub>50</sub>). From this I<sub>50</sub> value, one can evaluate the binding affinity of each of these compounds for cal-

modulin. As can be seen, short-aminoalkyl chain analogs of W-7, such as A-3, A-4, A-5, and I-240 all were less potent than W-7 in displacing [<sup>3</sup>H]W-7, but the long-aminoalkyl chain analog, A-6, was more potent.

W-5, a dechlorinated derivative of W-7 was seven times less potent than W-7 in displacing [<sup>3</sup>H]W-7. These results show that the ability of naphthalenesulfonamide derivatives to displace labeled W-7 from calmodulin correlated with the length of aminoalkyl chain of naphthalenesulfonamide and the chlorination of the naphthalene ring.

**The effect of naphthalenesulfonamide derivatives on Ca<sup>2+</sup>-dependent phosphorylation of platelet actomyosin.** Platelet actomyosin was analyzed by SDS-polyacrylamide gel electrophoresis after incubation of the actomyosin with [γ-<sup>32</sup>P]ATP in the presence of 100 μM Ca<sup>2+</sup>. The major peak of <sup>32</sup>P radioactivity corresponds to the 20,000-dalton protein, as shown in Fig. 1A, and this phosphoprotein was identified as 20,000-dalton MLC, using urea-glycerol polyacrylamide gel electrophoresis, as shown in Fig. 1B. In this gel system (11, 12), the light chains of myosin have a greater electrophoretic mobility than most other proteins and the phosphorylated 20,000-dalton MLC (position b) of Fig. 1B migrates slightly faster than the unphosphorylated one (from position a of Fig. 1B). In the presence of 100 μM Ca<sup>2+</sup>, >90% of 20,000-dalton MLC migrated in the faster position (position b of Fig. 1B). The quantitative determinations of the amount of <sup>32</sup>P incorporated into the 20,000-dalton MLC revealed that 0.80–0.95 mol of <sup>32</sup>P/mol of MLC was incorporated in the presence of Ca<sup>2+</sup>. In the absence of Ca<sup>2+</sup> (5 mM EGTA), a small amount of <sup>32</sup>P incorporation into 20,000-dalton MLC (Ca<sup>2+</sup>-independent phosphorylation) was observed (Fig. 1A and 1B). This actomyosin fraction contained ~50 U of calmodulin/mg protein. This Ca<sup>2+</sup>-dependent phosphorylation of MLC was not enhanced by addition of exogenous calmodulin from bovine brain to a concentration of 1 μM, suggesting that the amount of calmodulin contained in this fraction is sufficient to produce maximal stimulation of platelet myosin light chain kinase. The treatment of platelet actomyosin with 100 μM W-7 caused a shift of a band to a position of the slower mobility (from position b to a of Fig. 1B). The shift in the mobility of the band was seen to be accompanied by decreased radioactivity in this region. W-7 produced a dose-dependent inhibition of Ca<sup>2+</sup>-dependent phosphorylation of 20,000-dalton MLC with an IC<sub>50</sub> value of 70 μM but this compound did not inhibit Ca<sup>2+</sup>-independent phosphorylation, as shown in Fig. 2. W-7-induced inhibition of Ca<sup>2+</sup>-dependent phosphorylation was reduced by addition of calmodulin (1 μM), so that the IC<sub>50</sub> value of W-7 increased to 120 μM from 70 μM. A-4 and W-5 inhibited less selectively Ca<sup>2+</sup>-dependent

