**HMN-709, a Chlorobenzenesulfonamide Derivative, Is a New Membrane-Permeable Calmodulin Antagonist**

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**Received March 28, 1996 Accepted July 12, 1996**

**ABSTRACT**—Our objective is to describe the basic chemical and biological properties of the new calmodulin antagonist HMN-709 (2-[N-(2-aminoethyl)-N-(4-chlorobenzenesulfonyl)]amino-N-(4-fluorocinnamyl)-N-methylbenzylamine). This newly synthesized compound was found to inhibit the Ca\(^{2+}\)/calmodulin-dependent activation of calmodulin kinase I, smooth muscle myosin light chain kinase and Ca\(^{2+}\)-phosphodiesterase with IC\(_{50}\) values of 1.57±0.21, 2.29±0.09 and 0.30±0.08 \(\mu\)M (mean±S.E.), respectively. This compound showed little or no effect on the Ca\(^{2+}\)/calmodulin-independent activation of protein kinase A, protein kinase C and basal phosphodiesterase. In addition, HMN-709 inhibited calmodulin kinase I competitively with respect to calmodulin (\(K_i=0.88\ \mu\)M) and non-competitively with respect to ATP. Affinity chromatography, with HMN-709-coupled Sepharose HP, showed that the compound bound to calmodulin in a Ca\(^{2+}\)-dependent manner and did not bind to calmodulin kinase I. These results suggest that HMN-709 antagonizes calmodulin by binding to Ca\(^{2+}\)/calmodulin. HMN-709 inhibited collagen-induced platelet aggregation with an IC\(_{50}\) value of 11.80±0.86 \(\mu\)M (mean±S.E.) without inhibiting phorbol 12,13-dibutyrate-induced aggregation at doses up to 12 \(\mu\)M. HMN-709 appears to be a new, membrane-permeable calmodulin antagonist that may be used for studying the involvement of calmodulin in cellular processes.

**Keywords:** Calmodulin, Calmodulin antagonist, IC\(_{50}\), Affinity chromatography, Platelet function

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Calmodulin (CaM), a ubiquitous Ca\(^{2+}\)-binding protein, is important for regulating a variety of intracellular processes that are dependent on Ca\(^{2+}\), including metabolism of cyclic nucleotides (1), protein phosphorylation/dephosphorylation (2, 3) and structural and functional changes in cytoskeletal proteins (4). CaM is also involved in the expression of growth-related genes (e.g., c-fos and c-myc) (5) and cell cycle progression (6). The binding of Ca\(^{2+}\) to CaM induces a conformational change in the protein and the Ca\(^{2+}\)-CaM complex becomes capable of binding to target proteins by hydrophobic interactions and of modulating their functions (7, 8). These hydrophobic interactions were proven when the structure of the ternary complex, Ca\(^{2+}\)/CaM/M13 (a peptide of skeletal muscle myosin light chain kinase), was determined (9). A variety of chemicals serve as CaM antagonists, including trifluoperazine (10), calmidazolium (11), Ro 22-4839 (12) and tamoxifen (13). They bind to CaM in a Ca\(^{2+}\)-dependent manner and inhibit the Ca\(^{2+}\)/CaM actions both in vitro and in vivo. In addition, a set of CaM antagonists including W-7 and W-9 has been generated from naphthalenesulfonamide derivatives (14). The recent elucidation of the crystal structure of the Ca\(^{2+}\)/CaM complex with trifluoperazine revealed that Ca\(^{2+}\)/CaM binds to the CaM antagonist in a similar shape to that observed in Ca\(^{2+}\)/CaM/M13 (9, 15). Pharmacological studies by Hidaka and co-workers suggest that CaM is involved in platelet aggregation and secretion (16), vascular contraction (17), cell proliferation (18) and the degranulation of neutrophils (19).

In this report, we compared the effects of HMN-709, one of the new chlorobenzenesulfonamide derivatives synthesized by our group, on several Ca\(^{2+}\)/CaM-dependent and -independent enzymes. The binding of HMN-709 to Ca\(^{2+}\)/CaM, as well as to a Ca\(^{2+}\)/CaM-dependent enzyme, was also investigated. We have now identified HMN-709 as a new CaM antagonist. Furthermore, we studied the effects of HMN-709 on platelet aggregation and the release of 5-hydroxytryptamine (5-HT) from platelets.

