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Mechanisms of vasoinhibitory effect of cardioprotective agent, CP-060S, in rat aorta

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Abstract

Vasoinhibitory effects of (−)-(*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 (CP-060S), a synthesized cardioprotective agent, were examined. In the rat aortic rings, the contractile responses to cumulative application of angiotensin II, [Arg^8]-vasopressin (vasopressin), or prostaglandin $F_{2\alpha}$ were inhibited by CP-060S in a concentration-dependent manner. The Ca^{2+}-induced contractions in the presence of vasopressin or prostaglandin $F_{2\alpha}$ were also inhibited by CP-060S in a concentration-dependent manner. The inhibitory effect of 10^{-5} M CP-060S on phenylephrine-induced contraction was as potent as that of 10^{-6} M nifedipine, and the combined addition of 10^{-6} M nifedipine and 10^{-5} M CP-060S showed the effect similar to that of 10^{-5} M CP-060S alone. In rat aorta loaded with a Ca^{2+} indicator, fura-PE3, 10^{-5} M CP-060S completely inhibited the high K^+-induced increase in cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and contraction. In contrast, 10^{-5} M CP-060S only partially inhibited the increase in $[Ca^{2+}]_i$ and contraction due to phenylephrine or prostaglandin $F_{2\alpha}$. In the presence of 10^{-6} M nifedipine, 10^{-5} M CP-060S did not inhibit the increase in $[Ca^{2+}]_i$ and contraction induced by prostaglandin $F_{2\alpha}$. In a Ca^{2+}-free medium, the phasic increases in contraction and $[Ca^{2+}]_i$ induced by phenylephrine were not affected by 10^{-5} M CP-060S. These results suggest that the vasoinhibitory effect of CP-060S in rat aortic rings is due mainly to the inhibition of L-type voltage-dependent Ca^{2+}-channels.

Keywords: CP-060S; Aorta, rat; Ca^{2+} channel, L-type voltage-dependent; Ca^{2+}, Cytosolic

1. Introduction

Antagonists of the L-type voltage-dependent Ca^{2+} channel have been demonstrated in clinical studies to have beneficial effects in the treatment of hypertension, angina pectoris and arrhythmia. A new generation of Ca^{2+} channel antagonists appears to be useful, not only in the treatment of cardiovascular disease, but also in disease of the central nervous system, including focal and global cerebral ischemia and age-related cognition deficits and epilepsy (Scriabine and Janis, 1990). It has recently been reported that (−)-(*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 (CP-060S), a recently synthesized cardioprotective agent, not only caused vasorelaxation of rat isolated aorta by inhibition of L-type Ca^{2+} channels, but also prevented the veratridine-induced Ca^{2+} overload in cardiomyocytes by the mechanism not related to Ca^{2+} entry blockade (Tamura et al., 1996; Adachi et al., 1999). The blockade of Na^+ channels or inhibition of Na^+/Ca^{2+} exchange was suggested to be a possible mechanism by which CP-060S inhibits the Ca^{2+} overload (Tamura et al., 1996; Adachi et al., 1999). CP-060S was also reported to inhibit ischemia- and reperfusion-induced arrhythmia in anesthetized rats (Koga et al., 1996) and to limit myocardial infarct size in anesthetized dogs (Suzuki et al., 1998a). In guinea pig papillary muscles and cardiomyocytes, CP-060S was reported to inhibit not only Ca^{2+} currents by interacting with three principal binding sites on the L-type Ca^{2+} channel, but also non-inactivating Na^+ currents

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(Suzuki et al., 1998b; Fukuzawa et al., 1997; Tanabe et al., 1997). In guinea pigs mesenteric arteries, CP-060S exhibited the profile of the L-type Ca^{2+} channel antagonists, similar to that of diltiazem and gallopamil, whereas higher concentrations of CP-060S inhibited the delayed K^+ channel currents (Ohya et al., 1997). All of these results suggest that CP-060S has multiple sites of action in both smooth and cardiac muscles.

To know the precise mechanism of vasodilator effects of CP-060S, in the present experiments, we examined the effect of this compound on muscle tension and cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) in the vascular smooth muscle of isolated rat aorta.

