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Actions of CP-060*S* on veratridine-induced Ca^{2+} overload in cardiomyocytes and mechanical activities in vascular strips

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Abstract

CP-060*S*, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate, is a novel cardioprotective drug which is designed to prevent Ca^{2+} overload and cause vasorelaxation. The effects of this compound were evaluated and compared with those of CP-060*R* (enantiomer of CP-060*S*,) and diltiazem (Ca^{2+} channel antagonist) in a veratridine-induced model of Ca^{2+} overload and vasorelaxation. After 5-min superfusion of veratridine (74 μM), intracellular free calcium concentrations ($[Ca^{2+}]_i$) of rat single cardiomyocytes, as measured with the fura-2 procedure, were greatly elevated, from 44 ± 5 nM to 3705 ± 942 nM, and subsequently generated cell contracture. Pretreatment of cardiomyocytes with more than 300 nM of CP-060*S* or CP-060*R* for 30 min provided almost complete protection against the veratridine-induced cell contracture; in CP-060*S*(1 μM)-treated myocytes, $[Ca^{2+}]_i$ were minimal and partially elevated from 42 ± 5 nM to 72 ± 14 nM after 5 min of veratridine superfusion. In comparison, diltiazem showed no protection below 1 μM and only partial protection at 10 μM. CP-060*S*, CP-060*R* and diltiazem all shifted the concentration-response curve for $CaCl_2$ to the right in a competitive manner in depolarized rat thoracic aorta. The pA_2 values of CP-060*S*, CP-060*R* and diltiazem were 9.16 ± 0.18, 8.24 ± 0.14 and 7.66 ± 0.09, respectively. Our results indicate that CP-060 behaves stereoselectively as a Ca^{2+} channel antagonist and non-stereoselectively to protect against veratridine-induced contracture. The latter effect suggests that Ca^{2+} entry blockade is not the mechanism by which CP-060*S* exerts cardioprotection.

Keywords: CP-060*S*; Ca^{2+} overload; Veratridine; Cardiomyocyte; Ca^{2+} channel; Smooth muscle, vascular

1. Introduction

It has been demonstrated in clinical studies that the L-type Ca^{2+} channel antagonists have beneficial effects in the treatment of hypertension, angina pectoris and arrhythmias. It has been also expected that these drugs would exert beneficial effects in patients treated after acute myocardial infarction, because they prevent the coronary vasospasms and decrease oxygen consumption in cardiac tissue. Furthermore, they have been shown to reduce infarct size in experimental animal models of ischemia-reperfusion (Bush et al., 1982; Reimer and Jennings, 1984).

In contrast, as described below, several clinical trials suggested that Ca^{2+} channel antagonists provided only limited therapeutic benefits in the patients with acute myocardial infarction. Treatment with nifedipine neither reduced infarct size as determined by enzyme levels (Sirnes et al., 1984) nor decreased the cardiac events (Wilcox et al., 1986). Furthermore, diltiazem exerted no overall effect on mortality or cardiac events in patients with previous infarction (The Multicenter Diltiazem Postinfarction Trial Research Group, 1988), and was only effective to prevent early reinfarction and severe angina after non-Q-wave infarction (Gibson et al., 1986). Treatment with verapamil after acute myocardial infarction reduced the number of major cardiac events only in patients without heart failure (The Danish Study Group on Verapamil in Myocardial Infarction, 1990).

In pathological conditions such as ischemia or reperfusion, it is considered that Ca^{2+} overload plays a critical role in the structural damage and functional failure (Bourdillon and Poole-Wilson, 1981; Borgers et al., 1987; Steen-

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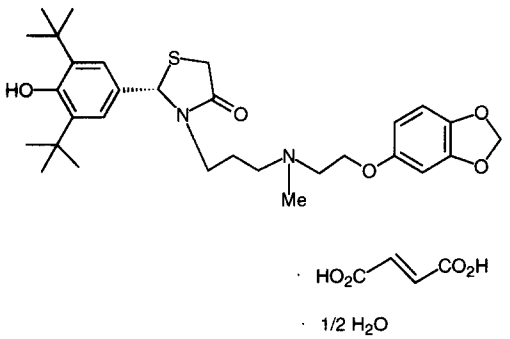

Fig. 1. Chemical structure of CP-060*S*, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate.

bergen et al., 1990). It is also reported that the Na^+/Ca^{2+} exchange, but not the L-type Ca^{2+} channel, represents an important pathway of Ca^{2+} overload preceded by Na^+ accumulation (Tani, 1990). These data suggest an important reason why Ca^{2+} channel antagonists showed only limited cardiac protection in the clinical trials mentioned above.