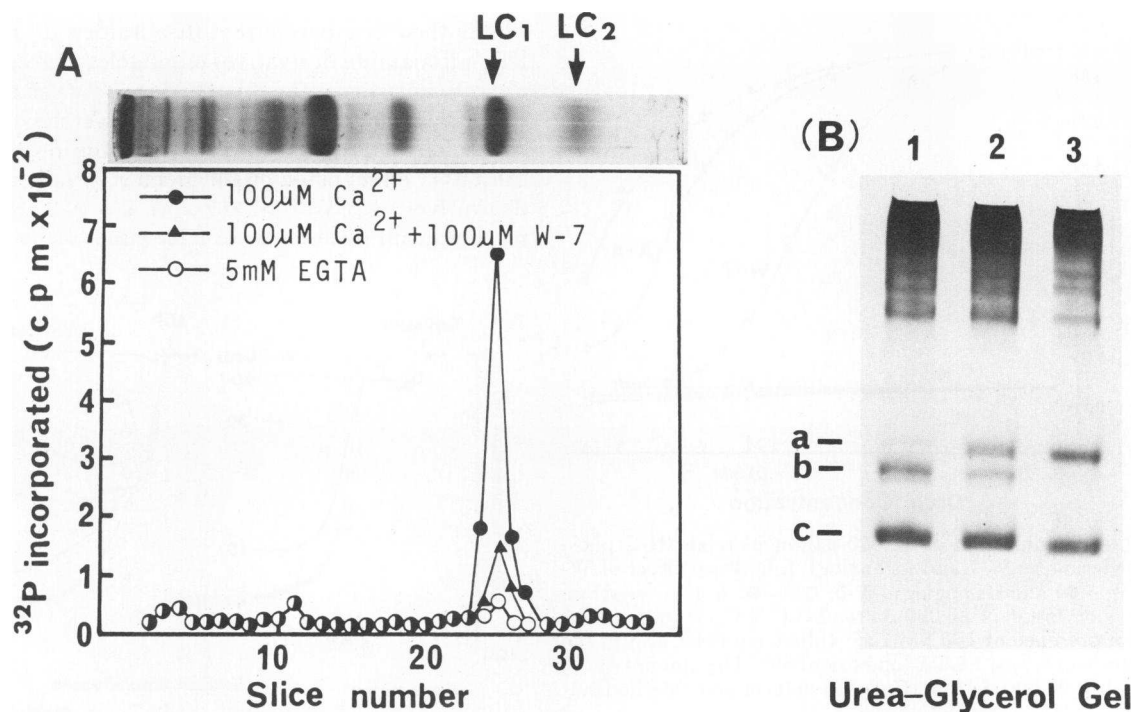


FIGURE 1 Effect of W-7 on distribution of  $^{32}\text{P}$  incorporated into platelet actomyosin. A. Distribution of  $^{32}\text{P}$  incorporated into platelet actomyosin after phosphorylation in the presence of 100  $\mu\text{M}$   $\text{Ca}^{2+}$  (●), 5 mM EGTA (○), or 100  $\mu\text{M}$  W-7 in addition to 100  $\mu\text{M}$   $\text{Ca}^{2+}$  (▲). LC<sub>1</sub> and LC<sub>2</sub> indicate the 20,000- and 15,000-dalton MLC of platelet myosin, respectively. Crude actomyosin was incubated under phosphorylation conditions as described in Methods, and phosphate incorporations were analyzed by 0.1% SDS 10% polyacrylamide gel electrophoresis. B. Urea-glycerol gel electrophoresis of the same samples as in Fig. 1 A. 1,  $\text{Ca}^{2+}$  100  $\mu\text{M}$ ; 2,  $\text{Ca}^{2+}$  100  $\mu\text{M}$  + W-7 100  $\mu\text{M}$ ; 3, 5 mM EGTA. a, the position of the unphosphorylated 20,000-dalton light chain; b, the position of the phosphorylated 20,000-dalton light chain; c, the 15,000-dalton light chain.

phosphorylation of platelet actomyosin in a dose-dependent manner, as shown by Fig. 2, and their  $\text{IC}_{50}$  values (in micromolar concentration) were 220 and 350, respectively. Comparing their  $\text{IC}_{50}$  values, W-5, a chlorine-deficient analog of W-7, was five times less potent than W-7.

