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INTERACTION OF THE DIHYDROPYRIDINE CALCIUM ANTAGONIST, CD-349, WITH CALMODULIN

MAKOTO TANAKA,* MAKOTO MURAMATSU, HIRONAKA AIHARA and SUSUMU OTOMO
Research Center, Taisho Pharmaceutical Co., Ltd., Saitama 330, Japan

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Abstract—The characteristics of the binding of the 1,4-dihydropyridine Ca^{2+} antagonist, 2-nitratopropyl 3-nitratopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine 3,5-dicarboxylate (CD-349), to calmodulin (CaM) and the effect of CD-349 on the Ca^{2+}/CaM-dependent enzyme, cyclic GMP (cGMP) phosphodiesterase (PDE), were investigated. CD-349 showed a Ca^{2+}-dependent binding to CaM, in equilibrium column binding studies. CD-349 inhibited the [^{3}H]CD-349 binding to CaM, at a concentration producing a 50% inhibition (IC_{50}) of 2.4 μM, whereas the CaM antagonist, trifluoperazine hydrochloride (TFP), stimulated the [^{3}H]CD-349 binding to CaM. Scatchard plot analysis of the binding of CD-349 to CaM revealed that the apparent dissociation constant (K_{app}) of CD-349 was 2.1 μM and the maximal number of binding sites (B_{max}) of CD-349 was 1.0 nmol/nmol CaM. In the presence of TFP, the K_{app} and B_{max} values of CD-349 binding to CaM were changed to 1.1 μM and 1.5 nmol/nmol CaM respectively. Although the CaM antagonists, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) and TFP, decreased and increased, respectively, the fluorescence intensity caused by 2-*p*-toluidinylnaphthalene-6-sulfonic acid (TNS)–CaM binding, CD-349 only slightly decreased the fluorescence of TNS bound CaM. CD-349 inhibited both basal and Ca^{2+}/CaM-activated cGMP PDE activity. However, CaM did not competitively antagonize the CD-349-induced inhibition of the Ca^{2+}/CaM-activated PDE activity. In addition, the kinetic study showed that CD-349 inhibited both basal and Ca^{2+}/CaM-activated cGMP PDE activity, competitively with cGMP, with almost the same inhibition constant (K_i). These results suggest that CD-349 binds to CaM, with Ca^{2+} dependency, at sites differing from those which bind to the CaM antagonist. The inhibitory activity of CD-349 on Ca^{2+}/CaM-dependent PDE does not seem to be due to a CaM antagonistic action.

It is now well established that chemically diverse groups of drugs, Ca^{2+} antagonists, block excitation–contraction coupling in cardiac and smooth muscle, possibly by inhibiting the slow channel mediated transmembrane Ca^{2+} influx into the cytoplasm [1, 2]. Biochemical actions of Ca^{2+}, in general, have been shown to be mediated by calmodulin (CaM)†, a Ca^{2+}-binding protein of ubiquitous occurrence, on smooth muscle contraction. This protein has been found to modulate the action of many enzymes after binding with and being activated by Ca^{2+} [3, 4]. CaM is an integral part of the contraction apparatus in smooth muscle and is responsible for the Ca^{2+}-dependent activation of myosin light chain kinase [5–7]. Many investigators reported that some Ca^{2+} antagonists act as CaM antagonists and are capable of binding and inhibiting CaM actions [8–14]. These Ca^{2+} antagonists may exert vasodilative actions mainly by blocking the Ca^{2+} influx and, in addition, through an intracellular mechanism such as the inhibition of the CaM-mediated process.

We found that our newly synthesized 1,4-dihydropyridine Ca^{2+} antagonist, 2-nitratopropyl 3-nitratopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine 3,5-dicarboxylate (CD-349), inhibits cyclic nucleotide phosphodiesterase (PDE) activity in the porcine coronary artery and bovine intrapulmonary blood vessels [15, 16]. This action was more remarkable in the presence of Ca^{2+}. The Ca^{2+} dependency of the inhibitory action of CD-349 may be caused by interaction between CD-349 and CaM since PDE is a Ca^{2+}/CaM-activatable enzyme.

We have now investigated interactions between CD-349 and CaM by making use of binding studies, and the inhibitory effect of CD-349 on the Ca^{2+}/CaM-dependent enzyme PDE was given attention.

