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A new and potent calmodulin antagonist, HF-2035, which inhibits vascular relaxation induced by nitric oxide synthase

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Abstract

HF-2035, 2-[*N*-(2-aminoethyl)-*N*-(2,4,5-trichlorobenzenesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine, was synthesized and its effects on calmodulin-dependent enzymes were investigated. HF-2035 inhibited calmodulin kinase I, calmodulin kinase II and myosin light-chain kinase with IC_{50} values of 1.3 μM, 1.6 μM and 68 μM, respectively. HF-2035 also inhibited the activity of recombinant rat neuronal nitric oxide synthase, one of the calmodulin-dependent enzymes, with a K_i of 0.78 μM. Partially purified nitric oxide synthase of rat brain was also inhibited by HF-2035 with an IC_{50} of 3.2 μM. Kinetic analysis indicated that this inhibitory effect of HF-2035 was competitive with respect to calmodulin. We examined the effects of HF-2035 on constitutive nitric oxide synthase in a bioassay using vascular strips of rabbit carotid artery with and without endothelium. HF-2035 inhibited acetylcholine- and calcium ionophore, A23187 (6*S*-[6α(2*S**,3*S**),8β(*R**),9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl] methyl]-4-benzoxazolecarboxylic acid)-induced relaxation of endothelium-intact strips with an ED_{50} of 1.5 ± 0.5 μM and 2.8 ± 1 μM, respectively. This compound, however, did not inhibit *N*-nitroso-*N*-morpholinoaminoacetonitrile (SIN-1A), an exogenous nitric oxide donor, -induced relaxation of endothelium-denuded strips. W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) inhibited acetylcholine-induced relaxation with an ED_{50} of 46 ± 7 μM, which was 30-fold less potent than HF-2035. HF-2035 was unable to inhibit the activity of the inducible form of nitric oxide synthase in isolated thoracic aorta of rat treated with *Escherichia coli* lipopolysaccharide. These findings suggest that HF-2035 is a new and potent calmodulin antagonist, and may be used as a mother compound to develop more selective inhibitors of constitutive nitric oxide synthase.

Keywords: HF-2035; W-7; Calmodulin antagonist; Vascular relaxation; Nitric oxide (NO) synthase inhibitor

1. Introduction

A variety of cellular functions are regulated by a family of related Ca^{2+}-binding proteins. Among these, calmodulin has attracted most attention since it is ubiquitous and activates a variety of enzymes, thereby exerting a pleiotropic effect on many cellular functions (Klee et al., 1980). There are many calmodulin-dependent enzymes such as protein kinases, phosphodiesterases, protein phosphatases, ATPase and nitric oxide synthases. The analysis of physiological functions of the calmodulin-dependent enzymes has been limited by the lack of highly specific inhibitors (Nairn and Picciotto, 1994). The calmodulin inhibitors, for example, W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), trifluoperazine or calmidazolium, will prevent activation of the calmodulin-dependent enzymes, but do not provide any specificity. Moreover, some inhibitors with poor penetration to the interior of cells are only of limited use (Stoclet et al., 1987). Evidence suggesting that various calmodulin-sensitive enzymes have different calmodulin binding sites (Klee and Vanaman, 1982) may provide us with an opportunity to develop agents that would inhibit selectively on a calmodulin-sensitive enzyme. Previously, we synthesized selective inhibitors of calmodulin kinase II such as KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (Tokumitsu et al., 1990) and KN-93, 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybezenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (Sumi et al., 1991). KN-62 exerts its inhibitory action through direct

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binding to the calmodulin binding site of the enzyme (Tokumitsu et al., 1990). KN-62 has been widely used in biological experiments for its selectivity and membrane permeability (Bading et al., 1993; Niki et al., 1993; Okazaki et al., 1994; Tsunoda et al., 1992).