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MATERIALS AND METHODS

Materials

HMM-709 was synthesized by a modification of the method of Hidaka et al. (20). W-7 was synthesized as described (20). The peptides, Syntide-2 (PLARTLSVA GLPGKK) (21) and smooth muscle myosin light chain (smMLC) peptide (KKRAARATSNVFA) (22), were synthesized and purified at Peptide Institute, Inc. (Osaka). Histone type III-S, VII-S, snake venom (Crotalus atrox) and phorbol 12,13-dibutyrate (PDBu) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DE-52 resin and P-81 paper were purchased from Whatman (Maidstone, Kent, England). The bacterial expression vector, pGex-2T, glutathione-Sepharose 4B and N-hydroxysuccinimide (NHS)-activated Sepharose HP were obtained from Pharmacia LKB Biotechnology, Inc. (Uppsala, Sweden). AG 50W-X4 resin and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Collagen was from Nycomed (München, Germany). [γ-32P]ATP (>5,000 Ci/mmol), [8-3H]cGMP (30 Ci/mmol) and aqueous counting scintillant (ACS II) were obtained from Amersham Corp. (Arlington Heights, IL, USA). [2,14C]-5-HT (56.9 mCi/mmol) was purchased from New England Nuclear (Boston, MA, USA). A cDNA of rat Ca2+/CaM-dependent protein kinase I (CaM kinase I) was kindly donated by Drs. A.C. Nairn and M.R. Picciotto (The Rockefeller University, New York, NY, USA). All other chemicals were reagent grade or better.

CaM was purified from bovine brain by the method of Yazawa et al. (23). DNA fragment encoding CaM kinase I or its COOH-terminal truncated mutant (mutant 1-293) was fused in-frame to glutathione S-transferase (GST)-coding region in pGex-2T. The plasmids were transformed into the bacterial strain, JM-109, and the recombinants expressed as a GST fusion protein and purified by affinity chromatography with glutathione-Sepharose 4B as described (24). Smooth muscle myosin light chain kinase (smMLCK) was prepared from chicken gizzard according to Adelstein and Klee (25). Ca2+/CaM-dependent (Ca2+/CaM-) and -independent (basal) cyclic nucleotide phosphodiesterase (PDE) were partially purified from rabbit brain by DE-52 column chromatography. The catalytic subunit of protein kinase A was prepared from bovine heart according to Beavo et al. (26). Protein kinase C was partially purified from rabbit brain by DE-52 column chromatography.

Protein kinase and PDE assays

CaM kinase I activity was assayed at 30°C for 10 min in a 50-µl mixture containing 35 mM HEPES-NaOH (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol, 0.01% (v/v) Tween 20, 50 µM Syntide-2, 100 µM [γ-32P]ATP (200 cpm/pmol), 480 ng of the enzyme, various amounts of compound as indicated, and either 0.5 mM CaCl2, 100 nM CaM or 1 mM EGTA. smMLCK activity was assayed at 30°C for 10 min in a 50-µl mixture containing 35 mM HEPES-NaOH (pH 7.5), 10 mM MgCl2, 1 mg/ml bovine serum albumin (BSA), 0.1% (v/v) Tween 80, 50 µM smMLC peptide, 100 µM [γ-32P]ATP (400 cpm/pmol), 150 ng of the enzyme, various amounts of compound as indicated, and either 0.5 mM CaCl2, 3 nM CaM or 1 mM EGTA. Reactions were initiated by the addition of ATP and terminated by pipetting 25 µl of the mixture onto P-81 paper as described by Roskoski (27). The paper was then washed with 75 mM phosphoric acid, dried and the radioactivity counted by Cerenkov counting. Mutant 1-293 activity was assayed in a similar mixture to that for CaM kinase I except that 1 mM EGTA and 270 ng of the enzyme were used. The activities were a linear function of the reaction time in each assay system.