METHODS

Materials. CD-349 was synthesized in the Research Center of the Taisho Pharmaceutical Co., Ltd. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), trifluoperazine hydrochloride (TFP), 2-*p*-toluidinylnaphthalene-6-sulfonic acid potassium salt (TNS), CaM-deficient cyclic nucleotide PDE from bovine brain, snake venom (*Crotalus atrox*) and Dowex 1×8 (chloride form) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]CD-349 (28 Ci/mmol) was synthesized by the Amersham Corp. (Arlington Heights, IL, U.S.A.). [^{3}H]Cyclic GMP (cGMP) (33.3 Ci/mmol) was purchased from New England

* Correspondence: Dr. Makoto Tanaka, Department of Pharmacology, Research Center, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Ohmiya, Saitama 330, Japan.

†Abbreviations: CaM, calmodulin; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; TFP, trifluoperazine hydrochloride; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid potassium salt; cGMP, cyclic GMP; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; K_{app}, apparent dissociation constant; B_{max}, maximal number of binding sites; and CD-349, 2-nitratopropyl 3-nitratopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine 3,5-dicarboxylate.

Nuclear (Boston, MA, U.S.A.). CaM from bovine brain and Sephadex G-50 superfine were purchased from the Wako Pure Chemical Co. (Osaka, Japan) and Pharmacia Fine Chemicals (Uppsala, Sweden) respectively.

Equilibrium binding. The binding of [^{3}H]CD-349 to CaM was determined by the gel permeation binding technique of Hummel and Dreyer [17] as adopted by Hidaka *et al.* [6]. CaM (180 μg) was applied to a Sephadex G-50 superfine column (1.0 × 23 cm) pre-equilibrated with buffer containing 20 mM Tris/HCl (pH 7.5), 20 mM imidazole, 3 mM $Mg(CH_3COO)_2$, 5% dimethyl sulfoxide (DMSO), and either 0.1 mM $CaCl_2$ or 2 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) with a definite concentration of CD-349 containing [^{3}H]CD-349 in trace amounts and at room temperature. The concentration of CD-349 after equilibrium was about 70% of the initial addition due to the adsorption of CD-349 to the Sephadex gel. Thus, the actual concentrations of CD-349 were calculated from radioactivity in the eluate, after equilibrium. The fraction volume and elution speed was 1.0 mL and 12 mL/hr respectively. After precipitation of CaM in 0.2-mL aliquots of each fraction by adding an equal volume of 20% trichloroacetic acid, CaM concentration was determined by measuring the protein concentration, according to Lowry *et al.* [18] with CaM as the standard. Radioactivity in 0.5-mL aliquots of each fraction was measured using a liquid scintillation spectrometer (TRI-CARB 4430, Packard Instrument Co., Downers Grove, IL, U.S.A.) in 10 mL of PCS Scintillator (Amersham Corp.).

Fluorescence measurement. Fluorescence measurements were carried out according to Tanaka and Hidaka [19], using a fluorescence spectrophotometer (RF-540, Shimadzu Corp., Kyoto, Japan). The reaction medium consisted of 50 mM Tris/HCl (pH 7.5), 10 μM $CaCl_2$, 10 μM CaM, 15 μM TNS, and drugs as indicated, in a total volume of 1.0 mL. Incubation was carried out for 60 min at 30° for purposes of equilibration. Emission intensity was measured at the wavelength from 380 to 550 nm, with excitation at 365 nm. CD-349 was dissolved in DMSO, and the experiments was carried out in the presence of 0.5% DMSO.

Assay procedure for cyclic nucleotide PDE activity. PDE activity was measured by the method of Beavo *et al.* [20] as described [15, 16]. The reaction was carried out at 37° for 2 min in medium (0.5 mL) containing 30 mM Tris/HCl (pH 7.5), 10 μM $CaCl_2$, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 M NaCl, 1 μM [^{3}H]cGMP, 50 μg bovine serum albumin (BSA), 0.004 units of CaM-deficient cyclic nucleotide PDE and 2.5 units CaM, as indicated. The reaction was terminated by boiling the tubes for 2 min. The [^{3}H]5'-GMP product was converted to [^{3}H]guanosine by additional incubation at 37° for 30 min with 50 μg of snake venom (*C. atrox*), as 5'-nucleotidase. Following the addition of carrier guanosine, the total contents of the tube were applied onto a Dowex 1×8 column (0.6 × 2.0 cm), guanosine was eluted with 8 mL of 0.1 M Tris/HCl (pH 7.5), and radioactivity in 1.0-mL aliquots was counted as described above.