Recently, nitric oxide synthase has received wide attention, since this enzyme is responsible for the production of nitric oxide. Nitric oxide is now generally accepted as a cell-signaling molecule participating in many physiological processes, such as regulation of vascular tone, platelet aggregation, neurotransmission and cytotoxic action of macrophages (Ignarro, 1991; Moncada et al., 1991; Lowenstein and Snyder, 1992; Nathan, 1992; Stamler et al., 1992). Three isoforms of nitric oxide synthase (isoforms I–III), which render many cells capable of synthesizing nitric oxide, have been described (Förstermann et al., 1994; Moncada and Higgs, 1993). Isoform I was classically found in neuronal cells, and isoform II is present in 'activated' macrophages, whereas isoform III exists in endothelial cells. Isoform I and III are present constitutively, but isoform II is inducible. The activity of isoforms I and III, but not II, is regulated by Ca^{2+} and calmodulin. A number of inhibitors of nitric oxide synthase with different pharmacological properties have been reported. Among these inhibitors, L-arginine-competitive inhibitors of nitric oxide synthase have been widely used as tools for elucidating the biological roles of nitric oxide (Furfine et al., 1993; Mayer et al., 1993; Narayanan and Griffith, 1994; Olken and Marletta, 1992, 1993). It will be beneficial to develop selective and potent inhibitors, because different isozymes play distinct roles in different tissues or biological systems. We now report on the pharmacological characterization of HF-2035, 2-[*N*-(2-aminoethyl)-*N*-(2,4,5-trichlorobenzenesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine, a newly synthesized benzenesulfonamide with toluidine structure (Fig. 1).

2. Materials and methods

2.1. Preparation of partially purified nitric oxide synthase

Sprague-Dawley rats (150–200 g) were killed by decapitation. Whole brains were quickly removed and frozen in liquid nitrogen. The frozen brains were homogenized in 5 volumes (w/v) of ice-cold homogenizing buffer containing 50 mM Tris/HCl (pH 7.8), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetate (EDTA), 1 mM *O,O'*-bis (2-aminoethyl) ethyleneglycol *N,N,N'-N'*-tetraacetic acid (EGTA), soybean trypsin inhibitor 10 mg/l and leupeptin 10 mg/l using a glass homogenizer with Teflon pestle and all subsequent procedures were carried out at 4°C. The homogenate was centrifuged at $10000 \times g$ for 60 min. The supernatant was loaded 2 ml min^{-1} onto diethylaminoethyl cellulose (Whatman DE 52) equilibrated with the above homogenizing buffer without leupeptin. The column was washed with equilibrating buffer for about 7 bed volumes of the column and eluted with 50 ml of a linear gradient of 0–0.5 M NaCl in homogenizing buffer. Active fractions were then collected and stored at −80°C, and employed as source for nitric oxide synthase.

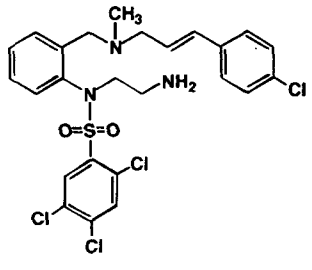

Fig. 1. Chemical structure of HF-2035.

2.2. Preparation of rat recombinant expressed nitric oxide synthase

Rat isoform I nitric oxide synthase cDNA was a kind gift from Dr. S.H. Snyder (Johns Hopkins University School of Medicine, Department of Neuroscience, Baltimore, MD, USA). A full-length open reading frame for the nitric oxide synthase was ligated into the baculovirus transfer vector pVL 1393 (Invitrogen, San Diego, CA, USA). Cultured Sf-9 cells were co-transfected with nitric oxide synthase cDNA/transfer vector and wild-type baculovirus DNA. Nitric oxide synthase-recombinant baculovirus were plaque-purified to homogeneity. Sf-9 cells were maintained as suspension cultures at 27°C. For expression studies, static cultures of Sf-9 cells were infected with nitric oxide synthase-recombinant baculovirus at 3 pfu/cell and grown for 36 h at 27°C as suspension culture in TNM-FH medium (Grace's insect medium containing 3.33 g/l lactalbumin hydrolysate and 3.33 g/l yeastolate) containing 10% FBS. The Sf-9 cell suspension cultures were harvested by centrifugation. The cell pellet was homogenized in 5 volumes of ice-cold buffer (50 mM Tris-HCl, pH 7.5 containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride) and centrifuged at $10000 \times g$ for 60 min at 4°C. The resultant supernatant was applied to 2',5'-ADP agarose affinity column. The column was washed with 0.5 M NaCl and eluted with 20 mM NADPH. Glycerol 20% was added to the eluate and kept at −80°C.

