A New Ca-Antagonist, CD-349, Binding to the Ca-Channel of Rat Myocardium and Brain and Hog Coronary Artery

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Abstract—The binding to a Ca-channel of a novel 1,4-dihydropyridine (DHP) Ca-antagonist, 2-nitratopropyl 3-nitratopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (CD-349), was studied in rat myocardium and brain and hog coronary artery, and the binding was compared with that of other DHPs. In rat myocardium, the binding reaction of $[^3H]$CD-349 was faster than that of nitrrendipine (NTD). The association rate constant of $[^3H]$CD-349 was about 10 times higher than that of $[^3H]$NTD. The dissociation rate was also higher than that of $[^3H]$NTD. Scatchard plot analysis of $[^3H]$CD-349 binding showed one high affinity site for CD-349. The dissociation constant ($K_d$) and maximum number of binding sites ($B_{max}$) were 333 pM and 286 fmoles/mg protein. They were almost the same as those for $[^3H]$NTD. $[^3H]$CD-349 and $[^3H]$NTD bindings were dose dependently inhibited by 1,4-DHPs: nifedipine, nimodipine, nicardipine and CD-349. The order of the inhibitory potency of these drugs was CD-349 $>$ nicardipine $>$ nimodipine $>$ nifedipine, when $[^3H]$CD-349 and $[^3H]$NTD were used as the ligands. Similar results were obtained for rat brain and hog coronary artery. $[^3H]$CD-349 binding was not changed by α- or β-adrenergic, cholinergic, histaminergic or serotonergic agents in rat myocardium. From these results, it is suggested that CD-349 binds to the Ca-channel reversibly with high affinity due to its high association rate for the site.

CD-349 (Fig. 1), a novel 1,4-DHP derivative, has a potent and long lasting vasodilatory effect on cerebral vessels in dogs (1). This compound has a Ca-antagonist-like pharmacological profile: it inhibits K-contraction, and the manner of the inhibition is similar to that of Ca-antagonists, nimodipine and nicardipine (1); it inhibits slow action potential responses in depolarized canine cardiac muscle (K. Tsuchida et al., unpublished data); and it inhibits $^{45}$Ca influx in guinea pig taenia coli (K. Momose et al., unpublished data). These effects strongly suggest that CD-349 is a Ca antagonist. CD-349 also demonstrated selective inhibitory effects on Ca-dependent cyclic nucleotide phosphodiesterase in blood vessels (2, 3).

High affinity binding sites for $[^3H]$1,4-DHPs, such as $[^3H]$NTD, $[^3H]$nimodipine and $[^3H]$nifedipine have been described for several tissues, and it has been suggested that these sites might be associated with Ca-channels (4–10). Numerous workers have suggested that the affinity of $[^3H]$1,4-DHP is related to the pharmacological potency of the compound (8–13).

In this study, we investigated the binding characteristics of CD-349 in rat myocardium.
and compared them with those in rat brain and hog coronary artery.

Materials and Methods

Myocardium and brain preparation

Male Wistar strain rats weighing 200–250 g were used. After decapitation, the heart and brain (except for the cerebellum) were immediately removed; and in the case of the myocardium, the heart was rinsed in cold saline following the removal of the lipid connected to the heart. The heart and brain tissues were homogenized with 10 vol. of 50 mM Tris-HCl buffer (pH 7.5) by 10 sec bursts at 25,000 rpm using an ULTRA TURRAX. The homogenate was filtered through silicon coated gauze and then centrifuged at 1,000×g for 10 min at 4 °C. The resultant supernatant was centrifuged at 48,000×g for 10 min, and the pellet was washed twice by means of centrifugation. The pellet finally obtained was suspended in 20 vol. of 50 mM Tris-HCl buffer (pH 7.5) and was used as the crude membrane fraction of the myocardium and brain for the following experiments.

