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INHIBITION OF CALMODULIN FUNCTION BY CV-159, A NOVEL DIHYDROPYRIDINE COMPOUND

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Abstract—1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine-dicarboxylic acid methyl 6-(5-phenyl-3-pyrazolyloxy)hexyl ester (CV-159), a new compound synthesized from dihydropyridine, was examined for its effect on calmodulin (CaM) function. The concentration of CV-159 producing 50% inhibition of Ca^{2+}/CaM activated myosin light chain kinase (MLC kinase) was 6.2 μM. The apparent K_i value of CV-159 was 0.8 μM for MLC kinase. On the other hand, the concentration of CV-159 producing 50% inhibition of Ca^{2+}/CaM activated cyclic nucleotide phosphodiesterase (Ca^{2+}-PDE) was 0.55 μM. CaM antagonized competitively the CV-159-induced inhibition of activation of both MLC kinase and Ca^{2+}-PDE. Interaction of CV-159 with CaM was also demonstrated by fluorescence studies using dansyl-CaM (5-dimethylaminonaphthalene-1-sulfonylated CaM). CV-159 produced a decrease in fluorescence intensity of dansyl-CaM, in a Ca^{2+}-dependent fashion, and the concentration of this drug producing 50% inhibition of dansyl-CaM fluorescence was 1.2 μM. However, the concentration of nicardipine producing 50% inhibition of MLC kinase exceeded 100 μM. CaM did not antagonize the nicardipine-induced inhibition of Ca^{2+}-PDE. These results suggest that the action of CV-159 is unique in that it inhibits both Ca^{2+}-PDE and MLC kinase, through interaction with calmodulin. CV-159 seems to be a different class of drug from known dihydropyridine compounds.

Ca^{2+} is a vital component of the intracellular signal system that regulates the activity of various cells. A large number of Ca^{2+}-regulated functions are mediated by a class of related Ca^{2+}-binding proteins. It is generally accepted that the contractile mechanism in skeletal muscle can be explained by the tropomyosin–troponin theory for the Ca^{2+}-regulation [1, 2] and troponin C has been shown to function as a Ca^{2+}-binding protein. On the other hand, the regulation of vascular smooth muscle contraction differs considerably. One of the most extensively studied regulatory mechanisms is the Ca^{2+}/calmodulin (CaM)‖-dependent phosphorylation of 20,000-dalton myosin light chain [3, 4]. CaM is a major Ca^{2+}-binding protein, present in all eukaryocytes, and its primary structure is highly conserved throughout the animal and plant kingdom [5]. The use of a specific antagonist should make for a rapid elucidation of CaM-dependent reactions. W-7, the CaM antagonist we synthesized, prevents superprecipitation and myosin ATPase activity of the smooth muscle actomyosin by inhibiting CaM-mediated MLC kinase [6]. Moreover, bepridil, a calcium antagonist, inhibits the CaM-induced activation of MLC kinase and displaces [^{3}H]W-7 binding to the CaM molecule [7]. One or another of the dihydropyridine derivatives interacts with CaM, but they do not have a CaM antagonistic action [8–10]. In the present study, we observed the effects of a newly synthesized dihydropyridine derivative (CV-159) on CaM-dependent enzymes such as MLC kinase and Ca^{2+}/CaM-dependent cyclic nucleotide phosphodiesterace (Ca^{2+}-PDE), and CaM conformation.

MATERIALS AND METHODS

CV-159 was synthesized in the Laboratory of Tokyo Tanabe Pharmaceutical Co., Ltd. (Tokyo, Japan), and the chemical structure is shown in Fig. 1. Nicardipine was purchased from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). A-3 was synthesized according to Hidaka *et al.* [11]. [$^{32}\gamma$-P]-ATP, cyclic [8-^{3}H]AMP, and cyclic [8-^{3}H]GMP were purchased from Amersham International Ltd. (U.K.). Trypsin (bovine pancreas), trypsin inhibitor (soybean) and snake venom (*Crotalus atrox*) were purchased from The Sigma Chemical Co. (St Louis, MO). All other reagents were of the highest grade available. CaM was isolated from bovine brain, as described previously [12]. MLC kinase was purified from chicken gizzard, according to Adelstein and Klee [13]. Myosin light chain, used as substrate for the kinase assay, was prepared according to the procedure by Perrie and Perry [14] and was free from CaM [15]. Ca^{2+}-PDE was partially purified from

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‖ Abbreviations used: CV-159, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine-dicarboxylic acid methyl 6-(5-phenyl-3-pyrazolyloxy)hexyl ester; CaM, calmodulin; MLC kinase, myosin light chain kinase; PDE, phosphodiesterase; dansyl-CaM, 5-dimethylamino-naphthalene-1-sulfonylated calmodulin; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate.