2.3. Determination of partially purified rat brain nitric oxide synthase or rat isoform I recombinant expressed constitutive nitric oxide synthase activity

Nitric oxide synthase activity was determined by conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (Bredt and

Snyder, 1990). Unless otherwise indicated, the standard reaction mixture contained 50 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 100 μM NADPH, 100 μM BH_4, 100 nM calmodulin, 3 μM L-arginine, approximately 170000–250 000 dpm of L-[2,3,4,5-^{3}H]arginine-HCl (40–70 Ci/mmol), and 20 μl of partially purified enzyme extract in a final incubation volume of 140 μl or 100 nM of recombinant constitutive nitric oxide synthase in a final incubation volume of 20 μl. Enzyme reactions were carried out at 37°C for 30 min then stopped with the addition of 800 μl and 180 μl Stop Buffer (20 mM sodium acetate, pH 5.5, 2 mM EDTA, 0.2 mM EGTA and 1 mM L-citrulline) for partially purified rat brain nitric oxide synthase and rat isoform I recombinant constitutive nitric oxide synthase, respectively. The reaction mixture generated above was applied over 1 ml of Dowex AG50W-X8, Na^+ form, 100–200 mesh which was pre-equilibrated with Stop Buffer. The eluate was collected in scintillation vials. Columns were eluted with an additional 1.6 ml of Stop Buffer. To each vial 6 ml of scintillation liquid was added prior to counting in a liquid scintillation spectrometer (Aloka, LSC 5100).

2.4. Kinase assay

Myosin light-chain kinase activity was measured under the conditions described by Kemp et al. (1987). Calmodulin kinase II activity was measured as described by Tokumitsu et al. (1990). The kinase activity of calmodulin kinase I was measured in a mixture (50 μl) containing 35 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.01% Tween 20, 0.5 mM $CaCl_2$, 50 μM syntide II, 9.6 μg/ml GST-calmodulin kinase I, 100 nM calmodulin, and 100 μM [γ-^{32}P]ATP (400 cpm/pmol) at 30°C for 5 min. The reaction mixture was spotted onto P-81 paper, followed by washing the paper in 75 mM phosphoric acid, as described by Yokokura et al. (1995).

2.5. Preparation of rabbit carotid arterial strips

Male rabbits weighing 2.5–4 kg were exsanguinated under anaesthesia with sodium pentobarbital (30 mg kg^{-1} intravenously). The internal carotid arteries were cleared of adherent loose connective tissue, cut into 3-mm rings and cut open longitudinally for tension recording. In another set of experiments, the endothelium was removed by gentle mechanical rubbing of the endothelial surface with a small, fine brush. The failure of acetylcholine (1 μM) to induce relaxation of strips was taken as an indication of the absence of a functional endothelium.

2.6. Preparation of rat thoracic aorta strips

The preparation was done according to Auguet et al. (1991). Male Sprague Dawley rats (150–200 g) were intraperitoneally injected with bacteria endotoxin lipopolysaccharide (10 mg kg^{-1}) or with solvent (saline, 1 ml kg^{-1}). Three hours later the endotoxin-treated animals displayed the signs of endotoxaemia including piloerection, diarrhoea, and lethargy. The rats were killed 6 h later by decapitation after anaesthesia with diethyl ether. The thoracic aorta was taken out and prepared as above.

2.7. Recording of mechanical response

The rabbit internal carotid vascular strips were vertically suspended in 20-ml organ baths filled with modified Krebs-Henseleit solution containing (mM): NaCl, 115; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and *d*-glucose, 10; ethylenediamine tetraacetic acid disodium salt (EDTA), 0.016 M. Mechanical activity was recorded isometrically by means of a force-displacement transducer (TB-612T; Nihon Kohden Kogyo Co., Tokyo, Japan). A tension of approximately 1 g was applied and the strips were equilibrated (60–90 min), adjusting the preload to 1 g every 30 min. The organ baths were kept at 37°C, and filled with warm (37°C) and oxygenated (95% O_2-5% CO_2) Krebs-Henseleit solution (pH 7.4) which was changed every 30 min. After equilibration, the strips were contracted with phenylephrine (1 μM), and acetylcholine (1 μM) was added to the baths to test the functional integrity of the endothelium. Only tissues which relaxed by more than 70% of the phenylephrine-induced tone after addition of acetylcholine were considered to have undamaged endothelium. Acetylcholine (1 μM) did not change the tone of endothelium-denuded strips. After extensive washing with fresh buffer, the strips were contracted with phenylephrine (1 μM). Before the start of the phenylephrine (1 μM)-induced contraction, indomethacin (10 μM) was added to prevent the production of endogenous vasoactive prostanoids. Once the plateau of contraction was obtained, the relaxant responses to cumulative addition of acetylcholine (1 nM–10 μM) or calcium ionophore, A23187 (6*S*-[6α(2*S**,3*S**),8β(*R**),9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl] methyl]-4-benzoxazolecarboxylic acid) (0.1 nM–1 μM) and *N*-nitroso-*N*-morpholinoaminoacetonitrile (SIN-1A), an exogenous donor of nitric oxide (1 nM–10 μM), were measured in endothelium-intact strips and endothelium-denuded strips, respectively. To examine the effect of HF-2035, or W-7, one of the calmodulin antagonist (Hidaka et al., 1978), on the vascular relaxation, the strips were treated with HF-2035 (1 μM, 3 μM, 10 μM), or W-7 (10 μM, 30 μM, 100 μM) for 30 min before acetylcholine, calcium ionophore, A23187 or SIN-1A-induced dose-dependent relaxation.

