Inhibition of Adenosine 3′,5′-Cyclic Monophosphate Phosphodiesterase by CD-349, a Novel 1,4-Dihydropyridine Derivative: Effect of EGTA on the Inhibitory Activity

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Abstract—CD-349 (2-nitratopropyl 3-nitratopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate) inhibited the activity of cyclic AMP phosphodiesterase (PDE) from the porcine coronary artery more effectively than that from the myocardium. Other dihydropyridines, nicardipine and nifedipine, were not tissue selective in inhibiting the cyclic AMP PDE from both sources. CD-349 inhibited cyclic AMP PDE noncompetitively with apparent inhibition constant (K_i) values of 6.6 and 4.6 μM for high and low affinity constant (K_m) enzymes, respectively. Basal activity of coronary arterial cyclic AMP PDE was decreased to approximately 65% of the control value by 0.2 mM ethylene glycol bis (β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA). In the coronary artery, the inhibition of cyclic AMP PDE by CD-349 was weakened in the presence of EGTA, while the inhibition of nicardipine and nifedipine were not affected. EGTA had no influence on the CD-349 induced inhibition of myocardial cyclic AMP PDE. Calmodulin antagonists such as trifluoperazine (TFP) gave substantially the same results as those with CD-349. These results indicate the relative selectivity of the coronary arterial cyclic AMP PDE inhibition by CD-349 and suggest that this selective inhibition is due to blockade of calcium/calmodulin-activated cyclic AMP PDE.

Dihydropyridines and structurally diverse groups of compounds, typified by nifedipine and verapamil, are classified as "calcium entry blockers" or "calcium antagonists". They are now used to treat some cardiovascular disorders such as angina, hypertension and cardiac arrhythmias (1-4). These drugs bind to calcium channels, block the voltage-dependent slow inward calcium flux through plasma membranes and exert potent vasodilative activity in vascular smooth muscle (3, 5, 6).

CD-349 is a novel 1,4-dihydropyridine derivative synthesized in our laboratory (Fig. 1). CD-349 had potent and long-lasting vasodilative and blood pressure decreasing activities following systemic administration in dogs (7). In crude membrane preparations of the rat cardiac muscle and the porcine coronary artery, CD-349 showed a dose-dependent inhibition of [3H]-nitrendipine binding. [3H]-CD-349 bound to the preparations with a K_d value almost the same as the inhibition constant (K_i) obtained from the displacement of [3H]-nitrendipine binding (8).

On the other hand, it has been shown that a 1,4-dihydropyridine derivative, nicardipine.
inhibited cyclic AMP phosphodiesterase (PDE) and increased cyclic AMP levels in the coronary artery (9). This mechanism also contributes to the vasodilative effect of the compound, since smooth muscle has been shown to be relaxed by cyclic AMP accumulation and subsequent activation of cyclic AMP-dependent protein kinase (10–13). Furthermore, it was found that nitrendipine and felodipine bound to calmodulin and inhibited the calmodulin-dependent process (14).

In the present study, we investigated the effect of CD-349 on cyclic AMP PDE activities in the porcine cardiac muscle and coronary arterial muscle, examining the possible involvement of calmodulin in the inhibiting effect of CD-349 on cyclic nucleotide PDE activity.

Materials and Methods

Materials: Porcine hearts of both sexes and various ages were obtained from a local slaughterhouse and put into ice-cold Tyrode’s solution. Dissection and homogenization of the blood vessels were completed within 3 hr after slaughter of the animals. CD-349, nicardipine and chlorpromazine were prepared in our laboratory. Nifedipine was purchased from Bayer and cyclic AMP, calmodulin, trifluoperazine hydrochloride (TFP) and snake venom (Crotalus atrox) were purchased from Sigma. [3H]-cyclic AMP (31.5 Ci/mmol) was purchased from New England Nuclear.