Table II shows that the potency in inhibition of  $\text{Ca}^{2+}$ -dependent phosphorylation of 20,000-dalton MLC increased with the length of the aminoalkyl chain of naphthalenesulfonamide. On the other hand, these compounds were not effective on  $\text{Ca}^{2+}$ -independent phosphorylation. The degree to which these compounds inhibited  $\text{Ca}^{2+}$ -dependent phosphorylation was directly related to their ability to bind to calmodulin.

**Inhibition of naphthalenesulfonamide derivatives on human platelet aggregation in vitro.** The effect of W-7 on human platelet aggregation induced by collagen (2  $\mu\text{g}/\text{ml}$ ), ADP (5  $\mu\text{M}$ ), epinephrine (1  $\mu\text{g}/\text{ml}$ ), sodium arachidonate (0.83 mM), thrombin (0.125 U/ml), or A-23187 (10  $\mu\text{M}$ ), is shown in Fig. 3. W-7 (10–

100  $\mu\text{M}$ ) inhibited in a dose-dependent manner platelet aggregation induced by various aggregation agents. When the preincubation time of platelets with W-7 was increased, more pronounced inhibition of aggregation was observed. The effect of increasing the amount of the aggregating agent could be blocked by a proportional increase in the amount of W-7. W-7 had no effect in a concentration up to 200  $\mu\text{M}$  on platelet agglutination induced by ristocetin (1.2 mg/ml). Collagen-induced release of serotonin associated with platelet aggregation was also inhibited by naphthalenesulfonamide derivatives, and the extent of the inhibition of the release was proportional to that of the inhibition of aggregation (Fig. 4).

Aggregation response were quantified both as the rate (slope) and maximum extent of aggregation, so that the alternations of the slope were similar to that of the maximum extent of aggregation. The concentration of these derivatives producing 50% inhibition of serotonin release agreed well with their concentra-

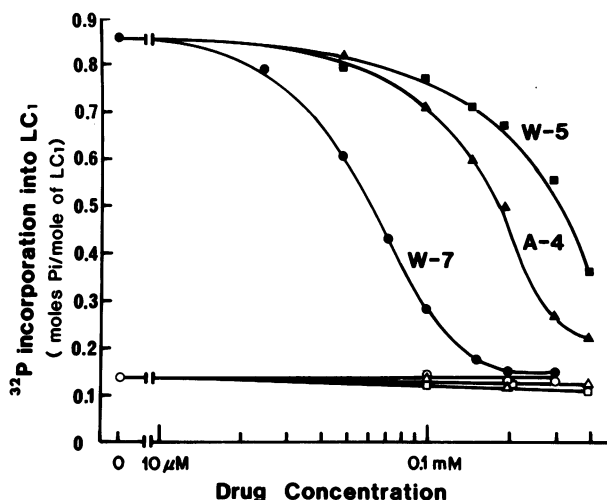


FIGURE 2 Inhibition of 20,000-dalton platelet MLC phosphorylation by W-7 and its analogs. Inhibitory effect of W-7 (○—●) and its analogs (W-5; □—■, A-4; △—▲) on phosphorylation of 20,000-dalton MLC (LC<sub>1</sub>) from platelets in the presence of 100  $\mu$ M Ca<sup>2+</sup> (filled symbols), and in the presence of 5 mM EGTA (open symbols). The amount of <sup>32</sup>P bound to 20,000-dalton MLC was determined (Methods).

tions producing 50% inhibition of the aggregation. When W-7 was added to PRP, no aggregation or slight serotonin release was observed above concentrations of 200  $\mu$ M. At higher concentrations of W-7 (300  $\mu$ M), there was spontaneous release of serotonin.

