In Vitro and ex Vivo Ca-Antagonistic Effect of 2-Methoxyethyl(E)-3-phenyl-2-propen-1-yl(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate (FRC-8653), a New Dihydropyridine Derivative

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(Received March 12, 1992)

The characteristics of calcium antagonism and vascular effect of 2-methoxyethyl(E)-3-phenyl-2-propen-1-yl(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate (FRC-8653) were investigated. FRC-8653 inhibited an increase in intracellular free calcium concentration during membrane depolarization in PC12 cells. FRC-8653 also inhibited the specific binding of $^3$H-nitrendipine to cardiac membranes, in a similar manner to nifedipine and nicardipine. FRC-8653 inhibited KCl- and CaCl₂-induced contractions in isolated rabbit aorta, but failed to affect noradrenaline-induced contraction. The vasorelaxing effect of FRC-8653 in rabbit aorta developed more slowly than that of nifedipine and nicardipine. In ex vivo experiment, the inhibitory effect of orally administered FRC-8653 against KCl-contraction in rat aorta lasted longer than that of nifedipine. These findings suggest that FRC-8653 dilates blood vessels by blocking calcium influx via dihydropyridine-sensitive, voltage-dependent calcium channels and that the vascular effects are slow in development and long in duration.

**Keywords** — 2-methoxyethyl(E)-3-phenyl-2-propen-1-yl(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate (FRC-8653); dihydropyridine; calcium-antagonism; vasorelaxing effect; fura-2; $^3$H-nitrendipine binding

Introduction

FRC-8653 is a new 1,4-dihydropyridine (DHP) compound synthesized in Fujirebio Inc. The chemical name is 2-methoxyethyl(E)-3-phenyl-2-propen-1-yl(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate. It has been shown that FRC-8653 has the hypotensive effect in experimentally hypertensive rats and dogs, and that its effect is slower in development and longer-lasting compared with those of nifedipine and nicardipine.¹ FRC-8653 also has inhibitory effects on Ca²⁺-influx by membrane depolarization in A10 cells,² and on Ba²⁺ inward current in the rabbit basilar artery.³

In the present study, we investigated the calcium antagonistic effect of FRC-8653 using cultured cells, cardiac membrane preparation and isolated blood vessels.

Materials and Methods

Male Japanese white rabbits (Tokyo Laboratory Animals Science Co., Japan), male Wistar rats (Japan SLC Inc., Japan), and male Sprague-Dawley (SD) rats (Charles River Japan, Inc., Japan) were used for the study.

Measurement of Intracellular Free Calcium Concentration — The intracellular free calcium was measured using a fluorescent calcium indicator, fura-2.⁴ PC12 cells, rat adrenal pheochromocytoma cells (ATCC No. CRL-1721), were suspended in HEPES physiological solution at 1.0 × 10⁶ cells/ml. The solution contained 120 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.0 mM CaCl₂, 25 mM HEPES, 10 mM glucose. The suspension was incubated with 2 mM fura-2/AM (Wako Pure Chemicals Ind., Japan) for 45 min at 37 °C. After washing the cells, a suspension at 1.0 × 10⁶ cells/ml was again prepared. Afterwards, the suspension was incubated with the test drug for 3 min at 37 °C.
and illuminated alternatively (48 Hz) at the excitation wavelength of 340 and 380 nm. Then the amount of 500 nm fluorescence induced by 340 nm excitation (F_{340}) and that induced by 380 nm excitation (F_{380}) were measured and the ratio of F_{340} to F_{380} was calculated automatically as an indicator of calcium concentration using a fluorimeter (CAF-100, Japan Spectroscopic). After membrane depolarization induced by 60 mM KCl, the change in the ratio was recorded. The results were expressed as inhibitory rate, taking the increase in ratio of the control group as 100%.