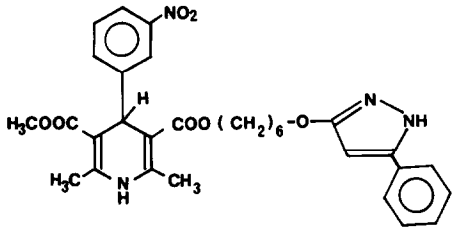

Fig. 1. Chemical structure of 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine-dicarboxylic acid methyl 6-(5-phenyl-3-pyrazolyloxy)hexyl ester (CV-159).

bovine brain by the method of Kincaid *et al.* [16]. Trypsin-treated MLC kinase and trypsin-treated Ca^{2+}-PDE was prepared as described [7, 17]. Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase were partially purified from human platelets, as described [18–20]. Dansyl-CaM was also prepared by the method of Kincaid *et al.* [21].

MLC kinase assay. MLC kinase activity was measured, as described [22]. Unless otherwise noted, the enzymatic reaction was assayed in a volume of 0.2 ml of 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 24 nM CaM, 0.2 mg/ml myosin light chain, 30 μM ATP, and 52 ng of MLC kinase at 30°.

Phosphodiesterase assay. Phosphodiesterase was assayed as described [23]. The reaction mixture (0.5 ml) contained 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.4 μM cyclic [8-^{3}H]AMP or cyclic [8-^{3}H]GMP, 0.1 mM $CaCl_2$ and 24 nM CaM or 1 mM EGTA and an appropriate dilution of the enzyme.

Dansyl-CaM fluorescence spectra. Dansyl-CaM fluorescence spectra were recorded using an Aminco-Bowman spectrophotofluorometer equipped with a high-stability xenon lamp scanning from 200 to 800 nm and measurements were conducted at 25° with a thermoregulator. Measurement of the CV-159-induced inhibition of dansyl-CaM fluorescence was carried out with an excitation wavelength of 345 nm and the emission spectra were recorded from 200 to 600 nm.

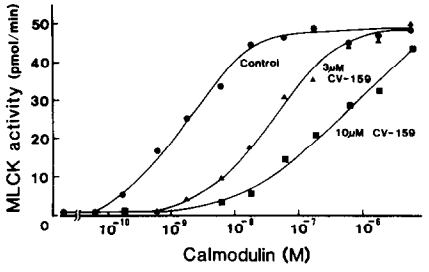

Fig. 3. Inhibition of the CaM stimulation of MLC kinase by CV-159. Activity of MLC kinase was assayed in the presence of 0.1 mM $CaCl_2$ and various concentrations of CaM: ●, in the absence of CV-159 (control); ▲, in the presence of 3 μM CV-159; ■, in the presence of 10 μM CV-159.

RESULTS

Inhibition of myosin light chain phosphorylation

The effects of CV-159 and nicardipine on phosphorylation of myosin light chain by MLC kinase were examined. The addition of CV-159 to the reaction mixture resulted in a marked inhibition of the Ca^{2+}/CaM-activated MLC kinase activity above 1 μM and the concentration of CV-159 producing 50% inhibition (IC_{50}) of activity was about 6.2 μM (Fig. 2). However, nicardipine had weaker effects on the Ca^{2+}/CaM-activated MLC kinase activity and the IC_{50} value of nicardipine-induced inhibition of the activity exceeded 100 μM. On the other hand, both compounds had little or no effect on the trypsin-treated MLC kinase activity, compared with A-3, an inhibitor of MLC kinase [24]. Figure 3 shows the effect of CaM on MLC kinase in the absence or presence of 3 μM and 10 μM CV-159. The CV-159-induced inhibition of MLC kinase activity was restored by the addition of excess CaM. When the inhibition of MLC kinase by CV-159 was analyzed by Dixon plots (Fig. 4), the resulting K_i value of CV-159 for the enzyme was 0.8 μM. Therefore, CV-159 may inhibit MLC kinase activity, in a competitive fashion with this concentration of CaM.