Enzyme preparations from coronary artery and myocardium: The right coronary, anterior descending and circumflex arteries were dissected from cold fresh porcine hearts and placed in cold saline. After removal of loosely connected fatty material, the tissue was weighed, minced with scissors and homogenized in 9 vol. of ice-cold buffer consisting of 40 mM Tris/HCl (pH 7.5), 0.25 M sucrose, 5 mM 2-mercaptoethanol and 5 mM MgCl2 with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany). Supernatant fractions were obtained by ultracentrifugation of this homogenate for 60 min at 100,000xg at 4°C. Following removal of the fat layer, the supernatant fractions were used as cyclic AMP PDE preparations. An enzyme preparation from the myocardium was obtained in the same manner.

Protein content was determined by the method of Lowry et al. (15) using bovine serum albumin as the standard.

Assay procedures for cyclic AMP PDE: PDE activity was determined by the method of Beavo et al. (16) with a slight modification. The standard reaction mixture consisted of 30 mM Tris/HCl (pH 7.5), 5 mM MgCl2, 1 mM 2-mercaptoethanol, 100 mM NaCl, 1 μM cyclic AMP (including 150,000 dpm [3H]-cyclic AMP), 50 μg bovine serum albumin, enzyme preparation (20–35 μg from the coronary artery or 125–160 μg from myocardium) and drugs. These enzymes caused less than 20% hydrolysis of cyclic AMP in the absence of the drugs. The total volume of the reaction mixture was 0.5 ml. The reaction mixture was incubated at 37°C for 2 min, with the protection from the light, and then heated in boiling water for 2 min (in order to stop cyclic AMP PDE activity). Ten μl of snake venom (Crotalus atrox, 5 mg/ml) was added to the cooled reaction mixture and incubated at 37°C for 30 min. Following an addition of 0.5 ml of 0.5 mM carrier adenosine, the total mixture was applied to a Dowex 1×8 (100–200 mesh, chloride form, Dow Chemical) column (0.6×2 cm) to separate adenosine and unhydrolyzed cyclic AMP. The column was eluted with 8 ml of 0.1 M Tris/HCl (pH 7.5), and the radioactivities in 1 ml aliquots were counted by a liquid scintillation spectrometer (Packard, TriCarb model 3375). Drugs were dissolved in dimethyl sulfoxide (DMSO), except for TFP and chlorpromazine. The final concentration of DMSO was adjusted to 2%, which slightly inhibited PDE activity (10% or less). Control values of cyclic AMP PDE activity were obtained in its presence. Under these experimental conditions, the reaction was found to be linear with time and enzyme protein concentration. The drugs studied did not inhibit the snake venom used in the assays. As a result, more than 90% of the substrate was hydrolyzed and dephosphorylated.

Results

Effects of CD-349 on cyclic AMP PDE activities: CD-349 inhibited porcine coronary arterial cyclic AMP PDE activities (Fig. 2,
Cyclic AMP Phosphodiesterase and CD-349

Fig. 2. Effect of CD-349 on cyclic AMP PDE from the porcine coronary artery and myocardium. Assays of cyclic AMP PDE activities were carried out as described in “Materials and Methods”, using the enzyme from the coronary artery (left panel) and the myocardium (right panel). Each point represents the mean of duplicate determinations. CD-349 (○), Nicardipine (●), Nifedipine (×).

Table 1. Effects of EGTA on basal cyclic AMP PDE from the porcine coronary artery and the myocardium

| Enzyme source | Cyclic AMP PDE activity (nmoles/mg protein/min) | Remaining activity (%) |
|---------------|-----------------------------------------------|------------------------|
|               | ‐EGTA                                       | 0.2 mM EGTA       |                        |
| Myocardium    | 1.04                                         | 1.01                  | 97.1                   |
| Coronary artery | 0.27                                         | 0.18                  | 66.7                   |

Values are the mean of 2 or 3 separate experiments.