TABLE II  
Effects of Naphthalenesulfonamide Derivatives on the Phosphorylation of 20,000-dalton MLC from Human Platelets in the Presence of 100  $\mu$ M Ca<sup>2+</sup> and in the Presence of 5 mM EGTA

Addition	Phosphorylation of 20,000-dalton MLC	
	5 mM EGTA	100 $\mu$ M Ca <sup>2+</sup>
$\mu$ M	mol of <sup>32</sup> P/mol of MLC	
None	0.14±0.01	0.89±0.04
A-3 (100)	0.13±0.01	0.84±0.02
A-4 (100)	0.13±0.02	0.77±0.03
A-5 (100)	0.14±0.01	0.64±0.02
I-240 (100)	0.15±0.01	0.40±0.04
W-7 (100)	0.14±0.01	0.28±0.01
A-6 (100)	0.13±0.04	0.15±0.03
W-5 (100)	0.14±0.009	0.81±0.03

Phosphorylation assays were performed as described in Methods. The reaction were performed at 25°C for 10 min. The amount of <sup>32</sup>P bound to 20,000-dalton MLC was determined as described under Methods. Each value represents the mean±SEM for four determinations from two separate experiments.

We then compared the IC<sub>50</sub> values of naphthalenesulfonamide derivatives on platelet aggregation in order to investigate the relationships between chemical structures and inhibitory action to platelet function. As shown in Fig. 5 and Table III, the order of the inhibitory effect based on the mean IC<sub>50</sub> values of these derivatives, was W-7 > A-5 > A-4 > A-3. However, no significant differences in IC<sub>50</sub> values of platelet ag-

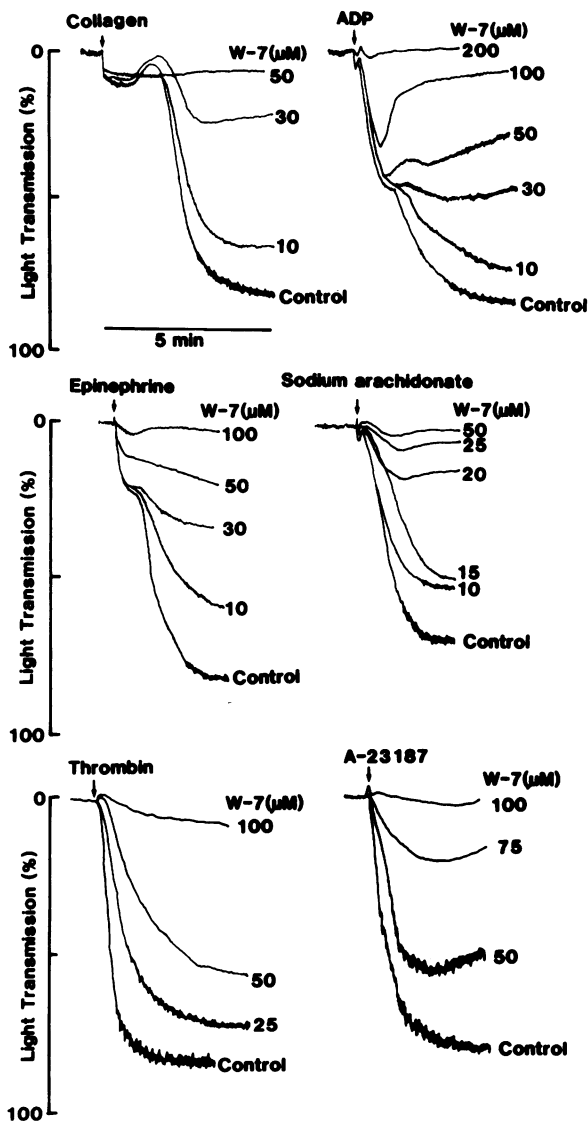


FIGURE 3 Inhibition by W-7 of human platelet aggregation. Effect of W-7 was examined in human platelet aggregation induced by collagen (2  $\mu$ g/ml), ADP (5  $\mu$ M), epinephrine (1  $\mu$ g/ml), sodium arachidonate (0.83 mM), thrombin (0.125 U/ml), and A-23187 (10  $\mu$ M). The platelets were preincubated without (control) or with W-7 (at final concentrations shown in figure) at 37°C with stirring for 3 min in the aggregometer cuvette before stimulation.