Hog coronary preparation

The membrane fraction of hog coronary artery was prepared as reported previously (14) according to the method of DePover et al. (8). The fraction was treated with neuraminidase (NUase) according to the method of Nagatomo et al. (15), since the treatment increased the protein linearity of [3H]NTD binding without changing the binding characteristics of [3H]NTD (14).

Binding assay

[3H]Labeled CD-349 and NTD were used as the ligands and were incubated with the membrane fraction for 10 and 20 min, respectively, at 25±1 °C in subdued light. The reaction was terminated by filtration on a Whatman glass filter (GF/B) under vacuum. The filter was rinsed with 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.5). After drying the filter, the radioactivity on the filter was measured with a Toluene-Triton scintillator (2 l toluene, 1 l triton X-100, 8 g PPO, 0.2 g POPOP). The radioactivity found in the presence of 1 μM non-labeled CD-349 and NTD was considered to be the nonspecific bindings of [3H]CD-349 and [3H]NTD, respectively, since no further inhibition was observed by adding 10^{-8} to 3×10^{-8} M CD-349 or NTD to the [3H]NTD binding assay mixtures. These values were subtracted from each experimental value of [3H]labeled ligand binding (total binding) to obtain the amount of specifically bound [3H]CD-349 or [3H]-NTD. The specific binding of [3H]CD-349 and [3H]NTD was about 30 and 45%, respectively, of the total bindings. Since the specific binding of [3H]labeled ligands was linearly increased, depending on the amount of protein, up to 1 mg/ml, 0.2–0.5 mg protein/ml was used for the experiment. Specific bindings of [3H]CD-349 and [3H]NTD reached a steady state after 10 and 20 min, respectively, and they were not changed by further incubation up to 30 min after the start of the reaction.

Competition assay: [3H]CD-349 and [3H]-NTD were used to determine the affinity of various DHPs by measuring the displacement of the specific binding of [3H]CD-349 and [3H]NTD. The concentration of the DHPs used was from 10^{-11} to 10^{-7} M. The inhibition constant (K_i) values were determined by the previously reported equation (16).

Kinetic studies: To measure the association rate constant, several different concentrations of [3H]CD-349 (97, 178 and 213 pM) and [3H]NTD (42, 73.7 and 164 pM) were used as the ligand. Dissociation rate constants of [3H]CD-349 and [3H]NTD were determined by measuring the [3H]CD-349 and [3H]-NTD bindings after adding the 10^{-6} M CD-349 and 10^{-6} M NTD, respectively.

Saturation assay: The K_d and B_{max} values for the binding site were determined by Scatchard plot analysis using a computer program, “SP123P” developed by Dr. Ono of Tokyo University for the PC9801 (NEC) personal computer.

Protein concentration was determined by the method of Lowry et al. (17) using bovine serum albumin as the standard.

Substances used

CD-349, nicardipine, nimodipine and nifedipine were synthesized in the department of organic chemistry at our research center. [5-Methyl-3H]NTD (S.A. 77.4 Ci/mmol) and [3H]CD-349 (S.A. 28 Ci/mmol) were obtained from New England Nuclear and Amersham, respectively. The various agonists
and antagonists were obtained from the following sources: noradrenaline bitartrate, atropine sulfate and histamine dihydrochloride, (Wako, Japan); yohimbine hydrochloride, DL-isoproterenol hydrochloride and DL-propranolol hydrochloride (Nakarai, Japan); chloropheniramine maleate and 5-hydroxytryptamine creatinine sulfate (Tokyo Kasei, Japan); and acetylcholine chloride (Daiichi, Japan). Phentolamine mesylate, prazosine hydrochloride, atenolol and clonidine hydrochloride were prepared from commercial products manufactured by Japan Ciba-Geigy (Japan), Pfizer Taito (Japan), Sumitomo (Japan) and Nippon Boehringer Ingelheim (Japan), respectively. Non-labeled drugs were dissolved in 50 or 100% ethanol or dimethylsulfoxide, and from 10 to 30 µl was applied to 2 ml of incubation medium.