2. Materials and methods

2.1. Mechanical responses

Male Wistar rats weighing 150–200 g were killed by rapid exsanguination under ether anesthesia. Thoracic aorta was isolated and adhering fat and connective tissues were removed. Ring preparations of the aorta were prepared (2–3 mm length) and each preparation was placed in a 25 ml organ bath containing physiological salt solution (in mM: NaCl 120.3, KCl 4.8, $CaCl_2$ 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 24.2, glucose 5.5) maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. A resting tension of 20 mN was applied to each muscle strip. The isometric tension was recorded through a force-displacement transducer (FT-03) connected to a six-channel Grass polygraph.

2.2. Cytosolic Ca^{2+} level

The thoracic aorta was isolated as described above and cut into helical strips (2–3 mm wide and 8–10 mm long). Cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) was measured using a Ca^{2+} indicator, fura-PE3, simultaneously with muscle tension as reported by Ozaki et al. (1987) and Sato et al. (1988). Muscle rings were loaded with 5×10^{-6} M acetoxymethyl ester of fura-PE3 (fura-PE3/AM) (Vorndranet et al., 1995) for 3–5 h in the presence of 0.02% cremophor EL at room temperature (23–25°C), and then placed in a tissue bath at 37°C. The muscle strip was illuminated alternately with 340 and 380 nm light, and the amount of 500 nm fluorescence induced by 340 nm excitation (F340) and that induced by 380 nm excitation (F380) was detected with a spectrophotometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). Ca^{2+}-free medium was made by removing $CaCl_2$ and adding 0.2 mM EGTA, unless otherwise stated.

2.3. Chemicals

The following chemicals were used: CP-060S (Chugai Pharmaceutical, Shizuoka, Japan), nifedipine, diltiazem,

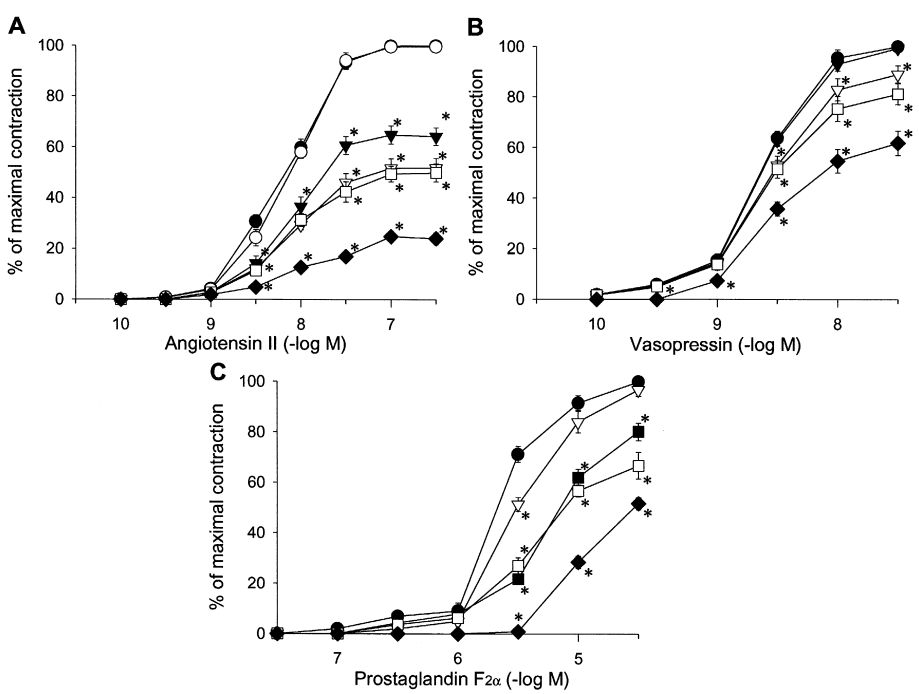

Fig. 1. Inhibitory effects of CP-060S on contraction elicited by angiotensin II, vasopressin, or prostaglandin $F_{2\alpha}$. The aortic rings were contracted by (A) angiotensin II (10^{-10}–3×10^{-7} M), (B) vasopressin (10^{-10}–3×10^{-8} M) and (C) prostaglandin $F_{2\alpha}$ (3×10^{-8}–3×10^{-5} M). CP-060S (●, control; ○, 10^{-9} M; ▼, 10^{-8} M; ▽, 10^{-7} M; ■, 3×10^{-7} M; □, 10^{-6} M; ◆, 10^{-5} M) was added to the bath 20 min before the addition of angiotensin II, vasopressin, or prostaglandin $F_{2\alpha}$. Data are expressed as the mean ± S.E.M. of five experiments. * Significantly different from respective controls ($P < 0.05$).