Therefore, we screened for a novel drug which prevents Ca^{2+} overload and has Ca^{2+} channel antagonistic activity, characteristics considered to be more convenient for patients with ischemic heart diseases such as angina pectoris and acute myocardial infarction. CP-060*S*, (−)-(*S*)-2-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-3-[3-[*N*-methyl-*N*-[2-(3,4-methylenedioxyphenoxy)ethyl]amino]propyl]-1,3-thiazolidin-4-one hydrogen fumarate (Fig. 1) potently prevented Ca^{2+} overload, was structurally novel, and relaxed vascular strips like a Ca^{2+} channel antagonist.

We therefore report for the first time on a compound, CP-060*S*, with dual actions, namely, vasorelaxation via Ca^{2+} channel antagonism and protection against veratridine-induced Ca^{2+} overload which is independent of Ca^{2+} channel antagonistic activity.

2. Materials and methods

2.1. Ca^{2+} overload in cardiomyocytes

Isolated ventricular myocytes were prepared from heart tissue of adult male Sprague-Dawley rats (Charles River Japan), weighing between 400 and 510 g according to a modified method of Powell et al. (Powell and Twist, 1976; Powell et al., 1980). After intravenous injection of 1000 U/kg heparin, the hearts were excised and perfused retrogradely through the aorta at 37°C with a flow rate of 10 ml/min. Initially the hearts were perfused for 5 min with Ca^{2+}-free Krebs-Henseleit buffer-A (KH-A; composition in mM: NaCl 118.5, $NaHCO_3$ 14.5, KCl 2.6, KH_2PO_4 1.18, $MgSO_4$ 1.18, glucose 11.1) gassed with 95% O_2-5% CO_2 (pH 7.4) at a flow rate of 10 ml/min to remove blood and subsequently for 20 min by recirculation of KH-A containing 25 μM $CaCl_2$, 375 U/ml collagenase (Yakuruto, Japan) and 0.1% bovine serum albumin (fatty acid-free, Sigma Chemical Co., USA). The ventricles were cut into small pieces, and incubated at 37°C for 10 min in the KH-A containing 25 μM $CaCl_2$, 250 U/ml collagenase and 1% bovine serum albumin. After the myocytes were dispersed by gentle agitation, the Ca^{2+} concentration in KH-A was raised to 1.0 mM by addition of $CaCl_2$. Finally, myocytes were transferred into Hepes-buffered physiological salt solution-A (PSS-A; composition in mM: NaCl 125.0, KCl 2.6, $CaCl_2$ 1.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 5.5, Hepes 10.0), and rod-shaped Ca^{2+} tolerant myocytes were obtained. All experiments using myocytes were performed at room temperature.

Ca^{2+} overload was induced by exposure to 74 μM veratridine in PSS-A (Ver Donck and Borgers, 1991). Drugs were added as pretreatment for 30 min before applying veratridine. More than 100 rod-shaped myocytes were used in the morphological study. The rod-shaped cells were counted twice, i.e. just before drug treatment and 5 min after veratridine application in the same area. The rod-shaped cell ratio was calculated as (the number of rod-shaped cells after veratridine application)/(the number of rod-shaped cells before drug treatment) × 100.

Cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using fura-2 as follows: myocytes were incubated with PSS-A containing 5 μM fura-2 acetoxymethyl ester (AM) (Molecular Probes, USA), 0.075% Pluronic F-127 and 1% bovine serum albumin for 30 min at room temperature in the dark. The cells were then washed three times with PSS-A to remove the external fura-2 AM. Rod-shaped myocytes with clear sarcomeres, sharp edges and no blebs, and considered to be intact were selected for measurement. The cells were placed on a glass cover-slip with Teflon wall (1.5 ml volume), and superfused at 1 ml/min with PSS-A. To determine the $[Ca^{2+}]_i$, a fura-2-loaded cell was illuminated with a 300 W xenon lamp through an interference and neutral density filter. The excitation wavelengths were 340 and 380 nm. Both filters had half-width of 10 nm. The fluorescence of a fura-2-loaded single isolated heart cell was imaged using a Nikon inverted microscope (TMD, 20× fluor objective, NA = 0.75). Images at 510 nm (half-width 40 nm) were obtained using a silicon intensified target (SIT) camera (C-2400-08, Hamamatsu Photonics KK, Japan) and stored in a digital imaging processor (Argus-100, Hamamatsu Photonics KK, Japan). The ratio image of each individual pixel was determined by dividing the image of fluorescence intensity at 340 nm by that at 380 nm after background subtraction. The autofluorescence of the myocytes was less than 10% of that of the fura-2-loaded cells.