**Results**

**Time courses of [3H]CD-349 and [3H]-NTD binding to the membrane fraction of rat myocardium:** The binding reaction of [3H]CD-349 was significantly faster than that of [3H]-NTD (Fig. 2). The binding reaction of [3H]-CD-349 to the cardiac membrane reached a steady-state in 5 min. On the other hand, [3H]NTD took about 20 min for the binding reaction to reach a steady-state, as reported previously (9, 14, 18).

**Association and dissociation rates for [3H]CD-349 and [3H]NTD to the binding sites:** The rate constants of association (k<sub>1</sub>) and dissociation (k<sub>2</sub>) were obtained from the equation: k<sub>obs</sub> = k<sub>1</sub>[L] + k<sub>2</sub> (k<sub>obs</sub>, observed apparent rate constant; L, concentration of ligand) using several concentrations of [3H]-CD-349 and [3H]NTD (Fig. 3). The association rate for [3H]CD-349 was 10.4 times

![Graph](image_url)

**Fig. 2.** Time course of specific [3H]CD-349 and [3H]NTD bindings to the membrane fraction of rat myocardium. [3H]CD-349 (28 and 98 pM) and [3H]NTD (147 pM) were incubated with the membrane fraction of rat myocardium for the indicated time at 25°C, and specific binding was measured.
higher than that for $[^3\text{H}]$NTD (Table 1). The dissociation rate of $[^3\text{H}]$CD-349 was 8.0 times higher than that of $[^3\text{H}]$NTD (Table 1). The dissociation rate was also determined by another method. The $k_2$ values were obtained by adding an excess of unlabeled CD-349 and NTD when the binding reached equilibrium. The values for CD-349 and NTD were almost half compared with those obtained from the association experiments. However, the dissociation rate for CD-349 was 8.3 times higher than that for NTD (Fig. 4). The $K_d$ ($k_2/k_1$) values computed from the above two methods were 230 and 111 pM for CD-349 and 329 and 140 pM for NTD, respectively (Table 1).

**Table 1.** Association and dissociation rate constants of $[^3\text{H}]$CD-349 and $[^3\text{H}]$NTD in rat myocardium

|                       | $[^3\text{H}]$CD-349          | $[^3\text{H}]$NTD          |
|-----------------------|-------------------------------|----------------------------|
| Association experiments | $k_1$ (min$^{-1}$/nM) | 3.566±0.434                 | 0.343±0.037                 |
|                       | $k_2$ (min$^{-1}$)       | 0.823±0.086                 | 0.103±0.002                 |
|                       | $K_d$ ($k_2/k_1$, nM)     | 0.230                      | 0.329                      |
| Dissociation experiments | $k_2$ (min$^{-1}$)       | 0.396±0.057                 | 0.048±0.003                 |
|                       | $K_d$ ($k_2/k_1$, nM)     | 0.111                      | 0.140                      |

Each value represents the mean±S.E.M. obtained from 3 separate experiments. $k_1$*: The values obtained from the association experiments were used.

Concentration dependency of $[^3\text{H}]$CD-349 and $[^3\text{H}]$NTD bindings to rat myocardium: A Scatchard plot analysis of the specific binding of $[^3\text{H}]$CD-349 showed that $[^3\text{H}]$CD-349 bound to the membrane with high affinity, as did $[^3\text{H}]$NTD (Fig. 5). The $K_d$ and $B_{\text{max}}$ values of $[^3\text{H}]$CD-349 were 330 pM and 286 fmoles/mg protein (Fig. 5). These values were almost the same as those of $[^3\text{H}]$NTD; the $K_d$ and $B_{\text{max}}$ values were 363 pM and 300 fmoles/mg protein (Fig. 5).