Membrane Preparations and Binding Assay — Eight week-old SD rats were killed by decapitation and the hearts were quickly removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose using a polytron (PT10-35, Kinematica). The homogenates were centrifuged for 10 min at 1000 \times g and the supernatant was centrifuged for 20 min at 48000 \times g. The resulting pellets were washed with 50 mM Tris-HCl buffer (pH = 7.4) and suspended to a final concentration of 100 mg wet weight/ml in Tris-HCl buffer. In a final volume of 1 ml, 0.1 ml of the membrane preparation was incubated with 2.3 \times 10^{-10} \text{M} ^3\text{H}-nitrendipine (85.9 Ci/mmol) and the drugs in Tris-HCl buffer for 90 min at 25 °C. The binding reaction was terminated by adding 3 ml of ice-cold Tris-HCl buffer and the mixture was then rapidly filtered under vacuum through Whatman GF/B glass fiber filters (Whatman) and washed three times with 3 ml of ice-cold Tris-HCl buffer. Assays were carried out in duplicate. The radioactivity retained was determined by liquid scintillation spectrometry. Specific binding was defined as that displaced by 10^{-5} \text{M} nifedipine. The results were expressed as percentages of the control binding.

Tension Measurement in Isolated Rabbit Aorta — The rabbits weighing 2.6—3.3 kg were sacrificed under anesthesia with pentobarbital Na. The thoracic aorta was removed, cleaned of surrounding connective tissues and cut into helical strips (5 \times 20 mm). The preparations were mounted in an organ bath containing 30 ml of physiological salt solution (PSS) gassed with 95% O_2 and 5% CO_2 at 37 ± 1 °C. PSS contained 118.2 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO_4\cdot7H_2O, 2.5 mM CaCl_2\cdot2H_2O, 1.2 mM KH_2PO_4, 24.8 mM NaHCO_3 and 10 mM glucose. The preparations were equilibrated for 2 h under resting tension of 1.5 g. The isometric tension was measured with a transducer (TB-612T, Nihon Kohden) and a carrier amplifier (AP-601G, Nihon Kohden), and was recorded on a recorder (WT-687G, Nihon Kohden). For the experiments of CaCl_2-induced contraction, modified PSS was used. It contained 80 mM KCl in equimolar replacement for NaCl and neither CaCl_2 nor Ca^{2+}-chelating agent was added. KCl, norepinephrine (NE) or CaCl_2 was cumulatively added to the organ bath to obtain the successive control contractions and then the preparation was washed. Afterwards, the preparations were incubated with the test drug for 90 min and KCl, NE or CaCl_2 were again cumulatively added. The contractile responses were expressed as percentages of the control contraction induced by 70 mM KCl, 3 \times 10^{-5} \text{M} NE or 1 mM CaCl_2, respectively. The test was performed in the shade and under a sodium lamp.

Investigation of the Time Course of Vasorelaxing Effect — The isometric tension measurement in rabbit aorta was carried out by the same method as described above. After the equilibrating period, sustained contractions were induced by 30 mM KCl and the test drugs were applied. The change of tension was continuously measured for up to 3 h. The tensions were expressed as percentages of the contraction induced by 30 mM KCl as 100%. In the preliminary experiment, it was confirmed that KCl-induced contraction was stable for more than 3 h.

Ex Vivo Effect of Orally Administered Drugs on KCl-Contraction in Rat Aorta — Wistar rats weighing 250—380 g were used for the test after fasting for 18 h. The animals were administered a single oral dose of 10 mg/kg of FRC-8653 or nifedipine. The thoracic aorta was removed at 1, 3, 7 or 24 h after administration, and cut into ring segments of 4 mm in length. The isometric tension measurement was performed as described above. After an equilibrating period under resting tension of 1 g, 50 mM KCl was added to the bath and the tonic contraction was measured. The tension measurement was performed within 2 h from the removal of
aorta.

**Drugs Used** — After dissolving FRC-8653 (Ajinomoto Co., Japan) in a 50% HCO-60 (Nikko Chemicals, Japan) ethanol solution, nifedipine (Sigma, St. Louis, MO) in a 1.5% polyvinylpyrrolidone K-30 (Gokyo Sangyo Inc., Japan) ethanol solution and nicardipine hydrochloride (Sigma) in distilled water, the solutions were diluted with distilled water. In the binding study, all drugs were dissolved in a 50% HCO-60 ethanol solution and diluted with distilled water. The 5% arabic gum suspension of drugs was used for oral administration.

**Statistical Analysis** — The results were expressed as mean ± S.E. The Student’s non-paired t-test was used for determining the significant difference between 2 groups, and any differences in variance were corrected with the Aspin-Welch’s method. Scheffe’s multiple comparison was used for the multiple intergroup test. p values less than 5% were considered to be significantly different.