The endotoxin-shocked rat thoracic aorta strips without endothelium were contracted with phenylephrine (1 μM), followed by addition of HF-2035 (1, 3, 10 μM) or aminoguanidine (300 μM). When the responses reached a steady stage, L-arginine (1 mM) was finally added to the organ bath to examine whether the activity was that of

inducible nitric oxide synthase, which requires L-arginine as substrate.

2.8. Statistical analysis

The results are expressed as means ± S.E. of the number (n) of observations. In organ bath experiments, n is the number of arterial strips used in a particular protocol with separate animals. Statistical differences between means were assessed by using one-factor analysis of variance (ANOVA). A P value less than 0.05 was considered to be statistically significant.

2.9. Drugs

HF-2035 and other benzenesulfonamide derivatives with a toluidine structure were prepared by a modification of the method previously described by Hidaka et al. (1984). NADPH, L-citrulline, L-arginine were bought from Sigma; (6*R*)-5,6,7,8-tetrahydrobiopterin from RBI (Research Biochemicals International). AG 50W-X8 cation-exchange resin (Bio-Rad); NACS204 high flash-point scintillation cocktail (Amersham); [^{3}H]L-arginine (Amersham); and lipopolysaccharide from *Escherichia coli*, 0111: B4 (Sigma). *N*-Nitroso-*N*-morpholinoaminoacetonitrile (SIN-1A) was kindly donated by Takeda Chemical Ind. (Osaka, Japan). Other chemicals were purchased and were of the highest grade available.

3. Results

3.1. Effect of HF-2035 on nitric oxide synthase activity in vitro

Using a partially purified nitric oxide synthase and recombinant expressed nitric oxide synthase, we investigated the nature of the inhibitory activity of HF-2035. HF-2035 inhibited the nitric oxide synthase activity of both preparations in a dose-dependent manner (Fig. 2). HF-2035 inhibited partially purified nitric oxide synthase of rat brain with an IC_{50} of 3.2 μM, and inhibited the activity of recombinant rat neuronal nitric oxide synthase with an IC_{50} of 1.99 μM. The inhibitory actions were not affected by various concentrations of L-arginine (data not shown). From the double-reciprocal plots shown in Fig. 3, HF-2035 was found to be a competitive inhibitor of calmodulin. Replots of the slope of the double-reciprocal plot versus the corresponding concentration of HF-2035 gave a K_i value of 0.78 μM.

3.2. Effect of HF-2035 on calmodulin-dependent kinases in vitro

The effects of HF-2035 on the phosphorylation activity of calmodulin-dependent kinases were examined. HF-2035 inhibited the activity of calmodulin kinase I with an IC_{50} of 1.3 μM (Table 1), and a K_i of 1.1 μM which was obtained from replots of the slope of double-reciprocal plot versus the corresponding concentration of HF-2035. HF-2035 also inhibited calmodulin kinase II and myosin light-chain kinase with IC_{50} values of 1.6 μM and 68 μM, respectively.

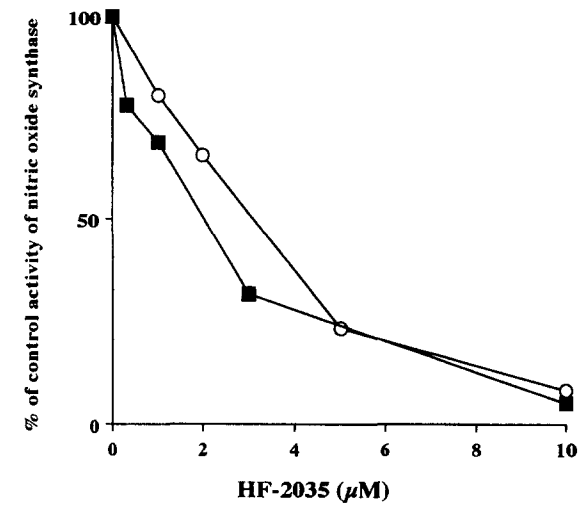

Fig. 2. Effect of HF-2035 on recombinant rat constitutive nitric oxide synthase and partially purified nitric oxide synthase. Partially purified rat brain constitutive nitric oxide synthase (○), and rat brain recombinant-expressed nitric oxide synthase (■) activities were measured in the presence of 100 nM nitric oxide synthase, 2 mM $CaCl_2$, 100 nM NADPH, 100 nM calmodulin, 100 nM BH_4, 3 μM L-arginine, 50 mM Tris-HCl, pH 7.6 and various concentrations of HF-2035 as shown. The reaction was initiated by adding [^{3}H]L-arginine (170000–250000 dpm). The nitric oxide synthase activity without addition of HF-2035 was taken as control activity (100%).