The activities of protein kinase A and C were assayed by modifications of the methods of Chijiwa et al. (28) and Inagaki et al. (29), respectively. The reaction for protein kinase A was carried out at 37°C for 5 min in a 0.2 ml mixture containing 25 mM Tris-HCl (pH 7.0), 10 mM MgCl2, 2 mM EGTA, 0.2 mg/ml histone type VII-S, 10 µM [γ-32P]ATP (150 cpm/pmol), adequately diluted enzyme and various amounts of compound as indicated. The reaction for protein kinase C was carried out at 37°C for 5 min in a 0.2-ml mixture containing 25 mM Tris-HCl (pH 7.0), 10 mM MgCl2, 0.2 mg/ml histone type III-S, 10 µM [γ-32P]ATP (150 cpm/pmol), adequately diluted enzyme, various amounts of compound as indicated and either 0.5 mM CaCl2, 50 µg/ml phosphatidylserine or 1 mM EGTA. The reactions were started by the addition of ATP and stopped by the addition of 1 ml of 20% (w/v) trichloroacetic acid (TCA), followed by the addition of 100 µl of 1 mg/ml BSA as a carrier protein. The reaction mixtures were centrifuged at 3,000 rpm for 10 min. The pellets were washed with 5% (w/v) TCA and the centrifugation was repeated. The washing cycle was carried out twice, and the pellet was dissolved in 1 ml of 1 M NaOH prior to Cerenkov counting. The activities were linear as a function of reaction time in each assay system.

PDE activity was measured by the method of Hidaka and Asano (30). The reaction was carried out at 30°C for 10 min in a 0.2 ml mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.1 mg/ml BSA, 0.4 µM [8-3H]-cGMP (3,800 dpm/pmol), adequately diluted enzyme, various amounts of compound as indicated, and either 0.2 mM CaCl2, 1.8 nM CaM or 1 mM EGTA. The reaction was initiated by the addition of cGMP and terminated by heating the mixture at 100°C for 5 min. The
5'-[^3]H]GMP product was converted to [^3]H]guanosine by another incubation at 30°C for 10 min with 20 μg snake venom. The mixture was loaded onto a column (0.5 × 2 cm) of AG 50W-X4 cation exchange resin and [^3]H]guanosine eluted with 2 ml of 3 M NH₄OH. The eluate was emulsified with 10 ml of ACS II, and the radioactivity was counted in a Beckman liquid scintillation counter.

The data were obtained from three independent experiments, each performed in duplicate.

**Coupling of HMN-709 to Sepharose HP**

HMN-709 (5.6 mg) was dissolved in a 50-ml coupling mixture containing 20 mM HEPES-NaOH (pH 7.0), 0.5 M NaCl, and 10% (v/v) N,N-dimethylformamide and coupled to NHS-activated Sepharose HP in a column (bed volume of 5 ml) according to the manufacturer's instructions. The coupling reaction was carried out at 4°C overnight with the ligand circulating through a closed system driven by a peristaltic pump. The coupling efficiency was not determined, and residual amine-reactive groups of the resin were blocked by 0.5 M ethanolamine.

**Assay for platelet aggregation and the release of 5-HT**

Human venous blood was freshly drawn from healthy donors who had not received any medication during the previous 4 weeks. Washed platelets were prepared by the method of Schmidt and Rasmussen (31) by using erythrocytes as a supporting cushion. Platelets were finally resuspended (5 × 10⁸ cells/ml) in a modified Tyrode-HEPES buffer containing 20 mM HEPES-NaOH (pH 7.4), 135 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂ and 0.1% dextrose. The washed platelet suspension was pre-incubated at 37°C for 2 min with various amounts of compound as indicated, and platelet aggregation was induced by the addition of either collagen or PDBu to final concentrations of 2 μg/ml and 100 nM, respectively. Platelet aggregation was carried out at 37°C in a volume of 300 μl and monitored photometrically with the 4-channel aggregometer, NBS Hema Tracer 601 (Niko Bioscience, Inc., Tokyo). Aggregation was quantified as its maximum and expressed as percentage relative to the modified Tyrode-HEPES buffer (100%). The data were obtained from three independent experiments, each performed in triplicate.