gregation among I-240, W-7, and A-6 were seen, although the capacity to compete for the specific binding of [<sup>3</sup>H]W-7 to calmodulin increased with the length of the aminoalkyl chain. W-5 is about four times weaker as an inhibitor of platelet aggregation than is W-7. The

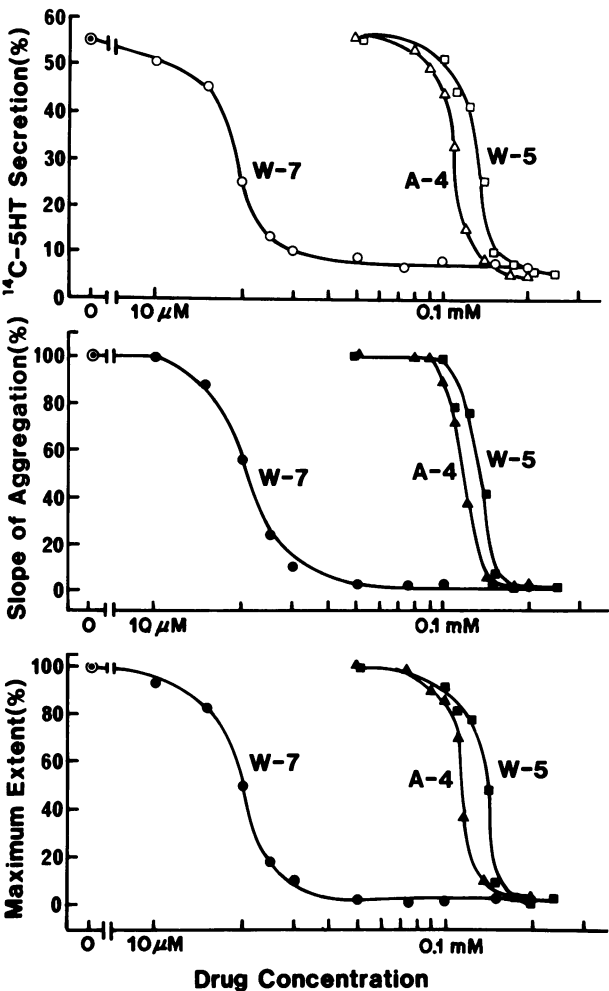


FIGURE 4 Effect of W-7 and its analogs on slope and maximum extent of platelet aggregation and serotonin release from platelet. Effects of W-7 (○ — ●) and its analogs (W-5; □ — ■, A-4; △ — ▲) on platelet aggregation (filled symbols) and [<sup>14</sup>C]serotonin release (open symbols) induced by collagen (2 μg/ml). (Methods). Aggregation response were quantified both as the slope of aggregation curve and the maximum extent of aggregation. The values of the slope represents the decrease in slope, expressed as a percentage of control. The values of the maximum extent were expressed as a percentage of the control. The control values of the aggregation are expressed as 100%. The release of serotonin was expressed as a percentage of the total amount of radioactivity originally contained in platelets. Each point is the mean of at least three experiments. The control values (○) of aggregation and release were obtained without addition of the compound.