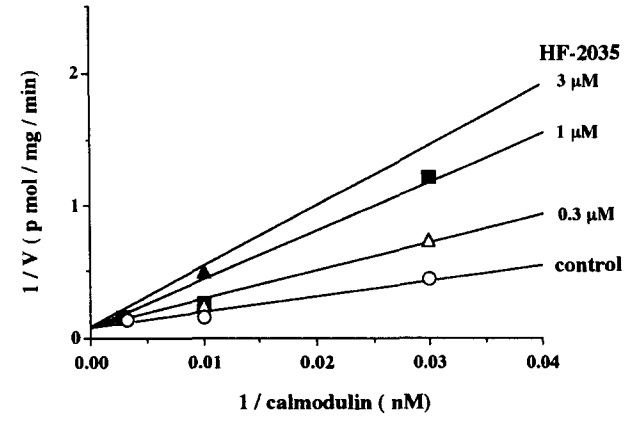

Fig. 3. Double reciprocal plots of inhibition of nitric oxide synthase activity by HF-2035. Rat brain constitutive nitric oxide synthase activity was measured in the presence of 3 μM L-arginine, 100 nM nitric oxide synthase, 2 mM $CaCl_2$, 100 nM NADPH, 100 nM BH_4, 50 mM Tris-HCl, pH 7.6 and various concentrations of calmodulin (30, 100, and 300 nM). The reaction was initiated by adding [^{3}H]L-arginine (170000–250000 dpm). Data are expressed as $(pmol/mg/min)^{-1}$ versus nM $calmodulin^{-1}$ in the absence (○) and presence of 0.3 μM (△), 1 μM (■) and 3 μM HF-2035 (▲).

Table 1
Pharmacological effects of HF-2035 and W-7

	IC_{50} (μM)				ED_{50} (μM)
	NOS	KI	KII	MLCK	Vascular relax.
HF-2035	1.99	1.3	1.6	68	1.5 ± 0.5
W-7	58.4	30	15	36.7	46 ± 7

Effects of HF-2035 and W-7 on the calmodulin-dependent enzymes were compared in vitro and in vivo. NOS (constitutive nitric oxide synthase, isoform I); KI (calmodulin kinase I); KII (calmodulin kinase II); MLCK (myosin light-chain kinase); and vascular relax. means the inhibitory effect on acetylcholine-induced vascular relaxation.

3.3. Organ bath experiments

3.3.1. Effect of HF-2035 on acetylcholine-, calcium ionophore A23187-, and SIN-1A-induced relaxation of rabbit carotid artery with and without endothelium

Acetylcholine (1 nM–10 μM) caused a concentration-dependent relaxation which is known to be dependent on endothelial generation of nitric oxide. Acetylcholine-induced relaxation was inhibited by increasing concentrations of HF-2035 (1, 3, 10 μM), with an IC_{50} of 1.5 ± 0.5 μM (Fig. 4). Maximum inhibition was observed at 3 μM and increases in concentration to 10 μM did not produce additional inhibition of the acetylcholine-induced relaxation. HF-2035 (1, 3, 10 μM) also inhibited the endothelium-dependent relaxation by the calcium ionophore A23187 (0.1 nM–1 μM), a receptor-independent activator of endothelial nitric oxide biosynthesis (Fig. 5). Two hours' washing of HF-2035-treated strips with the Krebs-Henseleit buffer restored the vasorelaxant activity of acetylcholine or the calcium ionophore A23187 (data not shown). HF-2035 did not block the relaxation of endothelium-denuded strips induced by *N*-nitroso-*N*-morpholinoaminoacetonitrile (SIN-1A) (1 nM–10 μM), an exogenous donor of nitric oxide (Fig. 6).