The release of 5-HT from platelets was measured by the method of Costa and Murphy (32). Briefly, the platelet suspension was incubated at 25°C for 60 min with [2-^14]C]-5-HT (0.1 μCi/ml suspension). These conditions permitted ~68% of the radioactivity to be taken up by the platelets. The [2-^14]C]-5-HT-loaded platelet suspension was pre-incubated at 37°C for 2 min with various amounts of compound as indicated, and the platelets were induced to release 5-HT by the 2 μg/ml collagen. The release action was carried out at 37°C for various periods of time as indicated and terminated by the addition of formaldehyde to a final concentration of 1.5% (v/v). Platelets were spun at 3,000 rpm for 5 min in a bench-top centrifuge, the supernatant emulsified by mixing with 2 ml of ACE II and the radioactivity counted in a Beckman liquid scintillation counter. The 5-HT release was expressed as a percentage, taking the 5-HT uptake into platelets as 100%. The data were obtained from three independent experiments, each performed in duplicate.

**Protein analyses**

SDS-PAGE was carried out according to Laemmli (33). Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue, followed by destaining in 10% (v/v) methanol and 10% (v/v) acetic acid. Gels were dried onto cellophane paper in vacuo. Apparent molecular masses on SDS-PAGE were determined by using SDS-PAGE molecular weight standards. Protein concentrations were determined by the Bradford assay (34) with BSA as a standard.

**RESULTS**

**Chemical properties**

HMN-709 is a new chlorobenzenesulfonamide derivative, whose chemical structure is given in Fig. 1. It is synthesized as a phosphate salt and soluble in either H₂O or 100% dimethylsulfoxide up to a tested concentration of 10 mM.

**Effects of HMN-709 on Ca²⁺/CaM-dependent and -independent enzymes**

HMN-709 inhibited the Ca²⁺/CaM-dependent activation of CaM kinase I, smMLCK and Ca²⁺-PDE with IC₅₀ values of 1.57±0.21, 2.29±0.09 and 0.30±0.08 μM.
(mean ± S.E.), respectively (Fig. 2, Table 1). On the other hand, the established CaM antagonist W-7 inhibited smMLCK and Ca\(^{2+}\)-PDE with IC\(_{50}\) values of 36.7 ± 3.8 and 7.10 ± 1.92 \(\mu\)M, respectively. CaM kinase I was inhibited to a lesser extent with an IC\(_{50}\) value above 30 \(\mu\)M (Table 1). The inhibitory action of HMN-709 was abolished by the addition of excess CaM, suggesting that this compound competes with Ca\(^{2+}\)/CaM upon enzyme activation (data not shown). In fact, HMN-709 inhibited a Ca\(^{2+}\)/CaM-dependent enzyme, CaM kinase I, competitively with respect to CaM with a \(K_{i}\) value of 0.88 \(\mu\)M and non-competitively with respect to ATP (Fig. 3). This competitive inhibition is also supported by the fact that the compound showed little or no effect on the Ca\(^{2+}\)/CaM-independent enzymes, protein kinases A and C (Table 1). In addition, HMN-709 showed much less inhibition of mutant 1-293, a COOH-terminal-truncated mutant of CaM kinase I, with an IC\(_{50}\) value of 52.6 ± 0.1 \(\mu\)M (Fig. 2a, Table 1). This mutant is assumed to have a Ca\(^{2+}\)/CaM-independent activity due to the deletion of the regulatory domain that encompasses the Ca\(^{2+}\)/CaM-binding site (24). HMN-709 only slightly inhibited basal PDE, with an IC\(_{50}\) value of 132 ± 16 \(\mu\)M (Fig. 2b, Table 1), while the basal PDE inhibitor, nicardipine, strongly inhibited the enzyme with an IC\(_{50}\) value of 1.04 ± 0.08 \(\mu\)M (data not shown). These results suggest that HMN-709 binds to Ca\(^{2+}\)/CaM, thereby inhibiting Ca\(^{2+}\)/CaM-dependent enzyme activation, or that the compound causes these inhibitions by directly acting on the Ca\(^{2+}\)/CaM-binding sites of the enzymes.

