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Short communication

Receptor occupation and pharmacokinetics of MPC-1304, a new Ca^{2+} channel antagonist, in spontaneously hypertensive rats

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Abstract

MPC-1304, (±)-methyl 2-oxopropyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarbonate, is a novel 1,4-dihydropyridine Ca^{2+} channel antagonist with potent and long-lasting antihypertensive effects. We characterized the ex vivo and in vivo binding properties of MPC-1304 to Ca^{2+} channel antagonist receptors in myocardial, aortic and brain tissues of spontaneously hypertensive rats (SHR) by radioreceptor assay using [^{3}H](+)-PN 200-110 ([5-*methyl*-^{3}H](+)-PN 200-110 (4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-1,4-dihydro-3-isopropylcarbonylpyridine-5-carboxylic acid methyl ester)). At 1 and 6 h after oral administration of MPC-1304 (10 mg/kg) in SHR, there was a significant decrease (48%) in the number of [^{3}H](+)-PN 200-110 binding sites (B_{max}) in myocardial membranes compared to control values. The plasma concentration of MPC-1304 in SHR correlated significantly with the occupation by this drug of myocardial Ca^{2+} channel antagonist receptors. The in vivo specific binding of [^{3}H](+)-PN 200-110 in particulate fractions of aorta of SHR was significantly reduced (74.8 and 37.9%, respectively) at 1 and 6 h after oral administration of MPC-1304 (3 mg/kg), while the myocardial [^{3}H](+)-PN 200-110 binding was decreased only at 1 h later. In these rats, there was little change in cerebral cortical [^{3}H](+)-PN 200-110 binding. In conclusion, MPC-1304 exerted more selective and sustained occupation in vivo of Ca^{2+} channel antagonist receptors in vascular tissues of SHR than in those of myocardial and brain tissues.

Keywords: MPC-1304; Ca^{2+} channel antagonist receptor; Receptor occupation; Spontaneously hypertensive rat (SHR)

1. Introduction

Ca^{2+} channel antagonists have been widely accepted for the treatment of hypertension and angina pectoris (Fleckenstein, 1983; Godfraind et al., 1986). Nifedipine, a prototype of 1,4-dihydropyridine derivatives, is well established as first-choice drug in various cardiovascular diseases (Murphy et al., 1989). MPC-1304, (±)-methyl 2-oxopropyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarbonate, is a novel 1,4-dihydropyridine Ca^{2+} channel antagonist with more potent and long-lasting vasodilating and hypotensive actions than nifedipine and nicardipine (Miyoshi et al., 1992). It was previously shown that MPC-1304 was the most potent hypotensive agent among representative 1,4-dihydropyridine derivatives when tested in conscious spontaneously hypertensive rats (SHR) and renal hypertensive dogs (Kanda et al., 1992).

In addition to the fact that [^{3}H]MPC-1304 binds to a single population of high-affinity binding sites in cardiac membranes, MPC-1304 showed a similar binding affinity to the 1,4-dihydropyridine receptor sites in brain, myocardial and vascular smooth muscle (Miyoshi et al., 1992). However, the in vitro receptor affinity of MPC-1304 does not appear to correlate with the pharmacological observation that this drug was 60 times more potent to inhibit the contractile response of aorta than of heart (Kanda et al., 1993; Okumura et al., 1993). Taking account of the pharmacokinetics and pharmacodynamics of MPC-1304, the measurement of

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its receptor binding affinity under more physiological condition may be important for the analysis of pharmacological effects. For this reason, we investigated the effect of oral administration of MPC-1304 on Ca^{2+} channel antagonist receptors in myocardial, brain and vascular smooth muscle tissues of SHR by using in vivo or ex vivo receptor binding techniques. We also examined the relationship between receptor occupancy and plasma concentration of this drug, according to previous reports by Yamada et al. (1992) and Uchida et al. (1995).

2. Materials and methods

2.1. Drug administration

Male SHR (11–15 weeks) were obtained from Charles River Japan (Atsugi, Japan). They were fasted for 16 h before the administration of drugs, and given MPC-1304 (3, 10 mg/kg) orally through a gastric tube. Control animals were given the vehicle. At 1–24 h after drug administration, the SHR were killed by bleeding from the descending aorta under light anesthesia with ethyl ether, and the myocardium and brain were perfused with 0.9% saline from the aorta. Then, both tissues were removed, and blood vessels were trimmed away. Plasma from rat blood was isolated by centrifugation, and stored at −80°C until the concentration of MPC-1304 was determined.