3.3.2. Effect of HF-2035 on the thoracic aorta from lipopolysaccharide-injected rats

In order to verify the effect of HF-2035 on inducible nitric oxide synthase induction, we measured its activity in a bioassay. We could detect inducible nitric oxide synthase activity by monitoring the tension of isolated rat aorta. Inducible nitric oxide synthase in rat vascular smooth muscle was induced by *E. coli* lipopolysaccharide 6 h before preparation. Lipopolysaccharide-treated endothelium-denuded aortic preparations, in which inducible nitric oxide synthase was expressed, produced extremely small responses to phenylephrine (1 μM) compared with those from non-treated rats. In the presence of phenylephrine (1 μM), aminoguanidine, but not HF-2035 produced a significant vascular contraction of the vascular strips. L-Arginine reversed aminoguanidine-induced contraction and reversed the tone developed with phenylephrine.

3.4. Comparison of the effects of HF-2035 and W-7 on calmodulin-dependent kinases (Table 1)

HF-2035 inhibited constitutive nitric oxide synthase, calmodulin kinase I, calmodulin kinase II and myosin light-chain kinase with approximately equal potency except for myosin light-chain kinase. W-7 inhibited the activity of constitutive nitric oxide synthase, calmodulin kinase I, calmodulin kinase II and myosin light-chain kinase with IC_{50} values of 58.4 μM, 30 μM, 15 μM and 36.7 μM, respectively. The inhibitory activity of W-7 was inferior to that of HF-2035 for the calmodulin-dependent

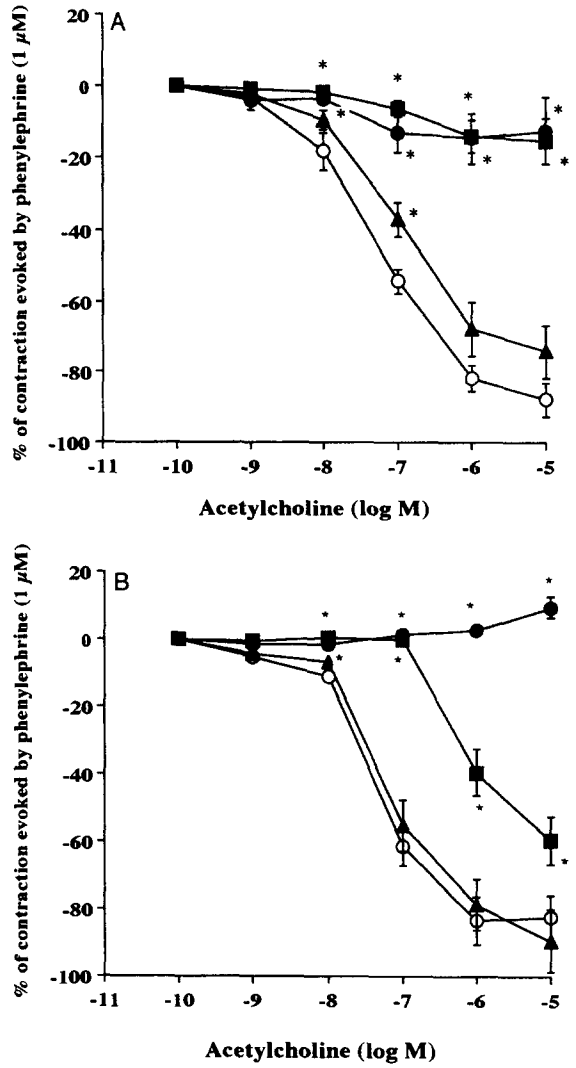

Fig. 4. Effect of (A) HF-2035 and (B) W-7 on acetylcholine-induced relaxation of rabbit carotid artery with endothelium. Acetylcholine (1 nM–10 μM) caused a concentration-dependent relaxation of endothelium-intact strips of rabbit carotid artery (○). The inhibition of acetylcholine-induced relaxation was observed (A) in the presence of 1 μM (▲), 3 μM (■) and 10 μM HF-2035 (●) or (B) in the presence of 10 μM (▲), 30 μM (■) and 100 μM W-7 (●). Values represent means ± S.E. (vertical bar) of the number (n) of separate experiments. Asterisks indicate significant differences from the control ($P < 0.0065$–0.0001, $n = 5$–9).