**Affinity chromatography with HMN-709-coupled Sepharose HP**

To elucidate the direct interaction between HMN-709 and either Ca\(^{2+}\)/CaM or the Ca\(^{2+}\)/CaM-dependent enzymes, affinity chromatography, with HMN-709-coupled Sepharose HP, was carried out. Elution of protein samples was monitored by measuring the absorption of light at 280 nm (Fig. 4). Representative fractions at different chromatographic steps were also analyzed by SDS-PAGE (inset). CaM was retained in the column in the presence of

![Diagram](image)

Fig. 2. Effects of HMN-709 on Ca\(^{2+}\)/CaM-dependent and -independent enzymes. Panel a: Effects of HMN-709 on CaM kinase I (○) and the Ca\(^{2+}\)/CaM-independent CaM kinase I, mutant 1-293 (●), are shown. Panel b: Effects of HMN-709 on Ca\(^{2+}\)/CaM-dependent (Ca\(^{2+}\)-) (○) and -independent (basal) PDE (●) are given. The activities of CaM kinase I, mutant 1-293, Ca\(^{2+}\)-PDE and basal PDE were 23 pmol of phosphate transferred per min, 7.6 pmol of phosphate transferred per min, 0.23 pmol of cGMP hydrolyzed per min and 0.42 pmol of cGMP hydrolyzed per min, respectively, in the absence of the compound and represented as the control values of 100%. Data are expressed as the mean ± S.E. of three independent experiments, each performed in duplicate. The data for concentrations above 10 \(\mu\)M are not included to highlight the differences between the effects of HMN-709 on Ca\(^{2+}\)/CaM-dependent and -independent enzymes.

| IC\(_{50}\) (\(\mu\)M) | HMN-709 | W-7 |
|-----------------|--------|-----|
| CaM kinase I    | 1.57 ± 0.21 | >30 |
| smMLCK         | 2.29 ± 0.09 | 36.7 ± 3.8 |
| Ca\(^{2+}\)-PDE | 0.30 ± 0.08 | 7.10 ± 1.92 |
| Protein kinase A| 91.3 ± 7.9 | 230 |
| Protein kinase C| >100 | 340 |
| Basal PDE      | 132 ± 16  | 120† |
| Mutant 1-293†  | 52.6 ± 0.1 | 200 |

Data are derived from three independent experiments, each done in duplicate and expressed as the mean ± S.E. *IC\(_{50}\) values are concentrations that produce a 50% inhibition of enzyme activity. †IC\(_{50}\) values reported in the literature (46 and 47, respectively). †Mutant 1-293 is a COOH-terminal truncated mutant of rat CaM kinase I, which possesses a Ca\(^{2+}\)/CaM-independent activity.
Ca\textsuperscript{2+} and eluted by buffer containing EGTA in exchange for Ca\textsuperscript{2+} (Fig 4, inset). CaM flowed through the column when loaded together with EGTA (data not shown). Interestingly, CaM kinase I, a Ca\textsuperscript{2+}/CaM-dependent enzyme, flowed through the column in the presence of Ca\textsuperscript{2+} (Fig. 4, inset). There is a slight possibility that excess enzyme was loaded and that a portion was retained in the column. However, the enzyme was not eluted with either EGTA or EGTA plus the denaturant, 6 M urea (Fig. 4, inset). These results indicate that HMN-709 binds, in a Ca\textsuperscript{2+}-dependent manner, to CaM and not to the Ca\textsuperscript{2+}/CaM-dependent enzyme.

**Effects of HMN-709 on platelet aggregation and the release of 5-HT**

HMN-709 inhibited collagen-induced platelet aggregation in a dose-dependent manner. A representative data set is given in Fig. 5a. The extent of inhibition varied among platelet preparations, although an IC\textsubscript{50} value of 11.80±0.86 \textmu M (mean±S.E.) was estimated from three independent experiments, each performed in triplicate. W-7 also inhibited the platelet aggregation, but to a lesser extent, with an IC\textsubscript{50} value of 17.45±1.39 \textmu M (data not shown). PDBu, a protein kinase C activating agent, caused platelet aggregation in a distinct time-dependent manner relative to collagen (Fig. 5b). This PDBu-induced platelet aggregation was not affected by HMN-709 at doses up to 12 \textmu M (Fig. 5b), which suggests that HMN-709 distinguishes between two different stimuli-coupled platelet aggregation processes and selectively inhibits the collagen-coupled process.