**Results**

**Effect on the Increase in Intracellular Free Calcium Concentration in PC12 Cells**

Figure 1 shows the inhibitory effect on KCl-induced increase in fura-2 fluorescence ratio (F340/F380) in PC12 cells. The results indicate that FRC-8653 as well as nifedipine and nicardipine dose-dependently inhibited the increase in intracellular calcium concentration by membrane depolarization. FRC-8653 was equipotent to nicardipine, but 10 times more potent than nifedipine.

**Displacement of 3H-Nitrendipine Binding to the Rat Heart Membranes**

The inhibition of 3H-nitrendipine binding by FRC-8653, nifedipine or nicardipine was determined. Displacement curves are shown in Fig. 2. The pattern of displacement of 3H-nitrendipine binding by FRC-8653 was similar to those of nifedipine and nicardipine which could completely inhibit 3H-nitrendipine binding at high dose.

**Inhibitory Effect on Vasocontraction**

As Fig. 3 shows, FRC-8653 as well as nifedipine and nicardipine significantly inhibited KCl-induced contractions in a dose-dependent manner. These drugs also inhibited CaCl2-induced contractions; potency of FRC-8653 was almost equal to nicardipine and greater than nifedipine. On the other hand, FRC-8653 failed to affect NE-induced contraction even at 10^-6 M. NE-induced contractions also unchanged in the presence of 10^-6 M of nifedipine or nicardipine (data not shown).

**Investigation of the Time Course of Vasorelaxing Effect**

Figure 4 shows the time-course of vasorelaxing responses to the drugs in isolated rabbit aor-

![Figure 1](image1.png)

Fig. 1. Inhibitory Actions of FRC-8653 (○), Nifedipine (□) and Nicardipine (△) on 60 mM KCl-Induced Ca^{2+}-Transients in PC12 Cells

![Figure 2](image2.png)

Fig. 2. Inhibitory Actions of FRC-8653 (○), Nifedipine (□) and Nicardipine (△) on 3H-Nitrendipine Binding to the Membrane Preparations from Rat Heart

Each point represents the mean ± S.E. of 4 experiments.
ta pre-contracted with 30 mM KCl. Although FRC-8653, $10^{-7}$ M, nifedipine, $10^{-7}$ M, and nicardipine, $10^{-8}$ M, produced the similar ex-
tent of vasorelaxation, the time course of responses was variable. The time required to relax by 40% in response to FRC-8653, nifedipine

Fig. 3. Inhibitory Effects of FRC-8653 (a), Nifedipine (b) and Nicardipine (c) on KCl- and CaCl2-Induced Contraction in Rabbit Aorta
Data are shown as mean ± S.E.
○ (left): control ($n = 18$), ○ (right): control ($n = 12$), ●: $10^{-10}$ M ($n = 6$), △: $10^{-9}$ M ($n = 6$), ▲: $10^{-8}$ M ($n = 6$), □: $10^{-7}$ M ($n = 6$).
and nicardipine were 76.9±6.9, 21.3±0.9 and 46.0±6.5 min, respectively. The time for FRC-8653 was significantly longer than those for nifedipine and nicardipine.

**Ex Vivo Inhibitory Effect of Orally Administered Drugs on KCl-Contraction in Rat Aorta**

Figure 5 shows the vasoconstriction caused by 50 mM KCl in rat thoracic aorta which was isolated at 1, 3, 7 or 24 h after oral administration of FRC-8653 or nifedipine. Compared with the developed tension in the preparation of the nontreated control group, the KCl-contractions in the preparations isolated at 1, 3 and 7 h after administration of FRC-8653 were significantly smaller. The inhibition was the greatest in the preparation isolated at 3 h. However, in the preparation isolated at 24 h, no significant difference from the control group was noted.

KCl-contraction after administration of nifedipine was significantly smaller only in the preparation isolated at 1 h after administration compared with the control. No inhibitory effect was noted in the preparation isolated at 3, 7 and 24 h after administration of nifedipine.