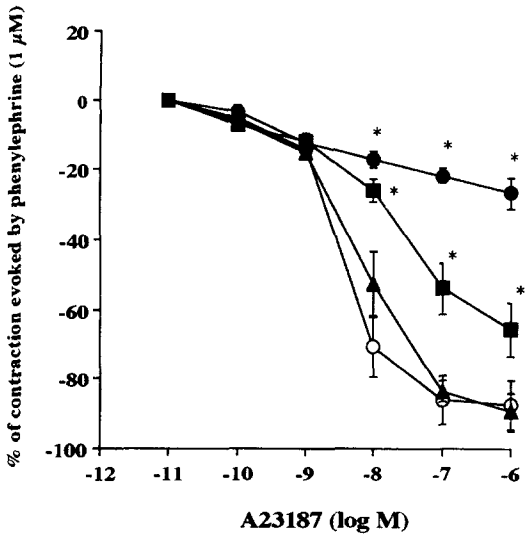

Fig. 5. Effect of HF-2035 on the calcium ionophore A23187-induced relaxation of rabbit carotid artery with endothelium. Calcium ionophore A23187 (0.1 nM–1 μM) caused a concentration-dependent relaxation of endothelium-intact strips of rabbit carotid artery (○). Calcium ionophore A23187-induced relaxation was inhibited by increasing concentrations of 1 μM (▲), 3 μM (■) and 10 μM (●) HF-2035. Values represent means ± S.E. (vertical bar) of the number (n) of separate experiments. Asterisks indicate significant differences from the control ($P < 0.0002$–0.0001, $n = 7$).

enzymes tested except for myosin light-chain kinase. Among these, the potency of HF-2035 was highest for constitutive nitric oxide synthase when compared with W-7, whereas the potency of HF-2035 was lower than that of W-7 for the inhibition of myosin light-chain kinase.

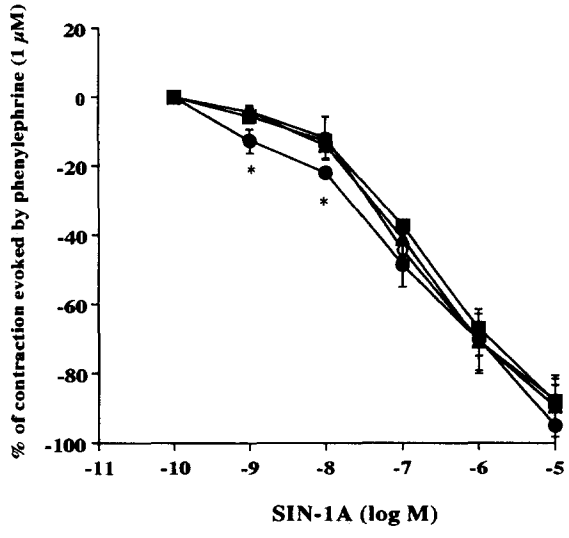

Fig. 6. Effect of HF-2035 on SIN-1A-induced relaxation of rabbit carotid artery without endothelium. SIN-1A (1 nM–10 μM) caused a concentration-dependent relaxation of endothelium-denuded strips of rabbit carotid artery (○). HF-2035 did not affect the relaxant responses induced by SIN-1A: 1 μM (▲), 3 μM (■) and 10 μM (●) HF-2035. Values represent means ± S.E. (vertical bar) of the number (n) of separate experiments. Asterisks indicate significant differences from the control ($P < 0.045$, $n = 8$).

4. Discussion

The present study demonstrated that HF-2035, a derivative of KN-93, potently inhibited constitutive nitric oxide synthases both in a bioassay and in vitro. HF-2035 inhibited not only constitutive nitric oxide synthase activity but also other calmodulin-dependent kinases, such as calmodulin kinase I, calmodulin kinase II, and myosin light-chain kinase (Table 1). The inhibitory effect of HF-2035 appears to be mediated through a calmodulin antagonist action (Fig. 3). These findings suggest that HF-2035 is a very potent calmodulin antagonist. When we compared HF-2035 to W-7 for potency and selectivity, we found that the inhibitory property of HF-2035 was superior to that of W-7 on nitric oxide synthase, calmodulin kinase I and calmodulin kinase II, whereas the activity of myosin light-chain kinase was inhibited very weakly by HF-2035. These observations suggest that HF-2035 has some selectivity on calmodulin-dependent enzymes. Moreover, ratios of the IC_{50} values of the two compounds on the four calmodulin-dependent enzymes suggest that HF-2035 preferentially inhibits nitric oxide synthase (Table 1). The K_i values of HF-2035 for nitric oxide synthase and calmodulin kinase I are also in agreement with the above conclusion that the effect of HF-2035 is relatively more potent on nitric oxide synthase. The inhibitory effect on nitric oxide synthase was not observed with KN-62 or KN-93 which is the mother compound of HF-2035 (unpublished observation). This finding suggests that HF-2035 is a distinct compound.