HMN-709 inhibited collagen-induced release of 5-HT from platelets in a dose-dependent manner. The extent resembled that to which it inhibited the collagen-induced platelet aggregation (Fig. 5c).

**DISCUSSION**

Many chemicals interact with CaM in a Ca\textsuperscript{2+}-dependent manner and antagonize Ca\textsuperscript{2+}/CaM actions. Calcium chelators such as EGTA may also be classified as CaM antagonists because they inhibit the binding of Ca\textsuperscript{2+} to CaM, thus inactivating CaM. Sasaki et al. reported that ruthenium red inhibits the formation of a Ca\textsuperscript{2+}-CaM complex by directly affecting the Ca\textsuperscript{2+}-binding sites of CaM and should also be designated as a CaM antagonist (35). However, since the concentrations needed to block Ca\textsuperscript{2+}/CaM-dependent and -independent processes are almost identical, the pharmacological efficacy of these compounds is limited. This concern is evident when studying trifluoperazine, calmidazolium and W-7, which possess particularly potent CaM antagonistic effects. These agents exhibited a selectivity of ~50-fold for the Ca\textsuperscript{2+}/CaM-dependent enzyme, Ca\textsuperscript{2+}-PDE, compared with the Ca\textsuperscript{2+}/CaM-independent enzyme, protein kinase C (36). This is consistent with our results, where W-7 in-
hibited Ca\(^{2+}\)-PDE and protein kinase C with IC\(_{50}\) values of 7.10±1.92 and 340\(\mu\)M, respectively (Table 1). More selective CaM antagonists need to be developed to further study the involvement of CaM in intracellular Ca\(^{2+}\) signaling pathways. In this respect, 5-iodo-1-C\(_8\) (N-(8-aminoctyl)-5-iodo-1-naphthalenesulfonamide), derived from W-7, has greatly improved potency as well as selectivity for Ca\(^{2+}\)/CaM (36). The IC\(_{50}\) value of this compound for Ca\(^{2+}\)-PDE is 300-fold less than that for protein kinase C (36). This compound was then used to clarify the importance of CaM in prolactin gene transcription (37).

We have pursued better CaM antagonists and have recently tested new benzenesulfonamide derivatives. Through our investigations, HMN-709 has been identified as another CaM antagonist. This compound has an IC\(_{50}\) value for Ca\(^{2+}\)-PDE that is >300-fold less than that for protein kinase C (Table 1). This selectivity appears almost comparable to that of 5-iodo-1-C\(_8\). However, our IC\(_{50}\) value of W-7 for Ca\(^{2+}\)-PDE was approximately 4 times lower than previously reported (Table 1) (38). This difference may well result from the use of different concentrations of CaM, 1.8 nM and 30 nM in this and the previous experiments, respectively (38). Thus, the IC\(_{50}\) value of HMN-709 for Ca\(^{2+}\)-PDE, 0.30±0.08 \(\mu\)M, may be corrected by the factor of 4 for a value of 1 \(\mu\)M or so. Even if it is the case, the selectivity of this compound for Ca\(^{2+}\)-PDE compared with protein kinase C is still much better than other CaM antagonists. HMN-709 inhibited CaM kinase I as well as, but to a much less extent, mutant 1-293 devoid of the Ca\(^{2+}\)/CaM-binding site of CaM kinase I (Fig. 2a, Table 1). This compound showed little or no effect on other Ca\(^{2+}\)/CaM-independent enzymes, protein kinase A, C, and basal PDE (Table 1). Therefore, the inhibition of mutant 1-293 may reflect the non-specific
Fig. 5. Effects of HMN-709 on platelet aggregation and the release of 5-HT. Panel a: Platelets were pre-incubated for 2 min without (control) or with the compound in concentrations ranging from 6 to 10 μM, indicated by the arrow. Aggregation was induced by adding 2 μg/ml collagen, indicated by the arrow head. Panel b: Similarly, platelet aggregation was induced by adding 100 nM PDBu in the presence of 8–12 μM HMN-709. The extent of aggregation was expressed as the percent increase in light transmission vs time, in which the light transmission of the buffer without platelets is taken as 100%. Panel c: [2-14C]5-HT-loaded platelets were pre-incubated without (○) or with 6 (△), 8 (□) or 10 μM (●) HMN-709. 5-HT release was induced by adding 2 μg/ml collagen. The release reaction was estimated by the percent increase in [2-14C]5-HT in the supernatant at the indicated times with the radioactivity in the platelets before the induction taken as 100%. Data are expressed as the mean ± S.E. from three independent experiments, each performed in duplicate.
effects of HMN-709 on the enzyme, which remains to be resolved. However, our present results indicate that this compound may be highly selective for Ca\(^{2+}\)/CaM-dependent enzymes when used at a dose of 10 \(\mu\)M or less.