The fura-2 340/380 ratios were converted into absolute Ca^{2+} concentrations with the following equation as reported by Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (Sf_2/Sb_2)$.

In this system, we were able to obtain the values of R_{min}, R_{max}, Sf_2 and Sb_2 by in vivo calibration according

to the method of Li et al. (1987) as follows: $R_{min} = 0.54$, $R_{max} = 9.17$, $Sf_2 = 117$ and $Sb_2 = 11$.

2.2. Vasorelaxant studies in rat aortic strips

Thoracic aortas were removed from male Sprague Dawley rats (370–500 g; Charles River Japan), dissected free from surrounding connective tissue, and cut into ring segments each about 2–3 mm long. Each strip of smooth muscle was mounted for isometric tension recording in an organ bath filled with 10 ml of Hepes-buffered physiological salt solution-B (PSS-B; composition in mM: NaCl 140.0, KCl 5.0, $CaCl_2$ 1.5, $MgCl_2$ 1.0, glucose 10.0, Hepes 5.0) or Krebs-Henseleit solution-B (KH-B; composition in mM: NaCl 119.0, KCl 4.8, CaCl2 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8, glucose 10.0). PSS-B was gassed with 100% O_2 and KH-B with 95% O_2-5% CO_2, and these solutions were maintained at pH 7.4 at 37°C. The strips were given a stretched tension of 2 g and allowed to equilibrate for more than 30 min. Isometric tension changes were monitored using an isometric transducer (Nihon Kohden Co., TB-611T) and recorded on a self-balancing potentiometric recorder (Yokogawa Co., 3066).

For the Ca^{2+} channel antagonism study, a contraction was first obtained by applying 60 mM K^+ PSS-B, then preparations were exposed to normal PSS-B for 20 min, to Ca^{2+}-free 60 mM K^+ PSS-B containing 0.1 mM EGTA for 20 min and to nominally Ca^{2+}-free 60 mM K^+ for 20 min. Cumulative concentration-response curves for $CaCl_2$ were then constructed. After washing of the preparations with PSS-B, second concentration-response curves to $CaCl_2$ were made in a similar manner except for pretreatment with CP-060*S* or CP-060*R* for 240 min or diltiazem for 60 min. Vehicle was also added as pretreatment in a time-matched way as control. Schild plots for competitive antagonism were made from the dose ratios (DR) of the agonist for four different relaxant drug concentrations so as to estimate the pA_2 value and the slope of the regression line for each organ segment (Arunlakshana and Schild, 1959).

Vasorelaxant effects against phenylephrine-, U46619- or 5-hydroxytryptamine (5-HT)-induced contractions were examined as follows. First, contractions were obtained by applying 1 μM phenylephrine, 10 nM U46619 or 10 μM 5-HT, then preparations were washed with normal KH-B. After 60 min, cumulative concentration-response curves to each agonist were made. Preparations were exposed to normal KH-B, then second concentration-response curves to each agonist were made after 240 min pretreatment with CP-060*S* or CP-060*R*, or for 60 min with diltiazem.

2.3. Drugs

The following substances were used: CP-060*S* and CP-060*R* (synthesized in our laboratory); diltiazem, *l*-phenylephrine, 5-hydroxytryptamine, A-23187 and veratridine (Sigma Chemical Co., USA); U-46619 (Cayman Chemical Co., USA).

2.4. Statistics

The values recorded were expressed as means ± S.E.M. (n = number of preparations). Protective effects against veratridine-induced morphological changes were analyzed using Student's *t*-test with Bonferroni correction. Comparisons of the multiple drug-treated groups vs. the vehicle-treated group in the experiments on $[Ca^{2+}]_i$ measurement were carried out using Dunnett's test. Differences with a P value of 0.05 or lower were considered significant.