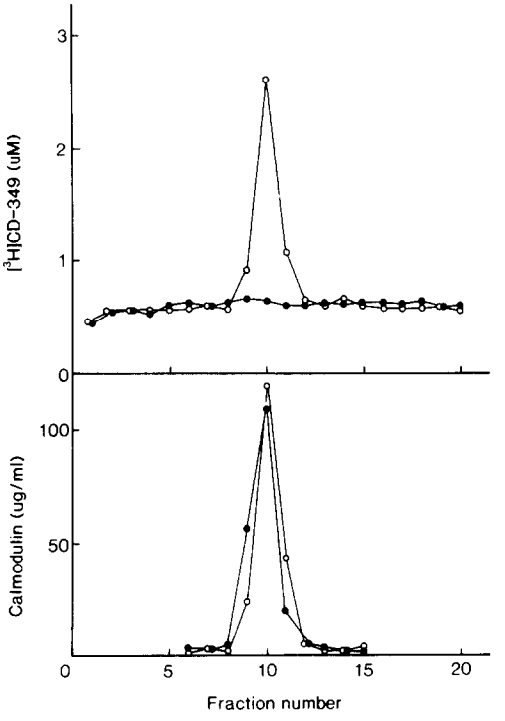

Fig. 1. Calcium-dependent [^{3}H]CD-349 binding to CaM. A Sephadex G-50 superfine column was pre-equilibrated with buffer containing either 0.1 mM Ca^{2+} (○) or 2 mM EGTA (●) and 0.5 μM [^{3}H]CD-349 as described in Methods. A typical chromatogram of the binding of [^{3}H]CD-349 to CaM is shown.

Data analysis. Saturation binding data were analyzed by Scatchard plots, and the apparent dissociation constant (K_{app}) and the maximal number of binding sites (B_{max}) were determined using a computer-assisted program, SP123, developed by Dr. H. Ono of The University of Tokyo for the PC-9801 (NEC, Tokyo, Japan) pesonal computer.

RESULTS

Equilibrium binding between [^{3}H]CD-349 and CaM. The binding of CD-349 to CaM was investigated using the equilibrium binding technique. The column was pre-equilibrated with buffer containing 0.5 μM [^{3}H]CD-349. Figure 1 illustrates a typical chromatogram of the binding of [^{3}H]CD-349 to CaM. Following the application of CaM to a column pre-equilibrated with a Ca^{2+}-containing (0.1 mM) buffer, the peak radioactivity of [^{3}H]CD-349 coincided with that of CaM. On the other hand, CaM applied to the column pre-equilibrated with a buffer in which EGTA (2 mM) replaced Ca^{2+} did not bind [^{3}H]CD-349. Since EGTA inhibited the formation of the CD-349–CaM complex, the binding seems to be Ca^{2+} dependent.

Effects of CD-349 and TFP on [^{3}H]CD-349 binding to CaM. [^{3}H]CD-349 binding to CaM was concentration-dependently inhibited by CD-349. The concentration producing 50% inhibition (IC_{50}) of

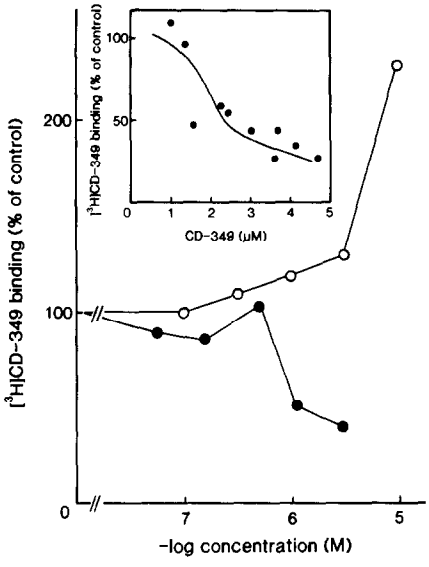

Fig. 2. Effects of CD-349 and TFP on [^{3}H]CD-349 binding to CaM. A Sephadex G-50 superfine column was pre-equilibrated with buffer containing 0.1 mM Ca^{2+}, 0.5 μM [^{3}H]CD-349 and non-labeled CD-349 (●) or TFP (○). The concentration of actual [^{3}H]CD-349 was calculated after equilibration. Data are from one experiment, done in duplicate. The inset also shows the CD-349 displacement curve in which the concentrations of CD-349 are changed within a narrow range. Control value (100%): 76.3 pmol/nmol CaM.