Effects of EGTA on the inhibition of cyclic AMP PDE activities by CD-349: Table 1 shows the effects of EGTA on coronary arterial and myocardial cyclic AMP PDE. The basal coronary arterial enzyme activity was partially inhibited, while that of the myocardium was not affected by addition of 0.2 mM EGTA. The effects of CD-349 to inhibit the coronary arterial cyclic AMP PDE were remarkably decreased by addition of 0.2 mM EGTA, especially when the concentration of CD-349 was low (Fig. 4). On the other hand, chlorpromazine, which is known to be a calmodulin antagonist, and nicardipine showed only slightly higher IC50 values in the presence of EGTA (11 and 20 μM for nicardipine, 250 and 400 μM for chlorpromazine in the absence and presence of EGTA, respectively). Similar results were obtained with nifedipine (data not shown). The IC50 values for CD-349 could not be obtained in these experiments because it was

left). IC50 values (μM) for CD-349, nicardipine and nifedipine were 7.6, 10 and 56, respectively. On the other hand, CD-349 showed less inhibitory effect against the myocardial cyclic AMP PDE, while nicardipine and nifedipine inhibited the activity with IC50 values (μM) of 32 and 200 (Fig. 2, right). Double-reciprocal plot analysis showed that 2 types of PDE activities, low and high affinity, were present in the coronary artery (Fig. 3). Both activities were inhibited by CD-349 and nicardipine. The apparent low and high K_m values obtained from the data illustrated in Fig. 3 were 1.5 or 1.7 μM and 125 or 175 μM, respectively. In the case of the low K_m activity, the K_i values of CD-349 and nicardipine were calculated to be 6.6 and 11.3 μM, respectively. For the high K_m activity, the K_i values were 4.6 μM for CD-349 and 5.7 μM for nicardipine.
Fig. 3. Double-reciprocal plots of the inhibition of low and high K_m cyclic AMP PDE enzymes from the porcine coronary artery. Assays of cyclic AMP PDE activities were carried out as described in "Materials and Methods", except that the concentration of cyclic AMP was varied. Cyclic AMP from 0.5 to 4 μM and 25 to 200 nM (inset) were used in assays for low and high K_m enzymes, respectively. Each point represents the mean of duplicate determinations. upper panel: control (○), 10 μM CD-349 (●). lower panel: control (○), 10 μM Nicardipine (●).

insoluble in water at concentrations over 10 μM. On the other hand, EGTA did not affect the inhibitory effect of CD-349 on myocardial cyclic AMP PDE (Fig. 5). The inhibitory effects of nicardipine and nifedipine were also not influenced by EGTA. The IC50 value for nicardipine was 23 μM both in the absence and presence of EGTA, and those for nifedipine were 110 and 150 μM, respectively.

Effect of CD-349 and TFP on the activity of cyclic AMP PDE: Probably because of the lack of specificity to calmodulin-dependent enzymes (17), the effect of chlorpromazine on the coronary arterial cyclic AMP PDE was not affected by EGTA (Fig. 4). Thus, further experiments were done using TFP, a more specific calmodulin antagonist (18, 19). As shown in Fig. 6, the inhibitory effects of both CD-349 and TFP on coronary arterial cyclic AMP PDE were weakened by 0.2 mM EGTA, but their effects on myocardial cyclic AMP PDE were not changed.

Discussion

We investigated the inhibitory effects of CD-349 on cyclic AMP PDE activity using unpurified enzymes from porcine coronary arteries and myocardium. As to the inhibitory action of 1,4-dihydropyridine derivatives on cyclic AMP PDE activity, Sakamoto et al. (9) reported that nicardipine inhibited cyclic AMP PDE from the canine basilar, carotid,
Fig. 4. Effect of EGTA on the inhibition of cyclic AMP PDE from the porcine coronary artery by CD-349. Assays of cyclic AMP PDE activities were carried out as described in "Materials and Methods", in the absence (left panel) and presence (right panel) of 0.2 mM EGTA. Each point represents the mean of duplicate determinations. CD-349 (○), Nicardipine (●), Chlorpromazine (×).

Fig. 5. Effect of EGTA on the inhibition of cyclic AMP PDE from the porcine myocardium by CD-349. Assays of cyclic AMP PDE activities were carried out as described in "Materials and Methods", in the absence (left panel) and presence (right panel) of 0.2 mM EGTA. Each point represents the mean of duplicate determinations. CD-349 (○), Nicardipine (●), Nifedipine (×).