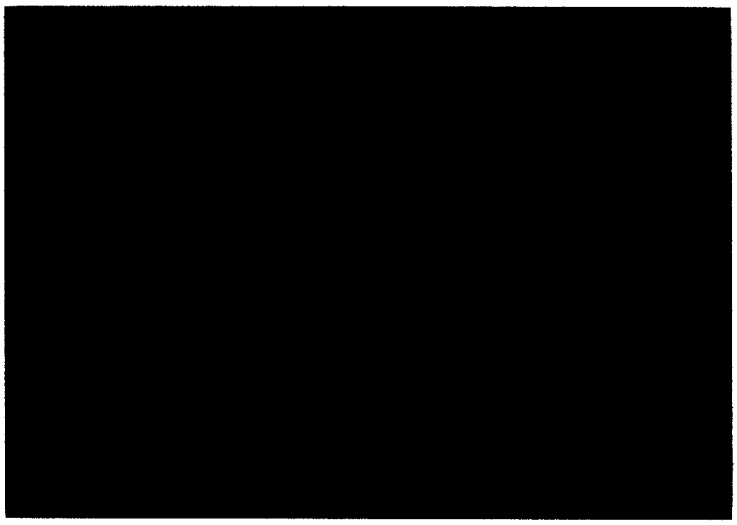

Fig. 2. Pseudocolor image of fluorescence ratios in a single rat ventricular myocyte. The color scale represents the $[Ca^{2+}]_i$. The myocyte was pretreated with either vehicle (left) or 1 μM CP-060*S* (right) for 30 min. $[Ca^{2+}]_i$ was measured just before (upper), 3 min after (middle) and 5 min after (lower) the 74 μM veratridine superfusion.

3. Results

3.1. Prevention of Ca^{2+} overload in cardiomyocytes

Fig. 2 demonstrates the pseudocolor images of fluorescence ratios in a single cardiomyocyte before and after veratridine superfusion. The color scale represents the $[Ca^{2+}]_i$. Exposure of quiescent myocytes to 74 μM veratridine caused elevation of $[Ca^{2+}]_i$ followed by hypercontraction in the following manner: 3 min after the veratridine treatment, $[Ca^{2+}]_i$ in the myocyte was markedly raised and the shape of the cell was shortened. At 5 min after treatment, $[Ca^{2+}]_i$ was further elevated and the myocyte hypercontracted. In contrast, pretreatment of 1 μM CP-060*S* almost completely prevented both the increase of $[Ca^{2+}]_i$ and change in morphology. Therefore, this model demonstrates the effect of Ca^{2+} overload in cardiomyocytes in vitro, and provides evidence for the protective effect of CP-060*S*.

The dose-response for the protective effects of CP-060*S*, CP-060*R* and diltiazem against veratridine-induced hypercontracture are shown in Fig. 3. Five minutes after the

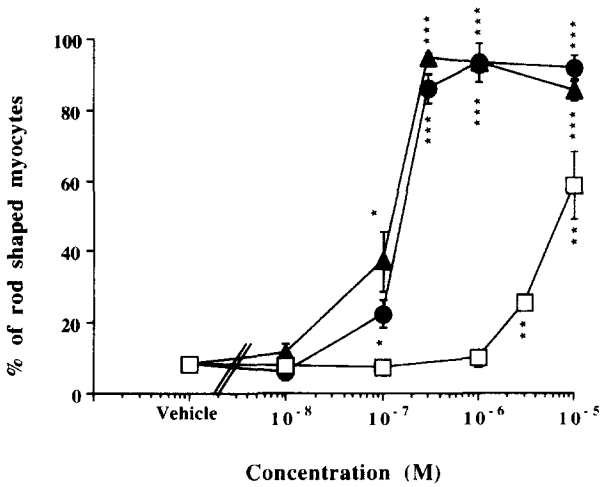

Fig. 3. Protective effects of CP-060*S* (closed circle), CP-060*R* (closed triangle) and diltiazem (open square) against veratridine-induced hypercontraction in rat cardiac myocytes. Each drug was added as pretreatment for 30 min. Morphological changes of the myocytes were observed 5 min after veratridine (74 μM) exposure. More than 100 myocytes were used in each experiment. Each point is the mean derived from five to six experiments with S.E.M. shown by vertical bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. vehicle-treated group.

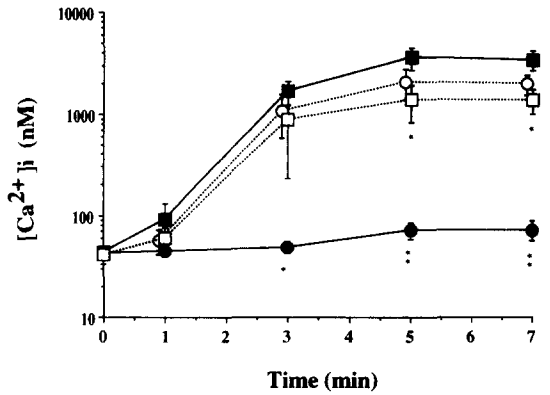

Fig. 4. Time course of the change in $[Ca^{2+}]_i$ in a single cardiac myocyte after veratridine superfusion. The myocyte was pretreated with 1 μM CP-060*S* (closed circle), 1 μM diltiazem (open circle), 10 μM diltiazem (open square) or vehicle (closed square) for 30 min. $[Ca^{2+}]_i$ in each cell was measured just before, 1 min, 3 min, 5 min and 7 min after the 74 μM veratridine superfusion. Each point is the mean derived from ten to thirteen cells with S.E.M. shown by vertical bars. * $P < 0.05$; ** $P < 0.01$ vs. vehicle-treated group.