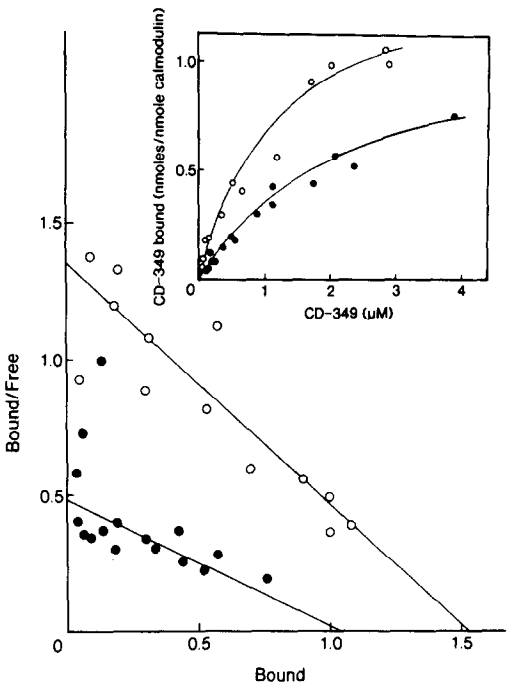

Fig. 3. Scatchard plot analysis of [^{3}H]CD-349 binding to CaM. A Sephadex G-50 superfine column was pre-equilibrated with buffer containing 0.1 mM Ca^{2+} and 0.25 to 10 μM [^{3}H]CD-349 (●) or 10 μM TFP (○). The concentration of actual CD-349 was calculated after equilibrium. Each point of this figure was from two sets of experiments. The inset shows the saturation curve of the binding. BOUND: nanomoles of CD-349 bound per nanomole of CaM. FREE: concentration of free CD-349.

CD-349 was 2.4 μM (Fig. 2). On the other hand, the CaM antagonist TFP (10 μM) markedly increased [^{3}H]CD-349 binding to CaM.

Scatchard plot analysis of CD-349 binding to CaM. A Sephadex G-50 column was pre-equilibrated with buffer containing various concentrations of CD-349 and a tracer level of [^{3}H]CD-349. CD-349 is insoluble at concentrations required to saturate completely the binding site of CaM. Under these conditions, the concentration used was limited to less than 4 μM (Fig. 3). Scatchard plot analysis demonstrated one high-affinity site for CaM, and the K_{app} and B_{max} were 2.1 μM and 1.0 nmol/nmol of CaM. The K_{app} was almost the same as the IC_{50} of [^{3}H]CD-349 binding to CaM obtained in a displacement experiment. As observed above, TFP (10 μM) enhanced CD-349 binding to CaM which resulted from an increase of both affinity (from 2.1 to 1.1 μM) amd B_{max} (from 1.0 to 1.5 nmol/nmol CaM).

Effect of CD-349 on the interaction between CaM and TNS. The effects of CD-349 and CaM antagonists, W-7 and TFP, on the increase in fluorescence intensity caused by the interaction of TNS to hydrophobic binding site on CaM are illustrated in Fig. 4. Strong fluorescence intensity was observed as the result of the binding of TNS (15 μM) to CaM (10 μM) in the presence of Ca^{2+}, as reported [19]. CD-349 (10 μM) slightly decreased the fluorescence intensity but did not change the wavelength which produced the maximal intensity, whereas W-7 (50 μM) and TFP (10 μM) markedly depressed and enhanced the fluorescence intensity, respectively, with a shift of