phenylephrine, angiotensin II, [Arg^8]-vasopressin (vasopressin), prostaglandin $F_{2\alpha}$ were obtained from Sigma (St. Louis, MO). Fura-PE3/AM was obtained from TEF LABS (Austin, TX).

2.4. Statistical analysis

Data are presented as the mean ± S.E.M. and statistically analyzed by using Student's *t*-test. Differences are considered significant at $P < 0.05$.

3. Results

In normal Krebs–Ringer solution, the cumulative addition of angiotensin II (10^{-10}–3×10^{-7} M; Fig. 1A), vasopressin (10^{-10}–3×10^{-8} M; Fig. 1B) or prostaglandin $F_{2\alpha}$ (3×10^{-8}–3×10^{-5} M; Fig. 1C) induced contractile responses in a concentration-dependent manner in rat aortic rings. Pretreatment of the aortic rings by CP-060S (10^{-9}–10^{-5} M) for 20 min inhibited the responses to these agonists in a concentration-dependent manner.

In rat aortic rings incubated in a Ca^{2+}-free medium containing vasopressin (10^{-8} M) or prostaglandin $F_{2\alpha}$ (5×10^{-6} M), a cumulative addition of Ca^{2+} (10^{-5}–10^{-2} M) induced contractile responses in a concentration-dependent manner (Fig. 2A,B).

In the experiment summarized in Fig. 3, the inhibitory effects of maximally effective concentrations of CP-060S and nifedipine were compared. In rat aortic rings, a cumulative addition of phenylephrine (3×10^{-9}–10^{-5} M) induced contractile responses in a concentration-dependent manner. Pretreatment of the aortic rings with CP-060S (10^{-5} M) or nifedipine (10^{-6} M) significantly inhibited

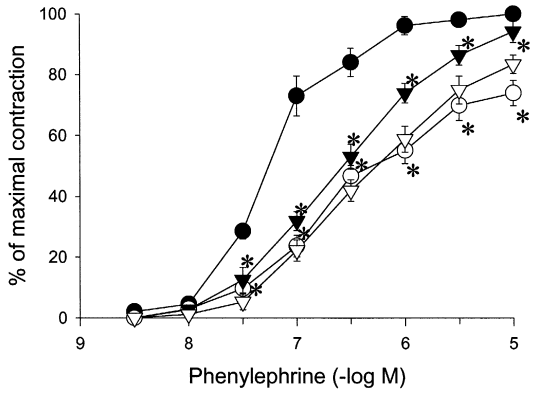

Fig. 3. . Inhibitory effects of CP-060S and nifedipine on contractile responses to phenylephrine. The aortic rings were contracted by phenylephrine (3×10^{-9}–10^{-5} M; ●). CP-060S (10^{-5} M; ○), nifedipine (10^{-6} M; ▼), and CP-060S (10^{-5} M) plus nifedipine (10^{-6} M; ▽) were added to the bath 20 min before the addition of phenylephrine. Data are expressed as the mean ± S.E.M. of five experiments. * Significantly different from respective controls ($P < 0.05$).

the responses to phenylephrine. The inhibitory effect of 10^{-5} M CP-060S was slightly but significantly stronger than that of 10^{-6} M nifedipine. Simultaneous addition of 10^{-5} M CP-060S and 10^{-6} M nifedipine showed the effect, which was not different from that of 10^{-5} M CP-060S or 10^{-6} M nifedipine alone.