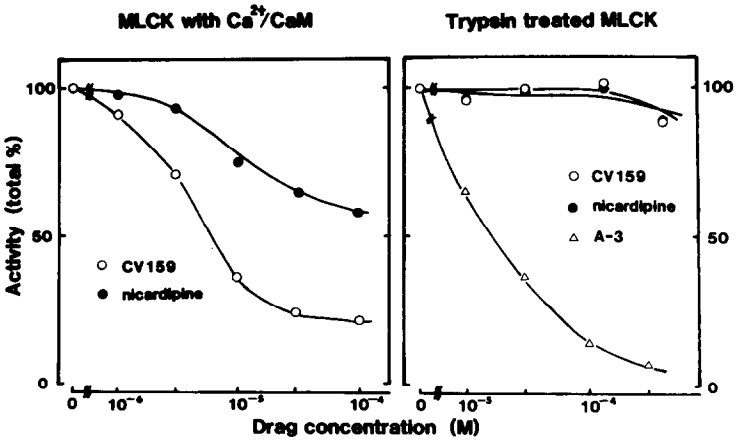

Fig. 2. Effect of CV-159 and nicardipine on myosin light chain phosphorylation. Isolated 20,000-dalton myosin light chain was phosphorylated by MLC kinase containing 24 nM CaM and 0.1 mM $CaCl_2$ (left panel), and by trypsin treated MLC kinase in 1 mM EGTA without Ca^{2+}/CaM (right panel). ○, CV-159; ●, nicardipine; △, A-3.

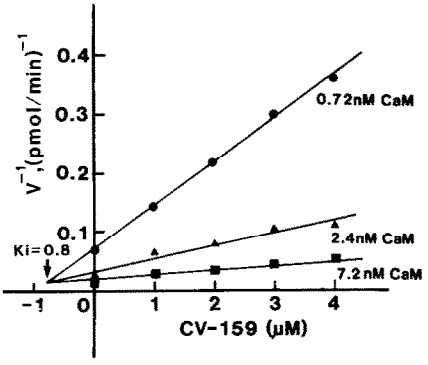

Fig. 4. Kinetic analysis of CV-159-induced inhibition of MLC kinase. Kinetic analysis of CV-159-induced inhibition of activation of MLC kinase was made using Dixon plots. MLC kinase activity was measured in the presence of 0.1 mM $CaCl_2$ and various concentrations of CV-159. Other assay conditions are described under Materials and Methods.

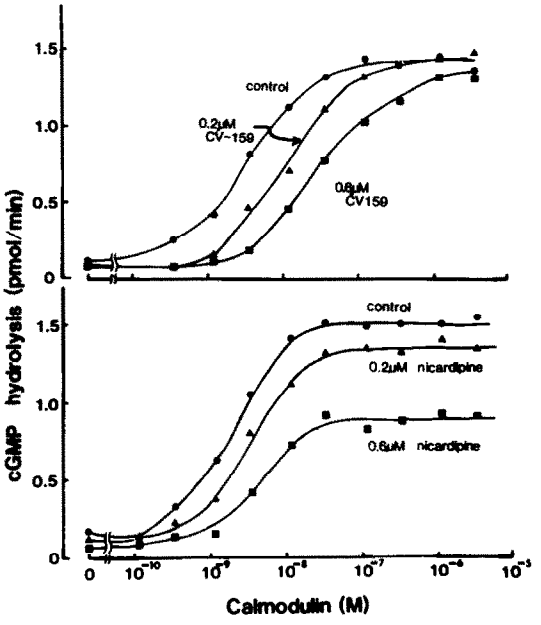

Fig. 5. Inhibition of the CaM stimulation of Ca^{2+}-PDE by CV-159 and by nicardipine. Ca^{2+}-PDE activity was measured using 0.4 μM cyclic [^{3}H]GMP as substrate in the presence of 0.1 mM $CaCl_2$ and various concentrations of CaM.

Effects of CV-159 and nicardipine on various phosphodiesterase activities

Both CV-159 and nicardipine were tested for their ability to inhibit the different forms of cyclic nucleotide phosphodiesterases (Table 1). Nicardipine proved to be a potent inhibitor of all the forms of phosphodiesterases, with the range of IC_{50} values being from 0.52 to 26 μM. Similar results were reported by Epstein *et al.* [9]. Although CV-159, a dihydropyridine derivative, was a potent and selective inhibitor of Ca^{2+}-PDE, this drug had essentially no effect on cyclic AMP or cyclic GMP phosphodiesterase below 300 μM. The CV-159 or nicardipine-induced inhibition of Ca^{2+}-PDE was tested further by examining the ability to alter the activation of Ca^{2+}-PDE by CaM (Fig. 5). In the case of CV-159-induced inhibition of Ca^{2+}-PDE, excess CaM could overcome this inhibition. However, CaM did not antagonize the effect of nicardipine on the Ca^{2+}-PDE activity. This indicated that the inhibition by nicardipine may be due to interaction of the drug with Ca^{2+}-PDE itself.