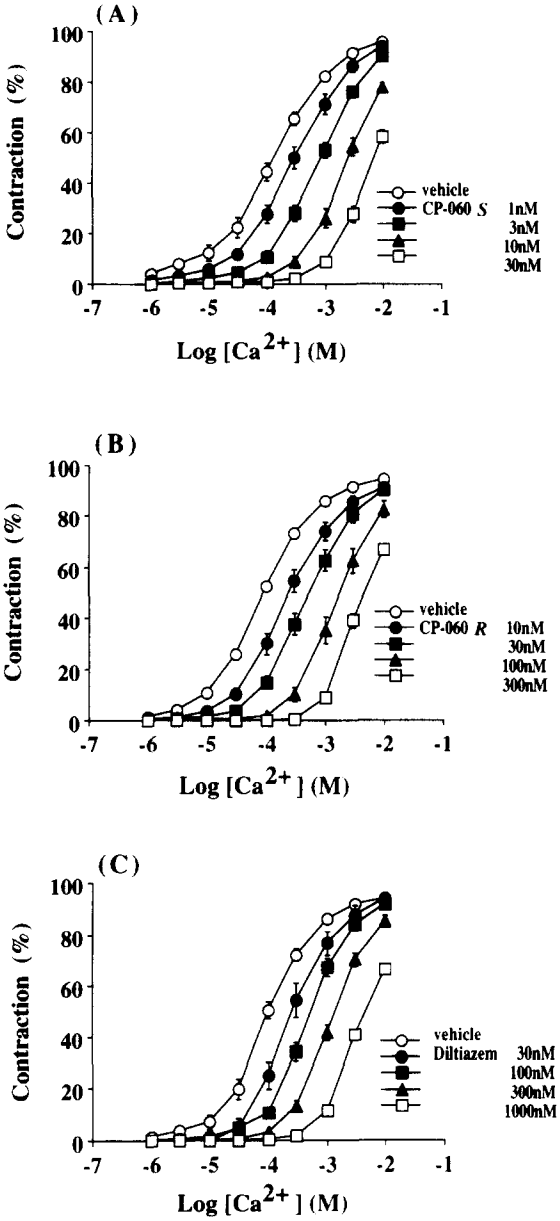

Fig. 5. Antagonism by CP-060*S* (A), CP-060*R* (B) or diltiazem (C) of Ca^{2+}-induced contractions of the depolarized rat aorta. CP-060*S* or CP-060*R* was applied 240 min and diltiazem was applied 60 min before the Ca^{2+} treatment. Each point is the mean derived from five experiments with S.E.M. shown by vertical bars.

exposure to veratridine the number of rod-shaped myocytes was largely decreased to 8.1 ± 2.3%. CP-060*S* and CP-060*R* inhibited the reduction of the number of rod-shaped cells in a concentration-dependent manner. Pretreatment with 0.1 μM CP-060*S* and CP-060*R* showed partial prevention against hypercontracture with rod-shaped cell ratio of 22.6 ± 4.0% and 37.3 ± 8.5%, respectively. Moreover, when cells were treated with more than 0.3 μM of CP-060*S* or CP-060*R*, more than 80% of the cells remained rod-shaped. In contrast, diltiazem below 1 μM did not show any significant protection, but only 58.9 ± 9.6% of the cells remained rod-shaped when 10 μM diltiazem was added as pretreatment.

The time course of the change in $[Ca^{2+}]_i$ after veratridine superfusion is shown in Fig. 4. In the vehicle-treated group, $[Ca^{2+}]_i$ was dramatically increased from 44 ± 5 nM to 3705 ± 942 nM after 5 min veratridine superfusion. Pretreatment with 1 μM of CP-060*S* significantly and almost completely inhibited this rise compared with the vehicle-treated group, i.e. $[Ca^{2+}]_i$ of CP-060*S* (1 μM)-treated myocytes showed only a slight elevation from 42 ± 5 nM to 72 ± 14 nM after 5-min veratridine superfusion. In contrast, 1 μM of diltiazem did not show significant prevention and 10 μM of diltiazem exerted a significant but only slight prevention, i.e. at 5 min after veratridine superfusion, the $[Ca^{2+}]_i$ of diltiazem (1 and 10 μM)-treated myocytes was increased from 41 ± 8 nM to 2143 ± 733 nM and from 41 ± 5 nM to 1398 ± 541 nM, respectively.