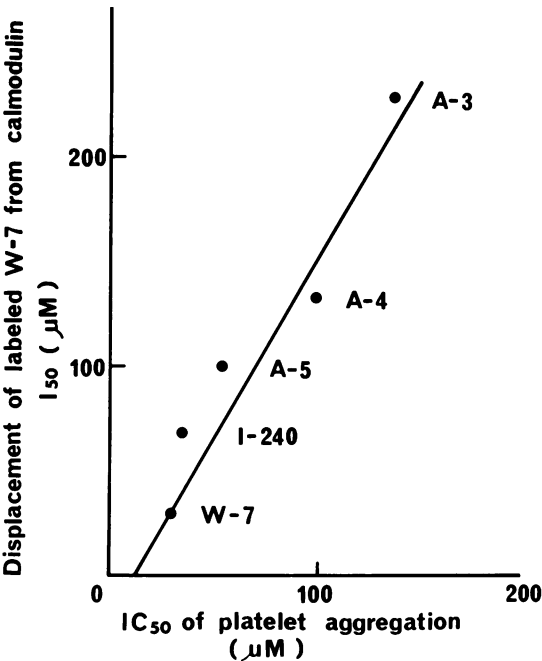


FIGURE 5 Correlation between the affinities of W-7 and its derivatives to calmodulin and their abilities to inhibit platelet aggregation. The data from Tables II and III are plotted with the concentration of the compound with displaced 50% of the labeled W-7 (*I*<sub>50</sub>) on the ordinate, and the mean *IC*<sub>50</sub> (micromolar) of the compound for inhibition of platelet aggregation induced by collagen (2 μg/ml) on the abscissa. The line of best fit was determined by regression analysis. The correlation coefficient was 0.94.

degree to which these naphthalenesulfonamide derivatives inhibit human platelet aggregation was directly related to their affinity to bind to calmodulin. This is shown graphically in Fig. 5, which demonstrates a significant positive correlation (*r* = 0.94).

TABLE III  
Inhibition of Naphthalenesulfonamide Derivatives of Human Platelet Aggregation In Vitro

Compound	No. of subjects	<i>IC</i> <sub>50</sub> *
		μM
A-3	4	137.7±16.4
A-4	5	99.4±7.0
A-5	4	54.5±12.7
I-240	5	35.2±10.7
W-7	11	32.0±4.2
A-6	6	40.0±7.0
W-5	6	137.8±6.2

Data are expressed as mean±SEM.  
\* *IC*<sub>50</sub> value was obtained visually from a plot of percent aggregation (maximum extent) vs. log concentration of each drug.

## DISCUSSION

Although it has been reported that calmodulin activates calcium-modulated enzymes, and regulates the assembly and disassembly of microtubules (17, 18), the physiologic significance of calmodulin has not been elucidated. One approach to understanding the biologic and physiologic function of calmodulin is a pharmacologic method using calmodulin antagonists that bind to calmodulin dependent on calcium and inhibit selectively  $\text{Ca}^{2+}$ -calmodulin-induced activation of enzymes. Previous studies showed that W-7 and its analogs selectively inhibit  $\text{Ca}^{2+}$ -dependent cyclic nucleotide phosphodiesterase (6, 7, 19),  $\text{Ca}^{2+}$ -dependent myosin light chain kinase from chicken gizzard and bovine aorta (6, 20, 21), and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  ATPase of erythrocyte ghost (22). We have recently demonstrated that W-7 inhibits the  $\text{Ca}^{2+}$ -activation of phosphodiesterase by direct binding to calmodulin (6). W-7 not only relaxed vascular strips (15, 19), but also inhibited platelet aggregation and secretion induced by various aggregating agents. However, the  $\text{IC}_{50}$  value of W-7 was indistinguishable for different aggregating agents, therefore this compound probably interacts through a common process in platelet aggregation.

It is generally assumed that the biological basis for contractile events in nonmuscle cells such as platelets is similar to the situation in smooth muscle. The regulation of actin-myosin interaction in platelets has been shown to involve phosphorylation-dephosphorylation of the 20,000-dalton MLC (23, 24). This phosphorylation increases the activity of actin-activated myosin ATPase (25), and the requirement of phosphorylation for actin-activated ATPase activity of myosin suggests that phosphorylation regulates platelet contractility (26). Platelet myosin light chain kinase has been identified as a  $\text{Ca}^{2+}$ -dependent enzyme that requires calmodulin for its activity (10, 27). Moreover, we have recently suggested that a calmodulin-mediated system, such as  $\text{Ca}^{2+}$ -dependent phosphorylation plays an important role in the phenomenon of platelet secretion (4). The present studies suggest that the binding affinity of our derivatives for calmodulin and the potency in inhibiting platelet aggregation depend on the chlorination at five position of the naphthalene ring, and also on the length of aminoalkyl chain. As a good correlation between the potency in inhibiting platelet aggregation and affinity to calmodulin was obtained, the inhibitory action of these compounds in platelet aggregation may be due to their effect on  $\text{Ca}^{2+}$ -calmodulin-dependent processes.