Interaction of CV-159 and nicardipine with dansyl-CaM

It has been reported that 5-dimethylamino-naphthalene-1-sulfonyl-calmodulin (dansyl-CaM) fluorescence can serve as a probe for the interactions between CaM and CaM-binding proteins or drugs [21, 25]. The fluorescence emission spectrum of dansyl-CaM was dramatically altered by the addition of Ca^{2+} and there was a "blue shift" from 525 to 495 nm and an increase in the intensity. Thus, we investigated the effects of CV-159 and nicardipine on dansyl-CaM fluorescence. In the absence of Ca^{2+}, the fluorescence intensities were little affected by either drug (data not shown). In contrast, in micromolar ranges, both CV-159 and nicardipine decreased the dansyl-CaM fluorescence in the presence of Ca^{2+} (Fig. 6). Johnson *et al.* reported that dansyl-CaM fluorescence was increased by felodipine, a dihydropyridine Ca^{2+} antagonist [25]. However, they did not report the effects of the other dihydropyridine derivatives on dansyl-CaM fluoresence. These observations suggest that the

Table 1. Inhibitions of various PDEs by CV-159 and by nicardipine

	IC_{50} (μM)*	
	CV-159	Nicardipine
Ca^{2+} PDE (with Ca^{2+}/CaM)†	0.55 ± 0.03	0.52 ± 0.02
Ca^{2+} PDE (trypsin treated)‡	1.0 ± 0.18	1.3 ± 0.35
cAMP-PDE§	>300	4.0 ± 0.34
cGMP-PDE§	>300	26 ± 5.6

* The IC_{50} value is defined as the concentration of the drug required to produce 50% inhibition of the enzyme activity and each value was mean ± SE of six experiments with six different populations.

† Ca^{2+}-PDE from bovine brain.

‡ Limited digestion of Ca^{2+}-PDE.

§ PDE from human platelets.

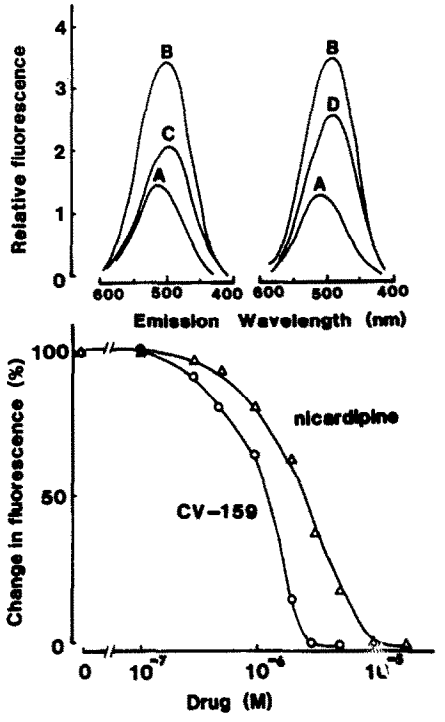

Fig. 6. Effect of CV-159 and nicardipine on dansyl-CaM fluorescence. Top panel: dansyl-CaM fluorescence spectra of 10 mM Mops (pH 7.0), 25 μM dansyl-CaM, 90 mM KCl, 1 mM EGTA (curve A). The subsequent addition of 2 mm $CaCl_2$ produced the increase observed in curve B and the subsequent addition of 2 μM CV-159 (curve C) or 2 μM nicardipine (curve D) produced inhibition of the Ca^{2+}-dependent fluorescence increase of dansyl-CaM. Bottom panel: dose-dependent inhibition of dansyl-CaM fluorescence by CV-159 and nicardipine. The percent of total decrease in dansyl-CaM fluorescence as a function of added CV-159 (○) and nicardipine (△).

effects of CV-159 or nicardipine on CaM differed from the effect of felodipine on CaM. The titration of 2.5 μM dansyl-CaM with CV-159 or nicardipine-produced fluorescence decreases are also given in Fig. 6 (lower panel). The half maximal bindings of CV-159 and nicardipine with dansyl-CaM were 1.3 and 2.4 μM, respectively. Studies on dansyl-CaM revealed that the affinity of CV-159 for dansyl-CaM was relatively higher than that of nicardipine.