3.2. Effects of CP-060S on Ca^{2+} concentration-response curves in rat aorta

$CaCl_2$ produced a concentration-dependent contraction of depolarized rat thoracic aorta. Limited solubility of $CaCl_2$ restricted its use at concentrations exceeding 10 mM.

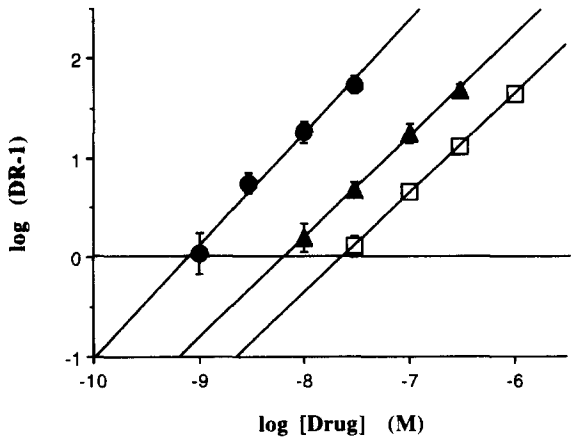

Fig. 6. Schild regression for CP-060*S* (closed circle), CP-060*R* (closed triangle) and diltiazem (open square). The plot was obtained by use of the DR calculated from EC_{50} values of the concentration-response curves in Fig. 5. Each point is the mean derived from five experiments with S.E.M. shown by vertical bars.

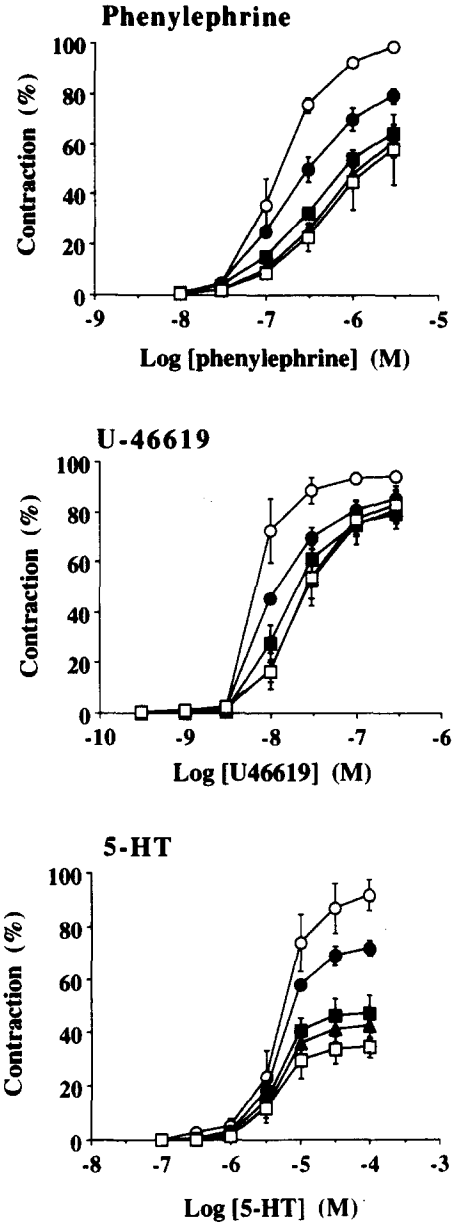

Fig. 7. Effects of CP-060*S* on cumulative concentration-response curves produced by phenylephrine, U-46619 and 5-HT in rat aorta. CP-060*S* was applied for 240 min before agonist treatment: vehicle (open circle); CP-060S 0.01 μM (closed circle), 0.1 μM (closed square), 1 μM (closed triangle), 10 μM (open square). Each point is the mean derived from three experiments with S.E.M. shown by vertical bars.

Since the vasorelaxant action of CP-060*S* and CP-060*R* started slowly, we tested the effect of duration of drug pretreatment. Maximum vasorelaxing actions occurred after 240-min pretreatment with both CP-060*S* and CP-060*R*, whereas this was only 60 min with diltiazem.