As to the inhibitory mechanisms involved, HMN-709 inhibits CaM kinase I competitively with respect to CaM and non-competitively with respect to ATP (Fig. 3). In addition, it binds to CaM in a Ca\(^{2+}\)-dependent manner as most CaM antagonists do (Fig. 4). These results indicate that the binding of HMN-709 to Ca\(^{2+}\)/CaM inhibits the Ca\(^{2+}\)/CaM-dependent activation of its related enzymes. In preliminary experiments, a 100,000 \(\times\)g supernatant from human platelet homogenates was screened by affinity chromatography with HMN-709-coupled Sepharose HP (data not shown). A specific polypeptide, which was retained in the column in the presence of Ca\(^{2+}\) and eluted with EGTA, showed a molecular mass of 22 kDa on SDS-PAGE and was stable upon heating to 90°C for 5 min. This polypeptide activated smMLCK in a Ca\(^{2+}\)-dependent manner. No polypeptide was eluted from this column with EGTA plus 6 M urea. These results suggest that the polypeptide is CaM and that HMN-709 binds selectively to CaM, in a Ca\(^{2+}\)-dependent manner, even in a crude extract of platelets.

The stimulation of platelets with collagen generates the phosphorylation of an endogenous polypeptide of 20 kDa (MLC) (39). This phosphorylation increases the activity of actin-activated myosin ATPase (40), and the resultant contraction of actomyosin mediates platelet activation such as aggregation and secretion (41). A pharmacological study by using W-7 suggests that CaM is involved in the phosphorylation of MLC in the activated platelets (16). Ca\(^{2+}\)/CaM can activate platelet MLC and the activated enzyme probably phosphorylates MLC (42). This scenario is supported by the evidence that ML-9, a selective MLCK inhibitor, inhibits collagen-induced platelet aggregation, 5-HT secretion, and the phosphorylation of MLC (43). Attention should also be paid to other CaM-dependent enzymes, Ca\(^{2+}\)-PDE and plasma membrane Ca\(^{2+}\) ATPase. These previous studies all support the importance of CaM in collagen-induced platelet activation. Conversely, phorbol ester directly activates protein kinase C, and it then phosphorylates a 40-kDa polypeptide, after which platelets aggregate (44). It is also documented that protein kinase C negatively modulates platelet aggregation by the phosphorylation of sites in MLC distinct from those targeted by MLCK (45). Clearly, the role of protein kinase C in platelet aggregation remains controversial. HMN-709 inhibited collagen-induced platelet aggregation in a dose-dependent manner (IC\(_{50}\) of 11.80 \(\pm\)0.86 \(\mu\)M) without affecting PDBu-induced aggregation (Fig. 5: a and b). This suggests that HMN-709 serves as a CaM antagonist and selectively inhibits the Ca\(^{2+}\)/CaM-dependent processes in platelets.

HMN-709 has been shown to be a potent and selective CaM antagonist. The involvement of CaM in cellular functions such as the expression of growth-related genes and cell cycle progression has recently been elucidated. HMN-709 may thus be useful for studying CaM further.

Acknowledgments
We thank Drs. A.C. Nairn and M.R. Picciotto (The Rockefeller University, New York, NY, USA) for the generous gift of the cDNA of rat CaM kinase I. This work was supported by grant-in-aids for encouragement of young scientists 00000337 (to H.Y.) and for scientific research 06404019 (to H.H.) from the Ministry of Education, Science, Sports and Culture, Japan.

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