In the fura-PE3 loaded rat aorta, high K^+ (72.7 mM), phenylephrine (10^{-6} M), or prostaglandin $F_{2\alpha}$ (10^{-5} M) induced rapid increase in $[Ca^{2+}]_i$, which was followed by a relatively sustained increase. These changes were accompanied by sustained increase in muscle tension (Fig. 4A). Addition of CP-060S (10^{-5} M) to the sustained phase of high K^+-induced responses induced rapid decrease in $[Ca^{2+}]_i$ to the level similar to that before the addition of high K^+. Contraction was also almost completely inhibited

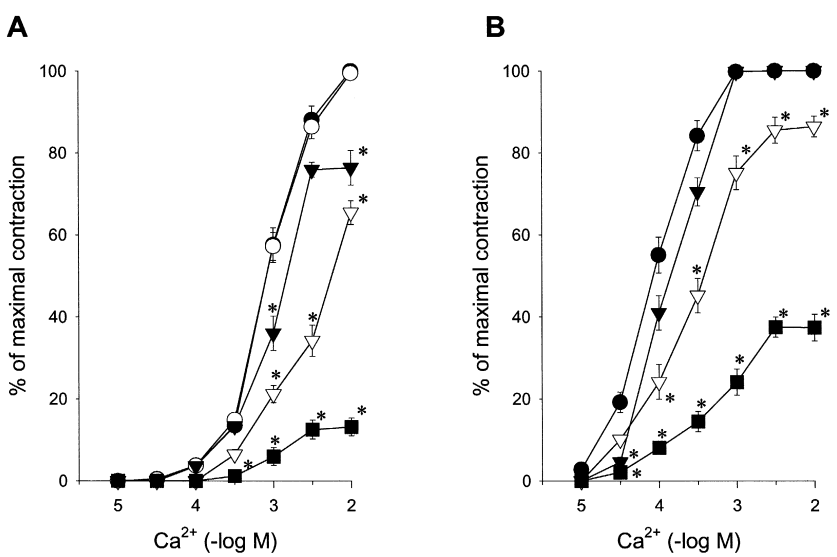

Fig. 2. The inhibitory effect of CP-060S on the contractile responses to Ca^{2+} in Ca^{2+}-free medium containing vasopressin or prostaglandin $F_{2\alpha}$. The aortic rings were incubated in Ca^{2+}-free medium containing (A) vasopressin (10^{-8} M) or (B) prostaglandin $F_{2\alpha}$ (5×10^{-6} M) for 30 min and then contracted by a cumulative addition of Ca^{2+}. CP-060S (●, control; ○, 10^{-8} M; ▼, 10^{-7} M; ▽, 10^{-6} M; ■, 10^{-5} M) was added to the bath 20 min before the addition of Ca^{2+}. Data are expressed as the mean ± S.E.M. of five experiments. * Significantly different from respective controls ($P < 0.05$).