CP-060*S* and CP-060*R*, as well as diltiazem, produced a parallel, rightward displacement of the concentration-response curves for $CaCl_2$ in depolarized rat thoracic aorta

(Fig. 5). The Schild plots for antagonism of $CaCl_2$ by CP-060*S*, CP-060*R* and diltiazem gave regression lines with slopes of 1.14 ± 0.12, 1.02 ± 0.07 and 1.01 ± 0.07 ($n = 5$, each), respectively, and these slopes were not significantly different from unity (Fig. 6). Thus, CP-060*S*, CP-060*R* and diltiazem behaved as competitive antagonists of Ca^{2+}-induced contraction with pA_2 values of 9.16 ± 0.18, 8.24 ± 0.14 and 7.66 ± 0.09 ($n = 5$, each), respectively.

3.3. Effects of CP-060S on agonist-induced contractions in rat aorta

Fig. 7 shows the inhibitory effects of CP-060*S* against phenylephrine-, U-46619- or 5-HT-induced contractions in rat aortic strips. Pretreatment of the tissues with CP-060*S* (0.01–10 μM) produced rightward displacement and decreased the maxima of the concentration-response curves for phenylephrine, U-46619 and 5-HT. Thus, CP-060*S* showed non-competitive inhibition against contractions induced by these agonists and the inhibitory effects of CP-060*S* on agonist-induced contractions were weaker than those on Ca^{2+}-induced contraction. CP-060*R* and diltiazem exerted similar but less potent inhibitory effects against contractions induced by these agonists (data not shown).

On the other hand, in aorta contracted with A-23187, a Ca^{2+} ionophore which transports Ca^{2+} as a lipid soluble complex through membranes (Schaffer et al., 1973), CP-060*S* (0.1–10 μM) or diltiazem (0.1–10 μM) had only weak vasorelaxant activity. In these preparations, however, sodium nitroprusside (0.3 μM) and forskolin (1 μM) which increase cyclic GMP (Ignarro and Kadowitz, 1985) and cyclic AMP (Seamon et al., 1981), respectively, evoked relaxation (Fig. 8).

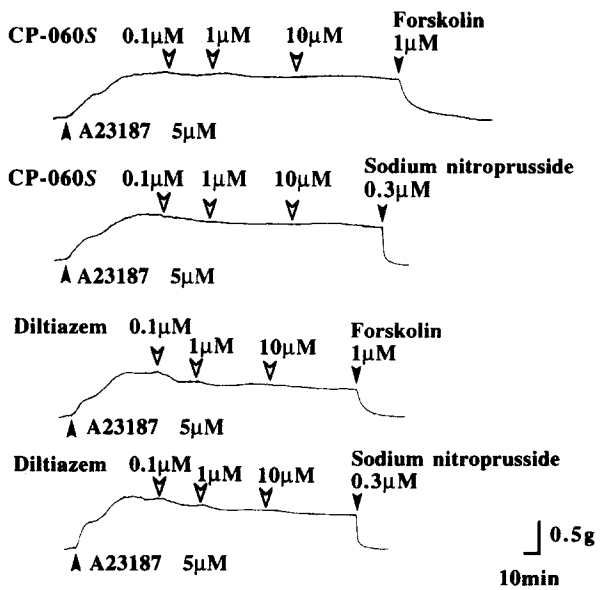

Fig. 8. Effects of CP-060*S* and diltiazem on A-23187-induced contraction in endothelium-denuded rat aortic strips.

4. Discussion

In the present study, we clarified the actions of CP-060*S* as a Ca^{2+} overload-preventing drug with an ability to relax vascular strips.

An important mechanism underlying the onset of ischemic or ischemia-reperfusion injury is considered to be the loss of Ca^{2+} homeostasis followed by Ca^{2+} overload, which plays a critical role in structural damage and functional failure (Bourdillon and Poole-Wilson, 1981; Borgers et al., 1987; Steenbergen et al., 1990). It is also suggested that Ca^{2+} overload is caused by a preceding intracellular Na^+ accumulation during ischemia or anoxia, and after reperfusion (Malloy et al., 1990; Tani and Neely, 1990; Van Echteld et al., 1991; Haigney et al., 1992). Several pathways for Na^+ influx have been postulated, one of which involves tetrodotoxin-sensitive non-inactivating Na^+ channels. Lysophosphatidylcholine (Burnashev et al., 1991, Undrovinas et al., 1992) which accumulates during ischemia and reperfusion (Sobel et al., 1978; Corr et al., 1987), and, oxygen radicals (Bhatnagar et al., 1990) cause long-lasting bursts of opening and prolong the inactivation process of the Na^+ channel. Hence, we utilized veratridine, which increases Na^+ influx by suppressing the inactivation process of Na^+ channels (Leibowitz et al., 1986; Sunami et al., 1993), as a tool for Na^+ loading into the myocyte and consequently produces Ca^{2+} overload.