Inhibition of $[^3\text{H}]$CD-349 and $[^3\text{H}]$NTD
Fig. 4. Time course of dissociations of specific [³H]CD-349 and [³H]NTD binding. [³H]CD-349 (69–180 pM) and [³H]NTD (74–88 pM) were used as ligands. [³H]CD-349 and [³H]NTD were incubated with membrane fractions of rat myocardium. A large excess of CD-349 (10⁻⁶ M) or NTD (10⁻⁶ M) was added at 20 or 60 min after the incubation, respectively. Each value represents the mean±S.E.M. obtained from 6 ([³H]CD-349) and 3 ([³H]NTD) separate experiments.

Fig. 5. Scatchard plot analyses of specific binding of [³H]CD-349 and [³H]NTD to membrane fractions of rat myocardium. Concentrations of [³H]CD-349 and [³H]NTD used were 26.6–1179.0 and 5.7–795.0 pM, respectively. Kd values of [³H]CD-349 and [³H]NTD were 333.0±80.1 pM and 363.0±67.6 pM, respectively. Bmax values of [³H]CD-349 and [³H]NTD were 285.6±19.4 and 300.0±15.0 fmole/mg protein, respectively. Each value represents the mean obtained from 5 ([³H]CD-349) and 4 ([³H]NTD) separate experiments.
bindings by 1,4 DHP Ca-antagonists: [3H]-CD-349 and [3H]NTD bindings were dose-dependently inhibited by the various 1,4-DHPs. The inhibition curves were all parallel with each other. The inhibition constant (Kᵢ) values were determined from the inhibition curves (Table 2). The Kᵢ values and the order of potency of the 1,4-DHPs obtained from the inhibitions of [3H]CD-349 binding were in good agreement with those obtained from the experiments on the inhibition of [3H]NTD binding. The inhibitory potencies of the 1,4-DHPs were CD-349 > nicardipine > nimodipine > nifedipine (Table 2). The Kᵢ values of CD-349 for [3H]CD-349 binding were almost the same as the Kᵢ values of the high affinity site obtained from Scatchard plot analysis and the association and dissociation experiments of [3H]CD-349.

Comparisons of inhibitory potencies of DHPs in rat brain and hog coronary artery: Kᵢ values of [3H]NTD in the brain and coronary artery were 0.34±0.1 and 0.22±0.06 nM, respectively. The Kᵢ value of [3H]NTD in the hog coronary artery was a little smaller than that in rat brain and myocardium.

[3H]NTD binding to the rat brain membrane fraction was completely inhibited by CD-349, nicardipine and nifedipine in a dose-dependent manner. The rank order for the inhibitory potencies of the DHPs was: CD-349 > nifedipine > nicardipine > nimodipine (Table 3). In hog coronary artery, CD-349 also had significantly higher potency than nicardipine (Table 3).

Effect of neurotransmitter related compounds to [3H]CD-349 binding in the myocardium: The effect of various agonists and antagonists on the binding of [3H]CD-349 was studied with doses of 10⁻⁶ and 10⁻⁵ M in rat myocardium (Table 4). There was no drug which inhibited or enhanced the binding of [3H]CD-349 among the stimulants and blockers of α- and β-adrenergic, cholinergic, histaminergic or serotoninergic agents. These results suggest that the binding of CD-349 is

| Table 2. Inhibition of [3H]CD-349 and [3H]NTD bindings by various DHPs in rat myocardium |
|-----------------------------------------------|
| [3H]CD-349 | [3H]NTD |
| IC50* | Kᵢ* | IC50* | Kᵢ* |
| CD349 | 0.22±0.06 | 0.18±0.05 (3) | 0.02±0.01 | 0.01±0.01 (3) |
| Nicardipine | 0.26±0.08 | 0.20±0.06 (3) | 0.06 | 0.05 (2) |
| Nimodipine | 0.58±0.17 | 0.38±0.07 (6) | 0.42±0.08 | 0.34±0.06 (4) |
| Nifedipine | 0.85±0.24 | 0.53±0.25 (3) | 2.20 | 1.78 (2) |