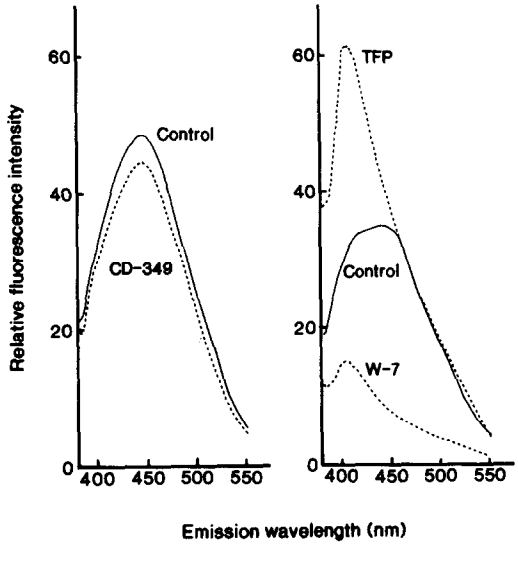

Fig. 4. Effects of CD-349, W-7 and TFP on the fluorescence induced by TNS binding to CaM. A fluorescence spectra of 15 μM TNS in 50 mM Tris/HCl (ph 7.5) buffer in the presence of 10 μM Ca^{2+} and 10 μM CaM, followed by the addition of CD-349 (10 μM). W-7 (50 μM) and TFP (10 μM). Recording was done at the wavelength from 380 nm to 550 nm. The excitation wavelength was 365 nm.

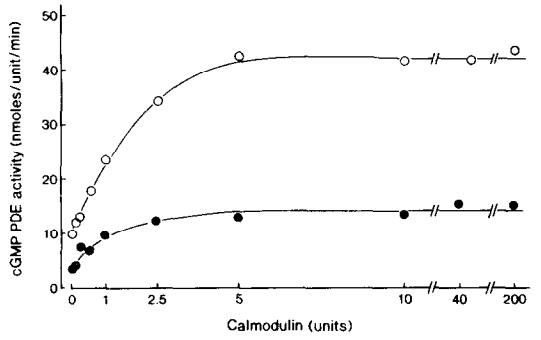

Fig. 5. Effect of CaM on CD-349-induced inhibition of Ca^{2+}/CaM-dependent PDE activity. Assay of PDE activity was carried out using CaM-deficient bovine brain cyclic nucleotide PDE with 1 μM cGMP in the absence (○) and presence (●) of 3 μM CD-349. Data are from one experiment, done in duplicate.

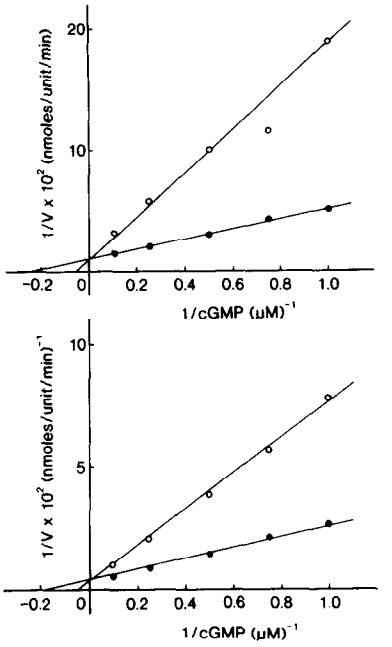

Fig. 6. Double-reciprocal plot analysis of CD-349-induced inhibition of Ca^{2+}/CaM-dependent PDE activity: The activities of basal (top) and Ca^{2+}/CaM (2.5 units)-activated (bottom) PDE as a function of the concentrations of cGMP in the absence (●) and presence (○) of 3 μM CD-349. Data are from one experiment, done in duplicate.

the maximal response toward the lower wavelength. These results suggest that CD-349 does not bind to the hydrophobic binding site of CaM which is exposed by the binding of Ca^{2+}, but does bind to the other side of CaM.

Effect of CaM on the inhibitory activity of CD-349 to Ca^{2+}/CaM-dependent cGMP PDE activity. CD-349 preferentially inhibits Ca^{2+}/CaM-activated PDE activity in crude enzyme preparations [15, 16]. To clarify whether the inhibition of Ca^{2+}/CaM-dependent PDE activity by CD-349 is due to inactivation of CaM via binding to the CaM as CaM antagonists do, the effect of CaM on the inhibitory activity of CD-349 to CaM-deficient PDE activity was studied. CaM caused a concentration-dependent activation of CaM-deficient PDE (Fig. 5), and full activation was observed at more than 5 units CaM/assay. CD-349 (3 μM) showed about 70% inhibition on the basal activity, and the inhibition could not be overcome by the increase of CaM up to 200 units/assay (i.e. 40-fold excess over that needed for the maximal activation).

Kinetic analysis of CD-349-induced inhibition of PDE activity. The activation of PDE by CaM was caused by the increase of V_{max} (from 95.2 to 250 nmol/unit/min), without change in the apparent K_m (from 4.2 to 5.0 μM) (Fig. 6). Double-reciprocal plot analysis of both basal and Ca^{2+}/CaM-activated PDE inhibition by 3 μM CD-349 in Fig. 6 indicates that the inhibition by CD-349 of both basal and Ca^{2+}/CaM-activated PDE activities was competitive with the substrate cGMP. The inhibition constants (K_i) of CD-349 for both basal and Ca^{2+}/CaM-activated PDE activities were 0.9 and 1.0 μM resepctively.