As shown in Fig. 2, elevation of $[Ca^{2+}]_i$ preceded cell contracture in veratridine-treated myocytes. This implies that the Ca^{2+} overload observed in this model is not caused by the non-selective pathways of Ca^{2+} influx due to membrane damage in hypercontracted cells. It is likely that Ca^{2+} overload is induced by the preceding Na^+ influx and subsequent activation of the Na^+/Ca^{2+} exchange, after which membrane damage and cell contracture occur.

The myocytes used in this study were quiescent and not electrically stimulated. Therefore, it is considered that the pathway of Ca^{2+} influx through the voltage-dependent Ca^{2+} channels makes a minor contribution to the Ca^{2+} overload in this model. Indeed, diltiazem showed weak protection against veratridine-induced Ca^{2+} overload; in contrast, CP-060*S* was potently protective, i.e. this agent inhibited both accumulation of $[Ca^{2+}]_i$ and hypercontracture. CP-060*R* also protected from hypercontracture caused by veratridine with similar potency to CP-060*S*, however, its Ca^{2+} channel antagonistic activity in smooth muscle was stereoselective. These results suggest strongly that Ca^{2+} entry blockade is not the mechanism by which CP-060*S* exerts protection. The possible mechanisms which protect from veratridine-induced injury could be blockade of Na^+ channels or inhibition of the Na^+/Ca^{2+} exchange. The mechanisms of CP-060*S* concerning cardioprotection are under further investigation.

It was reported that a compound, R56865, which inhibits the veratridine-induced Ca^{2+} overload (Ver Donck and Borgers, 1991), could protect from ischemia- and reperfusion-induced arrhythmia in the rat (Garner et al., 1990) and rabbit (Verscheure et al., 1995), and could reduce the infarct size of regionally ischemic reperfused porcine hearts (Klein et al., 1995).

Further investigations are required to evaluate the cardioprotective effects of CP-060*S* in vivo and to clarify its precise mechanisms for preventing Ca^{2+} overload. It is expected, however, that, as with R56865, CP-060*S* may also potently protect against ischemia- and reperfusion-induced injury.

In addition to its inhibitory effects on Ca^{2+} overload, CP-060*S* as well as diltiazem, produced competitive inhibition of the Ca^{2+}-induced contractions of the depolarized rat aorta, which suggests that it also acts as a Ca^{2+} channel antagonist. Furthermore, CP-060*S* showed non-competitive inhibition against the contractions induced by phenylephrine, U-46619 and 5-HT in rat thoracic aorta. The inhibitory effects on agonist-induced contractions were less potent than those of Ca^{2+}-induced contraction. Also, CP-060*S* failed to relax the A-23187-induced contraction in rat aortic strips. These observations further substantiate the Ca^{2+} channel antagonistic action of CP-060*S* as will be explained.

The Ca^{2+}-induced contraction in depolarized muscle is produced mainly due to the Ca^{2+} influx from the extracellular medium through voltage-dependent Ca^{2+} channels of the plasma membrane. Contractions induced by agonists such as phenylephrine, U-46619 and 5-HT are, however, considered to occur via the following three routes: activation of receptor-operated non-selective cation channels followed by activation of voltage-dependent Ca^{2+} channels (Nelson et al., 1990); activation of Ca^{2+} influx by a newly discovered messenger named CIF (Ca^{2+} influx factor) that is released when Ca^{2+} stores are empty (Randriamampita and Tsien, 1993); Ca^{2+} release from sarcoplasmic reticulum caused by inositol 1,4,5-trisphosphate (Somlyo et al., 1985). The contraction induced by A-23187, a Ca^{2+} ionophore which transports Ca^{2+} as a lipid soluble complex through a membrane, does not occur *via* voltage-dependent Ca^{2+} channels (Asano and Hidaka, 1984). Therefore, Ca^{2+} channel antagonists inhibit the contraction induced by Ca^{2+} in depolarized strips more strongly than that induced by agonists, and may not inhibit the contraction induced by A-23187, as was the case with CP-060*S*.

In general, Ca^{2+} channel antagonists either do not affect, or even increase the clinically seen mortality and myocardial reinfarction rate. One reason is that Ca^{2+} channel antagonists cannot protect from Ca^{2+} overload as mentioned above. Although the mechanisms are not yet clarified, it is clear that CP-060S potently protects from Ca^{2+} overload independently from Ca^{2+} channel antagonism.

In conclusion, we have described a novel anti-ischemic drug, CP-060*S*, which possesses not only a Ca^{2+} overload-preventing activity but also Ca^{2+} channel antagonistic activity. It is expected that this agent will be developed to treat ischemic diseases.

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