**Discussion**

It is well known that calcium antagonists of DHP type block the calcium influx through the voltage dependent calcium channel (VDC), binding to the DHP-sensitive site in this channel. In this context, FRC-8653 has been shown to block KCl-induced $^{45}$Ca$^{2+}$ influx into A10 cells and Ba$^{2+}$ inward current in the rabbit basilar artery. The present study further confirmed that FRC-8653 inhibits KCl-induced increase in the intracellular free calcium concentration in PC12 cells; the potency of FRC-8653 was almost equal to nicardipine and greater than nifedipine. Additionally, the present study with isolated rabbit aorta indicated that FRC-8653 blocks VDC without affecting the receptor-operated calcium channel. These results

**Fig. 4.** Time Course of Vasorelaxing Actions of 10$^{-7}$ M FRC-8653 (C), 10$^{-7}$ M Nifedipine (□) and 10$^{-8}$ M Nicardipine (Δ) on Rabbit Aorta Pre-contracted by 30 mM KCl

Each point represents the mean ± S.E. of 6 experiments.

**Fig. 5.** *Ex Vivo* Inhibitory Action of FRC-8653 (a, n = 6) and Nifedipine (b, n = 4) on 50 mM KCl-Induced Contraction in Rat Aortic Preparation Isolated at 1, 3, 7 and 24 h after Oral Administration of 10 mg/kg Drugs

Each value represents the mean ± S.E. of 6 experiments.

a) $p<0.05$, b) $p<0.01$: compared with the value of nontreated control group (C).
suggest that the effect of FRC-8653 may be ascribed substantially to the blockade of VDC.

Concerning the binding site of DHP derivatives, it has been reported that \(^3\)H-nitrendipine binding site in smooth muscle is the site which mediates the calcium-antagonism\(^6,7\) and that the DHP receptor/calcium channel complex from the heart was purified.\(^8\) In the present study, FRC-8653 dose-dependently blocked the specific binding of \(^3\)H-nitrendipine to the cardiac membrane preparation. The pattern of the displacement curve of FRC-8653 was similar to those of nifedipine and nicardipine. The results suggest that FRC-8653 binds to the DHP-receptor located on cell membranes. Thus, FRC-8653 may act through the same mechanism as nifedipine and nicardipine.

However, the time course of the cardiovascular effects of FRC-8653 differed from those of nifedipine and nicardipine. In the present study, the \textit{ex vivo} inhibitory effect of orally administered FRC-8653 on KCl-contraction of rat thoracic aorta developed more slowly and lasted for a longer period compared with nifedipine. The results were well consistent with the time course of the hypotensive effect of FRC-8653 orally administered to conscious normotensive rats.\(^1\) In both cases, the maximum effect of FRC-8653 was observed at 3 h after administration and a significant effect persisted until 7 h after administration. On the other hand, the effect of an equihypotensive dose of nifedipine reached its maximum at 1 h after administration and disappeared within 3 h after administration. Since the plasma concentration of FRC-8653 started to decrease 1 h after 10 mg/kg oral administration to rats (unpublished data), some factors other than the rate of absorption from the digestive tract or the rate of elimination from blood might have contributed to the characteristic time course of the effect of FRC-8653.

In the KCl-contracted rabbit aorta, FRC-8653 required a longer time to complete the vasorelaxing effect, compared with nifedipine and nicardipine at the doses that produced a similar extent of relaxation. Although further experiments are necessary to make clear the mechanism of the slow development of the effect of FRC-8653, it can be speculated that the rate of penetration of FRC-8653 to the site of action might be slower than nifedipine and nicardipine. In this context, it has recently been proposed that the most important determinants of the approach of DHP derivatives to their active site are their rates of partition into and diffusion within the lipid bilayer.\(^9\) On the other hand, the \textit{in vitro} effect of FRC-8653 has been shown to be relatively resistant to wash-out; the inhibition by FRC-8653 of the KCl-induced contraction of isolated rat mesenteric artery persisted for more than 7 h after its removal.\(^2\) Therefore, long duration of effects of FRC-8653 may be due to its slow rate of dissociation from the DHP-sensitive site. Such a mechanism of long duration of effect has been suggested for the DHP derivatives of long-acting type such as amlopidine,\(^10\) nisoldipine\(^11\) and benidipine.\(^12\)

In summary, the present study demonstrated that FRC-8653 may inhibit vasocontraction by blocking calcium influx \textit{via} DHP-sensitive VDC and that the vascular effect of FRC-8653 is slower in development and longer in duration compared with those of nifedipine and nicardipine.

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