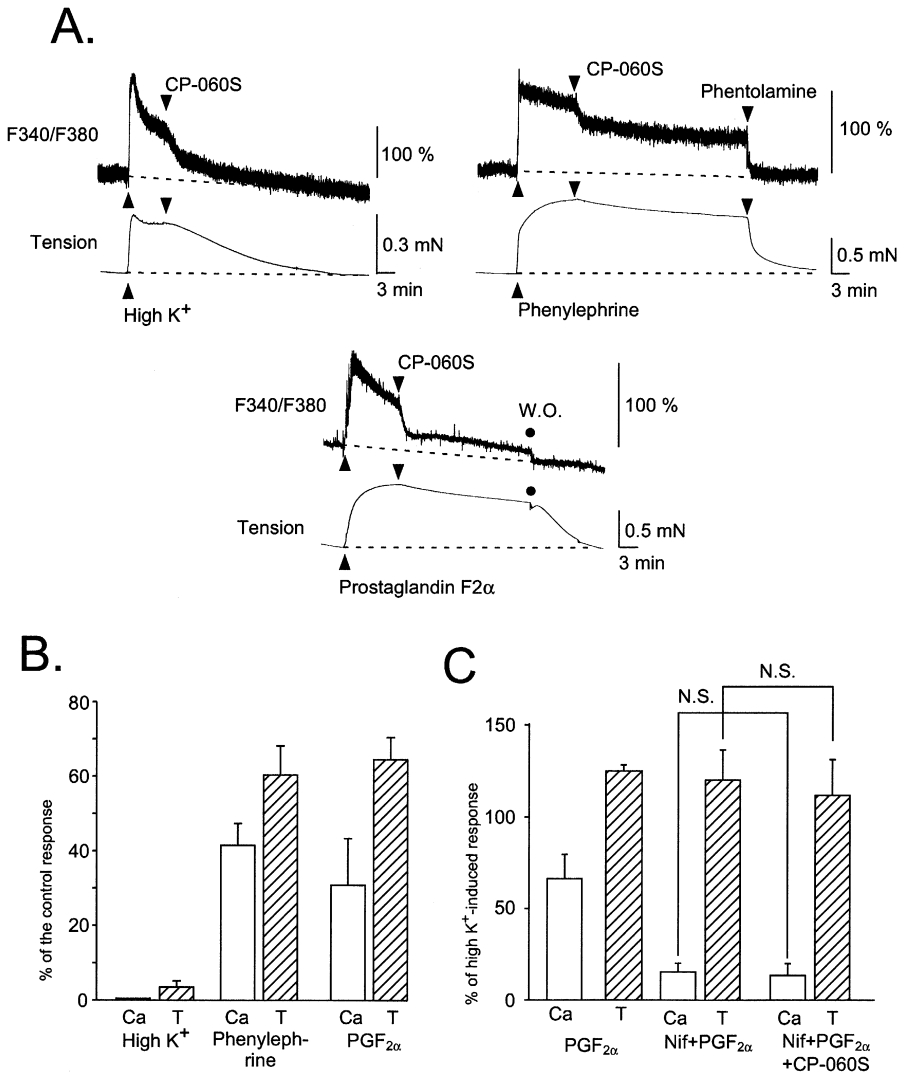

Fig. 4. Inhibitory effect of CP-060S on the increase in $[Ca^{2+}]_i$ and contraction due to high K^+, phenylephrine or prostaglandin $F_{2\alpha}$. (A) Typical recordings of the inhibitory effect of CP-060S on the increase in $[Ca^{2+}]_i$ (F340/F380) and muscle contraction (tension). The fura-PE3-loaded aorta was stimulated by high K^+ (72.7 mM), phenylephrine (10^{-5} M), or prostaglandin $F_{2\alpha}$ (10^{-5} M). After the changes in $[Ca^{2+}]_i$ and muscle tension reached a steady level, CP-060S (10^{-5} M) was applied. Phentolamine: 10^{-5} M. W.O.: Wash out. (B) The responses remaining 20 min after the application of CP-060S, taking the response before the addition of CP-060S (control response) as 100 %. (C) 10^{-5} M prostaglandin $F_{2\alpha}$-induced responses, 10^{-5} M prostaglandin $F_{2\alpha}$-induced responses in the presence of nifedipine (10^{-6} M), or the effect of CP-060S (10^{-5} M) in the presence of prostaglandin $F_{2\alpha}$ (10^{-5} M) and nifedipine (10^{-6} M), taking the responses to high K^+ (72.7 mM) measured before the addition of agonist or inhibitors as 100%. Data are expressed as the mean ± S.E.M. of four experiments. N.S.: not significantly different. $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$. Nif: nifedipine. Ca: $[Ca^{2+}]$; T: tension.

by 10^{-5} M CP-060S although the time course of this inhibition was slower than that of $[Ca^{2+}]_i$ (Fig. 4A,B).