coronary and femoral arteries. Furthermore, they observed that nicardipine increased the level of cyclic AMP in canine arterial strips. The inhibitory action of CD-349 on cyclic AMP PDE activity was stronger than that of nicardipine in the porcine coronary artery, whereas that on the PDE from the myocardium was less. CD-349 showed a higher selectivity for coronary arterial cyclic AMP PDE, while nicardipine and nifedipine had no such selectivity. On the other hand, there was a qualitative difference between these two enzymes. A remarkable decrease in cyclic AMP PDE activity was observed in coronary arterial PDE in the presence of EGTA, but not in myocardial PDE. In spite of the remarkable inhibitory effect of CD-349 on coronary arterial cyclic AMP PDE in the absence of EGTA, a decrease of inhibiting effect was observed in its presence. The effect of CD-
Fig. 6. Effect of EGTA on the inhibition of cyclic AMP PDE from the porcine coronary artery and the myocardium by CD-349 and TFP. Assays of cyclic AMP PDE activities were carried out as described in "Materials and Methods", in the absence (open symbols) and presence (filled symbols) of 0.2 mM EGTA using cyclic AMP PDE from the coronary artery (left panel) and the myocardium (right panel). Each point represents the mean of duplicate determinations. CD-349 (○), Trifluoperazine (△).

Honda and Imamura (20) reported that phenothiazines significantly inhibited the activity of cyclic AMP PDE from the rat cerebral cortex, but insignificantly affected that of the cyclic AMP PDE from the heart. They observed that calcium ions were necessary for this inhibition, but for the action of other well known PDE inhibitors, such as papaverine and theophylline, calcium ions were not necessary (21). On the basis of these findings, they suggested that phenothiazines inhibit calcium-activator-activated enzyme, but do not inhibit its basal activity. Nowadays, the activator has been found to be calmodulin, and the mechanism of inhibitory action of phenothiazines on the calcium/calmodulin-activated PDE has also been clarified by Levine and Weiss (22). They have found that phenothiazines bind to calmodulin in the presence of calcium, blocking the binding of calcium-calmodulin complexes to PDE. In our study, a similar calcium dependency was observed with CD-349 with respect to its inhibition of cyclic AMP PDE activity in the porcine coronary artery.

The removal of calcium ions from the calcium/calmodulin-activated PDE system attenuates the PDE activity (23). Cheung (24) speculated that the inhibition observed in the presence of EGTA is due to chelation of less firmly bound calcium, and the activity which remains resistant might be due to that which is more firmly bound. The lack of effects of EGTA on basal myocardial cyclic AMP PDE activity may be explained by this hypothesis. The present results suggest that at least 30-40% of the coronary arterial cyclic AMP PDE is in a calcium/calmodulin sensitive form, and that potent inhibition of this form of enzyme by CD-349 explains the selectivity of CD-349 for a calcium/calmodulin sensitive form of PDE in the coronary arteries.

The effects of calcium antagonists including 1,4-dihydropyridine derivatives on the cardiovascular system has been considered due to the blockade of calcium entry into the smooth muscle cells, even in the case of nicardipine, a potent PDE inhibitor (25, 26). Some 1,4-dihydropyridine calcium antagonists, such as nitrendipine and felodipine, appear to inhibit the phosphorylation of smooth muscle myosin light chains by binding to calmodulin and inhibiting the activation of myosin light chain kinase (14), a process which seems to be essential for induction of smooth muscle contraction (27–
Relaxation of smooth muscle induced by binding to calmodulin and by inhibition of calmodulin-dependent myosin light chain phosphorylation had been also observed with calmodulin antagonists, TFP and W-7 (30, 31). This study showed that CD-349 inhibited calmodulin-dependent cyclic AMP PDE in a manner different from other 1,4-dihydropyridine calcium antagonists, and a similar influence of EGTA on the inhibitory effects of CD-349 and TFP was observed in the cyclic AMP PDE in the coronary artery. In our study, [3H]-CD-349 showed calcium ion dependent binding to calmodulin with a stoichiometry of one molecule of CD-349 to one molecule of calmodulin and a Kd value of about 2 μM (M. Tanaka et al., unpublished observation). From these findings, the different effects of CD-349 on the calcium/calmodulin-dependent cyclic nucleotide PDE system might have a role at least in part in the muscle relaxation, besides the main mechanism of a calcium antagonist, which is inhibition of the calcium influx.

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