We further examined the pharmacological effects of HF-2035 on isoforms of nitric oxide synthase in a bioassay using vascular smooth muscle with and without endothelium. In strips of rabbit carotid artery with intact endothelium, HF-2035 blocked the vasorelaxant action of acetylcholine which generates nitric oxide in endothelial cells. The inhibitory action of HF-2035 was not due to the inhibition of the transduction mechanism of acetylcholine at the receptor, because HF-2035 also blocked the vasorelaxant action of the calcium ionophore A23187, a receptor-independent activator of nitric oxide synthase in endothelial cells. Moreover, in strips denuded of endothelium, HF-2035 did not inhibit the relaxation induced by SIN-1A, an exogenous donor of nitric oxide, independently of nitric oxide synthase, showing that the action of HF-2035 was not due to the inhibition of the vasorelaxant action of nitric oxide in vascular smooth muscle. Collectively, these results suggest strongly that, in the rabbit carotid artery, HF-2035 blocks the endothelial biosynthesis of nitric oxide. In addition, acetylcholine- or calcium ionophore A23187-induced relaxation was regained when HF 2035 had been thoroughly washed out with fresh buffer, suggesting that the effect of HF-2035 is reversible. W-7 abolished acetylcholine-induced vascular relaxation, and the concentration this required was 30-fold higher than that required for HF-2035 to produce the same effect (Fig.

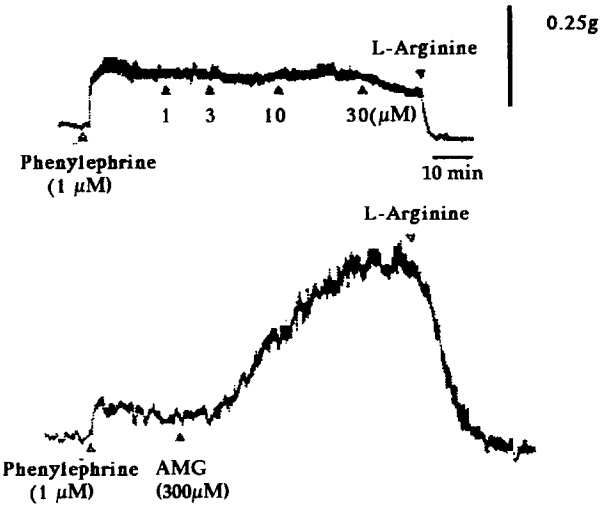

Fig. 7. Effect of HF-2035 on inducible nitric oxide synthase in rat thoracic aorta without endothelium. Reduced tonicity of the phenylephrine (1 μM)-induced contraction was observed in lipopolysaccharide-treated preparations. Aminoguanidine (AMG) or HF-2035 was added to the bath 60 min after phenylephrine. The different effects of aminoguanidine and HF-2035 were observed on reversal of the decrease in vascular responsiveness to phenylephrine in endothelium-free strips of endotoxin-shocked rats. The reversing effect of L-arginine was also observed. The same results were observed on at least three different animals.

4A and B). These results suggest that HF-2035 has similar potency both in a bioassay and in in vitro experiments concerning nitric oxide synthase activity.

Another isoform of nitric oxide synthase, isoform II, is induced in vascular smooth muscle by lipopolysaccharide. With lipopolysaccharide treatment, hyporeactivity to phenylephrine of the aortic strips without endothelium was observed (Fig. 7). This may have been due to sustained production of nitric oxide in smooth muscle by inducible nitric oxide synthase, since aminoguanidine, the selective inhibitor for inducible nitric oxide synthase (Misko et al., 1993), potentiated phenylephrine-induced vasocontraction of the lipopolysaccharide-treated strips. Such potentiation was not observed with HF-2035. Thus, we confirmed the calmodulin-dependent action of HF-2035 on nitric oxide synthase by the lack of its effect on isoform II which is independent of Ca^{2+}/calmodulin. In the present study, we did not use other calmodulin antagonists such as trifluoperazine and fendiline because Illiano et al. (1992) reported that the experiments could not be performed with these drugs at concentrations high enough to inhibit calmodulin activity without deeply affecting vascular tone in canine coronary artery.

According to these observations from the present study, we propose that biological study of nitric oxide synthase can be carried out with HF-2035, which is membrane permeable. Inhibition of nitric oxide synthesis in vivo has begun to reveal the extent of its physiological roles. Ongoing investigations and screening of drugs derived from HF-2035 should provide a clue to discover a potent and selective nitric oxide synthase inhibitor which binds directly to the calmodulin binding site of the enzyme.

In conclusion, our results suggest that HF-2035 may be used as a potent inhibitor of endothelial constitutive nitric oxide synthase and neuronal constitutive nitric oxide synthase. The inhibitory action of HF-2035 is competitive with that of calmodulin which is essential for the catalytic activity of the enzyme.

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