Addition of CP-060S to the sustained phase of phenylephrine or prostaglandin $F_{2\alpha}$-induced responses also inhibited $[Ca^{2+}]_i$. The decrease in $[Ca^{2+}]_i$ due to 10^{-5} M CP-060S was 58.5 ± 5.9% for phenylephrine or 69.2 ± 12.6% for prostaglandin $F_{2\alpha}$. Thus, CP-060S showed less inhibitory effect on $[Ca^{2+}]_i$ stimulated by phenylephrine or prostaglandin $F_{2\alpha}$ than that stimulated by high K^+. CP-060S also inhibited the contractions induced by phenylephrine or prostaglandin $F_{2\alpha}$. However, the inhibitory effects were also smaller than that on high K^+-induced contraction (inhibitory of contraction was 30.7 ± 7.8% for phenylephrine-induced contraction or 35.6 ± 6.0% for prostaglandin $F_{2\alpha}$-induced contraction). Comparing the inhibitory effect of CP-060S on $[Ca^{2+}]_i$ and contraction induced by phenylephrine and prostaglandin $F_{2\alpha}$, $[Ca^{2+}]_i$ was more strongly inhibited than contraction (Fig. 4A,B). Portions of the phenylephrine-stimulated $[Ca^{2+}]_i$ and contraction, which were not inhibited by CP-060S, were abolished by phentolamine (10^{-5} M). The CP-060S-resistant portion of $[Ca^{2+}]_i$ and contraction simulated by prostaglandin $F_{2\alpha}$ were abolished by washing the muscle with normal physiological salt solution.

To further examine the mechanism of CP-060S, effects of CP-060S on contraction and $[Ca^{2+}]_i$ were examined in the rat aorta pre-incubated with nifedipine. As shown in Fig. 4C, 10^{-5} M prostaglandin $F_{2\alpha}$-induced increase in

Table 1
Effect of CP-060S on the phenylephrine-induced phasic contraction and transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium

	Contraction[a]	$[Ca^{2+}]_i$[b]
Control	52.4 ± 9.9	128.5 ± 14.7
CP-060S	89.9 ± 11.3	143.2 ± 19.8

[a] The fura-PE3-loaded aorta was incubated in a Ca^{2+}-free medium for 1 min and then phenylephrine (3×10^{-6} M) was added. CP-060S was added 20 min before the addition of phenylephrine.

[b] Percentage of contraction and $[Ca^{2+}]_i$, respectively, of the responses to high K^+ in normal Ca^{2+} medium measured 25 min before the addition of phenylephrine. Each value represents mean ± S.E.M. of five experiments.

$[Ca^{2+}]_i$ and contraction of 66.1 ± 13.1% and 123.6 ± 3.5%, respectively ($n = 4$), of the responses to high K^+ measured before starting the experiment. Twenty minutes after the application of 10^{-6} M nifedipine, 10^{-5} M prostaglandin $F_{2\alpha}$ also induced increase in $[Ca^{2+}]_i$ and contraction to 15.2 ± 2.4% and 118.3 ± 16.7%, respectively, $n = 4$, of the responses to high K^+ measured before the addition of nifedipine. After the prostaglandin $F_{2\alpha}$ responses became stable in the presence of nifedipine, 10^{-5} M CP-060S was applied for 20 min. However, CP-060S did not induce additional inhibition on $[Ca^{2+}]_i$ and contraction ($[Ca^{2+}]_i$ and contraction remaining in the presence of CP-060S were 13.5 ± 3.2% and 110.4 ± 19.8%, respectively, $n = 4$; Fig. 4C).

In the fura-PE3 loaded rat aorta incubated in a Ca^{2+}-free medium for 1 min, addition of phenylephrine (3×10^{-6} M) induced a phasic contraction and transient increase in $[Ca^{2+}]_i$. As shown in Table 1, phenylephrine-induced transient increase in $[Ca^{2+}]_i$ was greater than that induced by high K^+, whereas the contraction was smaller than that induced by high K^+, as reported previously (Sato et al., 1988). Pretreatment of the aorta with CP-060S (10^{-5} M) for 20 min changed neither contraction nor $[Ca^{2+}]_i$ (Table 1). Nifedipine (10^{-6} M) or diltiazem (10^{-5} M) also did not affect the phasic contraction induced by phenylephrine (10^{-6} M) in Ca^{2+}-free medium (data not shown).