DISCUSSION

Biochemical and clinical studies of dihydropyridine calcium channel blockers have led to molecular characterization of the dihydropyridine binding property to target proteins. Boström *et al.* reported that felodipine altered the ^{113}Cd-nuclear magnetic resonance spectrum of CaM [8]. Felodipine also affected the dansyl-CaM fluorescence, as reported by Johnson and Witteneuer [25]. Moreover, nicardipine and nimodipine will inhibit CaM-dependent cyclic nucleotide phosphodiesterase (Ca^{2+}-PDE) [9]. However, Thayer and Fairhurst suggested that nicardipine binds to another site on CaM, as does chlorpromazine, pimozide, or Ca^{2+} [10]. We examined the effects of two types of the dihydropyridine derivatives (CV-159, and nicardipine) on another type of CaM-dependent enzyme (MLC kinase). Nicardipine could interact with CaM, as revealed in the dansyl-CaM studies. However, the addition of excess CaM did not overcome the inhibition of Ca^{2+}-PDE by nicardipine, and nicardipine inhibited various types of cyclic nucleotide phosphodiesterases. Moreover, the IC_{50} value of inhibition of MLC kinase by nicardipine exceeded 100 μM. We reported that nifedipine, a dihydropyridine derivative, interacted directly with Ca^{2+}-PDE and did not affect the MLC kinase activity [26]. These observations suggest that most dihydropyridine Ca^{2+} channel blockers are potent inhibitors of Ca^{2+}-PDE and may not inhibit calmodulin activity. Movsesian *et al.* reported that turkey gizzard MLC kinase was inhibited by felodipine and nitrendipine [27]. They also described Ca^{2+}-dependent binding of nitrendipine to CaM. But, they did not define whether excess CaM could overcome the inhibition of MLC kinase. CaM concentration is important to clarify the CaM-antagonistic actions of drugs. CV-159 is also a dihydropyridine derivative, but the chemical structure of the drug differs from other dihydropyridine derivatives, such as nicardipine, nifedipine and felodipine. CV-159 inhibited not only Ca^{2+}/CaM-activated Ca^{2+}-PDE, but also trypsin treated Ca^{2+}-PDE, which was active without Ca^{2+}-CaM (Table 1). However, the inhibition of Ca^{2+}/CaM-activated Ca^{2+}-PDE by CV-159 disappeared in the presence of excess CaM. These observations suggest that this novel compound preferentially binds to CaM, when these two proteins, CaM and Ca^{2+}-PDE, exist at same time. Ca^{2+}/CaM-activated MLC kinase was strongly inhibited by CV-159, at a micromolar range, with no inhibition of the trypsin treated MLC kinase, which was active in the absence of Ca^{2+}/CaM. The interaction of a hydrophobic probe with CaM, in a Ca^{2+}-dependent fashion, has been reported [28, 29], and naphthalenesulfonamide derivatives and antipsychotics are CaM antagonists, which bound CaM, Ca^{2+}-dependently, as revealed in fluorescence studies [25]. However, some drugs were reported to bind CaM molecule in the presence of Ca^{2+}, with no inhibition of the CaM-dependent activities of enzymes [9]. In the present work, we obtained evidence that both nicardipine and CV-159 interact with dansyl-CaM and inhibit the fluorescence intensity, in the presence of Ca^{2+}. However, nicardipine did not antagonize the CaM-activated MLC kinase activity. On the other hand, the dihydropyridine calcium antagonist felodipine did not inhibit the dansyl-CaM fluorescence, rather the fluorescence was amplified [25]. These findings may be due to different drug binding sites on CaM. Fluorescent studies also suggest a cooperative interaction between CV-159 and CaM, while the Dixon plots are consistent with 1 : 1. However, the concentrations of CaM used in fluorescent studies much higher than that in kinetic analysis, and further examination will be needed to explain the discrepancy.

Our findings suggest that the newly synthesized dihydropyridine drug, CV-159, is a potent CaM antagonist which has a different binding site on CaM,

as compared to nicardipine, trifluoperazine, W-7 and prenylamine. This drug should enable precise definitions of the CaM–enzyme interactions.

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