DISCUSSION

CaM has three or four hydrophobic binding sites which are exposed by Ca^{2+} binding to and by conformational change of CaM [10, 12, 19, 21], and the calculated numbers of Ca^{2+}-dependent binding sites on CaM of W-7, TFP and TNS are 3 [6], 2 [22] and 2 [23] sites per molecule. Concerning the binding site of drugs, including CaM antagonists on CaM, it has been reported that the potent CaM antagonists calmidazolium, TFP and W-7 bind to hydrophobic sites exposed by the binding of Ca^{2+} to CaM, whereas the binding site of some other drugs, felodipine and prenylamine, which possess Ca^{2+} antagonistic actions, differs from that of CaM antagonists [6, 24–27]. In this study, CD-349 only slightly decreased the fluorescence intensity induced by complex formation with TNS and CaM, in the presence of Ca^{2+}. However, equilibrium binding experiments showed that CD-349 binds to CaM, Ca^{2+}-dependently. Scatchard plot analysis of the binding of CD-349 to CaM showed that the maximal binding capacity of CaM is one molecule of CD-349 per molecule of CaM, and the K_{app} of CD-349 is 2.1 μM. This K_{app} of CD-349 to CaM is almost the same as the IC_{50} obtained from the inhibition of both PDE activity [15, 16] and CD-349-CaM binding by the drug. Bostorom *et al.* [8] reported that CaM has one to two binding sites for felodipine and the K_{app} is 1–10 μM. Movsesian *et al.* [13] reported that the K_{app} of nitrendipine is 52 μM and that the dihydropyridines bind to CaM at the site exposed by the binding of Ca^{2+}, and bind CaM antagonists or CaM-dependent enzymes. Taking all these data into consideration, CD-349 has a reasonable K_{app} for CaM at which the CaM-mediated process, myosin P-light chain phosphorylation by myosin light chain kinase, is inhibited by felodipine and nitrendipine [13].

The present study showed that TFP enhanced but W-7 depressed the increase of fluorescence intensity induced by TNS–CaM–Ca^{2+} complex formation.

Furthermore, Scatchard plot analysis of the stimulation of [^{3}H]CD-349 binding to CaM by TFP demonstrated that TFP increases both the affinity and B_{max} of the CD-349 binding. These results suggest that the binding sites of TFP differ from those of W-7 itself, while W-7 and TNS bind to a similar portion of CaM. The binding sites of CD-349 and TFP also differ, despite the same requirement of Ca^{2+} for the binding of these drugs to CaM. It may be that the binding of TFP to CaM increases the affinity of CD-349 to CaM and exposes another site which could interact with CD-349.

The functional significance of the binding of CD-349 to CaM was assessed by studying the effect of CD-349 on the Ca^{2+}/CaM-activated PDE. CD-349 inhibited both basal and Ca^{2+}/CaM-activated PDE activity, and the inhibitory potency remained unchanged in the presence of excess CaM. These findings suggest that CD-349 has no effect on the interaction between PDE and CaM, and that the inhibition of Ca^{2+}/CaM-activated PDE activity by CD-349 is not the result of binding to and inactivation of CaM. Double-reciprocal plot analysis of the inhibitory action of the enzyme showed that CD-349 inhibited both basal and Ca^{2+}/CaM-activated PDE activities, in a competitive manner with respect to the substrate, cGMP. These results indicate that CD-349 may inhibit Ca^{2+}/CaM-dependent PDE activity by direct effects on the enzyme, even though CD-349 binds to CaM in a Ca^{2+}-dependent manner and shows a good correlation with K_{app} of CaM binding and IC_{50} of the CaM-dependent enzyme activity. This would be consistent with findings that the inhibition of PDE by the dihydropyridines is due to a direct interaction of the drugs with Ca^{2+}/CaM-dependent PDE [28].

In conclusion, the present study suggests that CD-349 binds to CaM, Ca^{2+}-dependently, but that the inhibition of PDE is not responsible for the binding to CaM. The inhibition of PDE by CD-349 is due to direct effects of CD-349 on Ca^{2+}/CaM-dependent PDE.

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