One additional discrepancy found in the platelet aggregation study vis à vis the binding studies is that A-6 and I-240 are equipotent with W-7 in blocking platelet aggregation, although their binding affinity to

calmodulin increased steadily with the length of the aminoalkyl chain. One possible explanation would be the difference in penetration of the compound through the platelet membrane, and this aspect is now being investigated. Nonetheless, collectively, our data suggest that the degree to which these naphthalenesulfonamides inhibit the human platelet aggregation in vitro parallels their binding affinity to calmodulin and their potency in inhibition of  $\text{Ca}^{2+}$ -dependent phosphorylation of 20,000-dalton MLC. Moreover, electron microscopic autoradiography of platelets incubated with radiolabeled W-7 has shown that this agent penetrates platelet membranes and is mainly localized in the platelet hyalomere (unpublished observation). We have recently reported that a hydrophobic probe, 2-p-toluidinylnaphthalene-6-sulfonate (TNS) interacts with calmodulin in the presence of  $\text{Ca}^{2+}$  and W-7 can suppress the TNS fluorescence (28). Although the possibility that W-7 binds to other hydrophobic proteins or lipids cannot be excluded, and that synthetic compounds generally have multiple functions, our data are in accord with the hypothesis that the W-7-induced inhibition of platelet aggregation associated with secretion may involve, at least in part, the prevention of calmodulin-mediated processes such as  $\text{Ca}^{2+}$ -dependent phosphorylation of 20,000-dalton MLC.

## ACKNOWLEDGMENTS

We thank Dr. T. Tanaka for the assistance of binding assay and M. Ohara, Kyushu University, for critical reading of the manuscript.

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Japan (1980).

## REFERENCES

1. Detwiler, T. C., I. F. Charo, and R. D. Feinman. 1978. Evidence that calcium regulates platelet function. *Thromb. Haemostasis*. **40**: 207-211.
2. Smoake, J. A., S. Y. Song, and W. Y. Cheung. 1974. Cyclic 3',5'-nucleotide phosphodiesterase. Distribution and developmental changes of enzyme and its protein activation in mammalian tissue and cells. *Biochim. Biophys. Acta*. **341**: 402-411.
3. Muszbek, L., J. Kuznicki, T. Szabo, and W. Drabikowski. 1977. Troponin C-like protein of blood platelets. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **80**: 308-312.
4. Nishikawa, M., T. Tanaka, and H. Hidaka. 1980.  $\text{Ca}^{2+}$ -calmodulin-dependent phosphorylation and platelet secretion. *Nature (Lond.)*. **287**: 863-865.
5. Weiss, B., and R. M. Levin. 1978. Mechanism for selectively inhibiting the activation of cyclic nucleotide phosphodiesterase and adenylate cyclase by antipsychotic agents. *Adv. Cyclic Nucleotide Res.* **9**: 285-303.
6. Hidaka, H., T. Yamaki, M. Naka, T. Tanaka, H. Hayashi, and R. Kobayashi. 1980. Calcium-regulated modulator protein interacting agents inhibit smooth muscle cal-