4. Discussion

In vascular smooth muscle, mechanism of contraction induced by membrane depolarization is different from that induced by receptor agonists (for review, see Karaki et al. 1997). Membrane depolarization opens the L-type Ca^{2+} channel to increase $[Ca^{2+}]_i$ and, thus, induces contraction. Therefore, L-type Ca^{2+} channel antagonists strongly inhibit the contractions induced by membrane depolarization. In contrast, receptor-agonists open not only the L-type Ca^{2+} channel, but also the non-selective cation channel (Karaki et al., 1988; Kuriyama et al., 1995). In addition, agonists, but not membrane depolarization, increase Ca^{2+} sensitivity of contractile elements by modulating the protein phosphatase via an activation of the GTP-binding protein (Nishimura et al., 1988; Kitazawa et al., 1991). The third difference is that agonists, but not membrane depolarization, activate phosphatidylinositol turnover to generate inositol trisphosphate, which releases Ca^{2+} from intracellular storage sites (Sato et al., 1988). Because L-type Ca^{2+} channel antagonists do not inhibit the mechanisms to open non-selective cation channel, to increase Ca^{2+} sensitivity of contractile elements or to release Ca^{2+} from intracellular store, these antagonists have less potent inhibitory effect on agonist-induced contraction than on depolarization-induced contraction.

Previously, it has been shown in rat aorta that although CP-060S inhibits the contractions induced by both membrane depolarization and receptor-agonists, including phenylephrine, 5-hydroxyrtyptamine and a thromboxane A_2 analog, U-46619, the inhibitory effect on depolarization-induced contraction was much greater than that on agonist-induced contraction (Tamura et al., 1996). It has also been reported that CP-060S inhibits the Ca^{2+}-induced contraction of the depolarized rat aorta in a competitive manner (Tamura et al., 1996). In the present study, it was found that CP-060S also inhibited the contractile responses induced by angiotensin II, vasopressin or prostaglandin $F_{2\alpha}$ in a concentration-dependent manner.

To further examine if CP-060S has the effects similar to those of the L-type Ca^{2+} channel antagonists, we measured $[Ca^{2+}]_i$ of vascular smooth muscle simultaneously with contraction using a fluorescent Ca^{2+} indicator, fura-PE3. It was found that 10^{-5} M CP-060S almost completely inhibited high K^+-induced increase in muscle tension and $[Ca^{2+}]_i$. This result supports the suggestion that CP-060S has an effect to inhibit the L-type Ca^{2+} channel. CP-060S also inhibited the increase in muscle tension and $[Ca^{2+}]_i$ due to prostaglandin $F_{2\alpha}$ and phenylephrine although the effects were smaller than on the changes due to membrane depolarization. Furthermore, the inhibitory effect of CP-060S on $[Ca^{2+}]_i$ was greater than that on contraction in the muscle stimulated with prostaglandin $F_{2\alpha}$ or phenylephrine. This result is consistent with that of L-type Ca^{2+} channel antagonists reported previously (Karaki et al., 1991).

To further compare the effects of CP-060S and L-type Ca^{2+} channel antagonists, we examined if CP-060S and a typical L-type Ca^{2+} channel antagonist, nifedipine, have additional inhibitory effects. Results in Fig. 3 indicated that CP-060S and nifedipine showed almost similar inhibitory effect on the phenylephrine-induced contraction and combined application of CP-060S and nifedipine did not show greater inhibitory effect than either CP-060S or nifedipine alone. Furthermore, we confirmed that the increases in muscle tension and $[Ca^{2+}]_i$ due to prostaglandin $F_{2\alpha}$ were inhibited by either CP-060S or nifedipine and combined addition of both CP-060S and nifedipine did not show additional inhibitory effects on muscle tension and $[Ca^{2+}]_i$. These results again support the suggestion that the

vasodilator effect of CP-060S is due to inhibition of the L-type Ca^{2+} channel.

Because these effects of CP-060S are similar to those of L-type Ca^{2+} channel antagonists, we examined if this inhibitor inhibits Ca^{2+} release from storage sites. For this purpose, we used the transient increase in $[Ca^{2+}]_i$ and phasic contraction induced by phenylephrine in a Ca^{2+}-free medium, which have been shown to be due to Ca^{2+}-release (Shibata et al., 1987; Sato et al., 1988). Result indicated that CP-060S has no effect on the transient responses to phenylephrine in a Ca^{2+}-free medium, suggesting that CP-060S does not have inhibitory effect on intracellular Ca^{2+} mobilization.

From these results, we conclude that the vasodilator action of CP-060S in the rat aorta is most likely due to the inhibition of the L-type voltage-dependent Ca^{2+} channels.

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