Concentration of [3H]CD-349 and [3H]NTD used were 19.5–373.6 pM and 57.9–935 pM, respectively. Each value represents the mean±S.E.M. obtained from 2–6 separate experiments. *×10⁻⁹ M. The number of experiments is indicated in parentheses.

| Table 3. Inhibition of [3H]NTD binding by various 1,4-DHPs in rat brain and hog coronary artery |
|-----------------------------------------------|
| | Rat brain | Hog coronary artery |
| | IC50* | Kᵢ* | IC50* | Kᵢ* |
| CD349 | 0.95±0.30 | 0.76±0.23 (4) | 0.41±0.12 | 0.30±0.08 (4) |
| Nicardipine | 2.03±0.35 | 1.64±0.23 (3) | 1.56±0.44 | 1.15±0.32 (4) |
| Nimodipine | 2.03±0.19 | 1.67±0.22 (3) | – | – |
| Nifedipine | 1.50±0.15 | 1.14±0.13 (4) | – | – |

Each value represents the mean±S.E.M. obtained from 3–4 separate experiments. Concentration of [3H]NTD used was 49–98 pM. Kᵢ and Bmax values were obtained from Scatchard analyses of [3H]NTD bindings. Rat brain: Kᵢ=0.34±0.10 nM, Bmax=97.2±14.9 fmoles/mg protein. Hog coronary artery: Kᵢ=0.22±0.06 nM, Bmax=77.3±4.8 fmoles/mg protein (14). *×10⁻⁹ M. The number of experiments is indicated in parentheses.
not influenced by any other neurotransmitters.

**Discussion**

This study showed that CD-349 binds to the cardiac membrane fraction in a specific, reversible and saturable manner with high affinity. In parallel studies with [3H]CD-349, the B\textsubscript{max} and K\textsubscript{d} of [3H]NTD were almost the same as for [3H]CD-349. The association and dissociation rates of [3H]CD-349 binding, however, were significantly faster than those of [3H]NTD.

Other substituted DHPs completely inhibited [3H]CD-349 and [3H]NTD bindings with the same rank order of potency: CD-349 > nicardipine > nimodipine > nifedipine. Concerning the difference of the K\textsubscript{i} value of CD-349 obtained from the displacement of [3H] CD-349 and [3H]NTD bindings, the value was smaller when [3H]NTD was used as the ligand. Other DHPs also had smaller K\textsubscript{i} values when [3H]NTD was used as the ligand. The K\textsubscript{i} value should be the same regardless of the ligand used, but the reason for the difference in this study is not yet clear.

CD-349 had high affinities for the brain and coronary artery, as well as for the myocardium. CD-349 showed potent relaxing effects on high potassium-, 5-HT- and PGF\textsubscript{2\alpha}-induced canine basilar artery contractions (1). In vivo, CD-349 decreased blood pressure and increased cerebral tissue blood flow (1). [3H]CD-349 binding was dose-dependently inhibited by NTD. [3H] NTD binding was also dose-dependently inhibited by CD-349. It has been reported that NTD binds to the DHP binding site of the Ca-channel (4-8, 18). Thus, it is suggested that the binding site of [3H]CD-349 is the DHP binding site of the Ca-channel. This idea might be supported by results showing that CD-349 has inhibitory effects on the slow action potential response in depolarized canine cardiac muscle (K. Tsuchida et al., unpublished observations) and on the 45Ca influx in the smooth muscle of guinea pig taenia coli (K. Momose et al., unpublished observations).

Taking these results together, it is suggested that CD-349 showed its pharmacological effects by acting on the Ca-channel as an antagonist with high affinity.