- cium-stimulated protein kinase and ATPase. *Mol. Pharmacol.* 17: 66-72.
7. Hidaka, H., T. Yamaki, T. Totsuka, and M. Asano. 1979. Selective inhibitors of  $\text{Ca}^{2+}$ -binding modulator of phosphodiesterase produce vascular relaxation and inhibit actin-myosin interaction. *Mol. Pharmacol.* 15: 49-59.
8. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244: 4406-4412.
9. Hummel, J. P., and W. Y. Dreyer. 1962. Measurement of protein-binding phenomenon by gel filtration. *Biochim. Biophys. Acta.* 63: 530-532.
10. Hathaway, D. R., and R. S. Adelstein. 1979. Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity. *Proc. Natl. Acad. Sci. U.S.A.* 76: 1653-1657.
11. Perrie, W. T., and S. V. Perry. 1970. An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* 119: 31-38.
12. Perrie, W. T., L. B. Smillie, and S. V. Perry. 1973. A phosphorylated light-chain component myosin from skeletal muscle. *Biochem. J.* 135: 151-164.
13. Born, G. V. R. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (Lond.)*. 194: 927-929.
14. Tangen, O., H. J. Berman, and P. Marfey. 1971. Gel filtration. A new technique for separation of blood platelets from plasma. *Thromb. Diath. Haemorrh.* 25: 268-278.
15. Hidaka, H., M. Asano, S. Iwadare, I. Matsumoto, T. Totsuka, and N. Aoki. 1978. A novel vascular relaxing agent, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide which affects vascular smooth muscle actomyosin. *J. Pharmacol. Exp. Ther.* 207: 8-15.
16. Kinlough-Rathbone, R. L., H. J. Reimers, and J. F. Mustard. 1976. Sodium arachidonate can induce platelet shape change and aggregation which are independent of the release reaction. *Science (Wash., D.C.)*. 192: 1011-1012.
17. Wang, J. H., and D. M. Weisman. 1979. Calmodulin and its role in the second-messenger system. *Curr. Top. Cell Regul.* 15: 47-107.
18. Wolff, D. J., and C. O. Brostrom. 1979. Properties and functions of the calcium-dependent regulator protein. *Adv. Cyclic Nucleotide Res.* 11: 27-88.
19. Hidaka, H., T. Yamaki, M. Asano, and T. Totsuka. 1978. Involvement of calcium in cyclic nucleotide metabolism in human vascular smooth muscle. *Blood Vessels.* 15: 55-64.
20. Hidaka, H., M. Naka, and T. Yamaki. 1979. Effect of novel specific myosin light chain kinase inhibitors on  $\text{Ca}^{2+}$ -activated  $\text{Mg}^{2+}$ -ATPase of chicken gizzard actomyosin. *Biochem. Biophys. Res. Commun.* 90: 694-699.
21. Kanamori, M., M. Naka, M. Asano, and H. Hidaka. 1981. Effects of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and other calmodulin antagonists (calmodulin-interacting agents) on calcium-induced contraction of rabbit aortic strips. *J. Pharmacol. Exp. Ther.* 217: 494-499.
22. Kobayashi, R., M. Tawata, and H. Hidaka. 1979.  $\text{Ca}^{2+}$ -regulated modulator protein interacting agents: inhibition of  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase of human erythrocyte ghost. *Biochem. Biophys. Res. Commun.* 88: 1037-1045.
23. Adelstein, R. S., M. A. Conti, and B. Barylko. 1978. The role of myosin phosphorylation in regulating actin-myosin interaction in human blood platelets. *Thromb. Haemostasis.* 40: 241-244.
24. Daniel, J. L., I. R. Molish, and H. Holmsen. 1981. Myosin phosphorylation in intact platelets. *J. Biol. Chem.* 256: 7510-7514.
25. Adelstein, R. S., and M. A. Conti. 1975. Phosphorylation of platelet myosin increases actin activation of myosin ATPase activity. *Nature (Lond.)*. 256: 597-598.
26. Lebowitz, E. A., and R. Cooke. 1978. Contractile properties of actomyosin from human platelets. *J. Biol. Chem.* 253: 5443-5447.
27. Dabrowska, R., and D. J. Hartshorne. 1978. A  $\text{Ca}^{2+}$ - and modulator-dependent myosin light chain kinase from non-muscle cells. *Biochem. Biophys. Res. Commun.* 85: 1352-1359.
28. Tanaka, T., and H. Hidaka. 1980. Hydrophobic regions function in calmodulin-enzyme interactions. *J. Biol. Chem.* 255: 11078-11080.