In this study, the K\textsubscript{d} values for [3H]NTD were 0.36, 0.34 and 0.22 nM in rat myocardium, brain and hog coronary artery, respectively. The K\textsubscript{d} value of hog coronary artery for [3H]NTD was not very different from those of other tissues. These data were mostly similar with those for the cortex, heart and ileum previously reported (19). In a study by Gould et al. (19), it was also found that the rank order of the inhibitory potencies of

|                      | [3H]CD-349 binding (% of control) |
|----------------------|----------------------------------|
|                      | \(10^{-6}\) M | \(10^{-5}\) M          |
| Noradrenaline       | 108.0±14.5  | 127.8±27.4         |
| Phentolamine        | 86.1±21.6   | 81.8±18.5          |
| Prazosine           | 135.4±31.2  | 118.6±41.6         |
| Atenolol            | 114.6±40.4  | 109.0±12.0         |
| Clonidine           | 95.0±5.8    | 113.9±8.4          |
| Yohimbine           | 93.0±9.8    | 108.6±25.1         |
| Isoproterenol       | 96.3±25.8   | 96.4±12.8          |
| Propranolol         | 93.4±33.7   | 135.9±31.2         |
| Acetylcholine       | 89.9±13.5   | 100.5±24.9         |
| Atropine            | 87.4±9.4    | 86.1±12.5          |
| Histamine           | 85.5±19.9   | 93.5±22.4          |
| Chlorpheniramine    | 121.5±31.6  | 139.5±27.4         |
| 5-Hydroxytryptamine | 71.7±21.1   | 91.4±24.0          |

Each value represents the mean±S.E.M. obtained from 3–6 separate experiments. [3H]CD-349 used as the ligand was 200–300 pM.

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Table 4. Effect of various agonists and antagonists on specific binding of [3H]CD-349 to rat myocardium
the DHPs among the different tissues was not different. They did not find any differences in the rank order of various DHP affinities for the binding site among the cortex, heart and ileum. In the present study, CD-349 had the highest affinity among the DHPs tested in the myocardium, brain and coronary artery. These data suggest that the sequence of the binding affinity of DHPs tested, including CD-349, does not change significantly in the different tissues. However, it might be difficult to clarify the tissue specificity of DHPs from the binding experiment carried out with the tissue homogenized preparation, because the action of DHPs on the Ca-channel and DHP binding to the Ca-channel are strongly voltage dependent, and these effects can only be found in intact cells or tissues of the heart and the blood vessel (20–24). The present study suggests that CD-349 binds to the DHP binding site with higher affinity in the cardiac muscle, the neuron, and the blood vessel, compared with the other DHPs used in this experiment.

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[^3H]DHP \text{ Ca-antagonists have a smaller dissociation constant compared with the ligands of other neurotransmitter receptors.}
\]

The association and dissociation rate constants of \[^3H\]CD-349 were significantly higher compared with those of \[^3H\]NTD. The binding affinity of a \[^3H\]Ca-antagonist is temperature dependent (25–28). The association and dissociation rates are presumably a reflection of the energy involved in the formation and separation of the ligand-receptor complex.

The association rate of Ca-antagonist binding to the Ca-channel is a temperature-dependent process, as is the dissociation of the complex (25, 26). Both are slower at lower temperatures, as are the other receptor bindings (29, 30). The association of \[^3H\]-NTD was found to be very rapid at 37°C, equilibrium being reached within minutes after the start of the binding (26). At 37°C, the half-time for the dissociation of a \[^3H\]-Ca-antagonist was 3 min and at 25°C, 18 min (26). The reaction at 37°C is similar to that of \[^3H\]CD-349 at 25°C in the present experiment. The Kd value of \[^3H\]NTD was increased by about 3 to 4 times by increasing the temperature of the incubation mixture from 0 to 37°C (28).

Though a thermodynamic study was not carried out in the present investigation, the above results together suggest that the binding of CD-349 is associated with a significant increase of entropy.

From the results that CD-349 bound to the Ca-channel and did not interact with other neurotransmitter receptors, it is suggested that the binding site of CD-349 is the DHP binding site.

In conclusion, these results suggest that CD-349 is a selective and reversible Ca-antagonist with high affinity to the Ca-channel due to the high association rate.

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