2.2. Receptor binding assay

The myocardial tissues from SHR were minced with scissors and homogenized with a Kinematica Polytron homogenizer in ice-cold 50 mM Tris-HCl buffer (pH 7.4). The myocardial homogenate was centrifuged at $500 \times g$ for 10 min, and the supernatant fraction was centrifuged at $40\,000 \times g$ for 15 min. The pellet was resuspended in the ice-cold buffer, and the suspension was centrifuged again at $40\,000 \times g$ for 15 min. The homogenate of cerebral cortex was centrifuged at $40\,000 \times g$ for 15 min. The pellet was washed twice by centrifugation and the resulting pellet was finally resuspended in the buffer to utilize in the binding assay. All steps were performed at 4°C. Protein concentration was measured according to the method of Bradford (1976) with bovine serum albumin as standard.

The binding assay of [^{3}H](+)-PN 200-110 was performed according to the methods of Lee et al. (1984) and Yamada et al. (1990, 1992). Briefly, the membranes (200–600 μg of protein) prepared from rat myocardium and brain were incubated with different concentrations (0.03–0.57 nM) of [^{3}H](+)-PN 200-110 in 50 mM Tris-HCl buffer. Incubation was carried out in the dark under sodium light for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, USA) through Whatman GF/B glass fiber filters and the filters were rinsed with ice-cold buffer. Tissue-bound radioactivity was determined in a liquid scintillation counter after the addition of scintillation fluid. Specific [^{3}H](+)-PN 200-110 binding was determined from the difference between counts in the absence and presence of 1 μM nifedipine.

In vivo labeling of Ca^{2+} channel antagonist receptors using [^{3}H](+)-PN 200-110 was performed as described by Yamada et al. (1992) and Uchida et al. (1995). MPC-1304 (3 mg/kg) was given orally to SHR at 1, 6 and 12 h before an i.v. injection of 555 kBq of [^{3}H](+)-PN 200-110 (66.0 ng) into the femoral vein under light anesthesia with ethyl ether. The blood was taken from the descending aorta of rats at 10 min after the administration of [^{3}H](+)-PN 200-110, and myocardial, aortic and brain (cerebral cortical) tissues were removed. These tissues were homogenized in ice-cold 50 mM Tris-HCl buffer, and the particulate bound radioactivity was determined by rapid filtration of homogenates over Whatman GF/C filters. Aliquots of the homogenate without filtering were measured to give the total radioactivity (bound + free). Total and particulate bound radioactivity was measured by liquid scintillation counting after the addition of scintillation fluid. Similarly, [^{3}H](+)-PN 200-110 (555 kBq) was injected i.v. to control and nifedipine (40 mg/kg i.p., 0.5 h pretreatment)-treated SHR, to determine total and non-specific binding in each tissue.

2.3. Determination of MPC-1304 in plasma

The plasma concentration of MPC-1304 was determined by high-performance liquid chromatography (HPLC) as previously described (Iida et al., 1991). Following the addition of a mixture of *n*-hexane and diethyl ether and sodium carbonate, the plasma sample was shaken mechanically. The organic layer was transferred to a test-tube and evaporated to dryness at 40°C under a nitrogen flow. The residue was dissolved in the HPLC mobile phase, and aliquots were injected into the HPLC column. The HPLC system consisted of a Shimadzu LC-6AD pump, a SIL-6B autoinjector and a SPD-6A UV-spectrophotometric detector (Shimadzu, Kyoto, Japan). The mobile phase, degassed by bubbling with helium, was prepared by mixing ethylene chloride, *n*-hexane and ethanol.

2.4. Data analysis

The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H](+)-PN 200-110 binding were estimated by Rosenthal analysis of the saturation data (Rosenthal, 1967). The ability of

MPC-1304 to inhibit specific [^{3}H](+)-PN 200-110 (0.24 nM) binding in vitro was estimated from the IC_{50} values, which are the molar concentrations of unlabeled drug necessary for displacing 50% of the specific binding (estimated by log probit analysis). A value for the inhibition constant, K_i, was calculated from the equation, $K_i = IC_{50}/(1 + L/K_d)$, where L equals the concentration of [^{3}H](+)-PN 200-110. The Hill coefficients for saturation data of [^{3}H](+)-PN 200-110 binding and for inhibition by MPC-1304 were obtained by Hill plot analysis.

The occupancy (%) of myocardial Ca^{2+} channel antagonist receptors by MPC-1304 was calculated with the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{MPC-1304})]/B_{max}(\text{control})\} \times 100$. Receptor occupancy by MPC-1304 was plotted against its plasma concentration (Cp) in SHR given the drug orally. The plot was found to obey Hill's equation:

$$\log[(B_{max} - B)/B] = r \cdot \log(\text{Cp}) - \log(K')$$

Statistical analysis was done with Welch's t-test and a one-way analysis of variance followed by Dunnett's test for single and multiple comparisons, respectively. Statistical significance was accepted at $P < 0.05$.

2.5. Drugs

[5-*methyl*-^{3}H](+)-PN 200-110 (4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-1,4-dihydro-3-isopropylcarbonylpyridine-5-carboxylic acid methyl ester (2605 GBq/mmol)) was purchased from DuPont-NEN Co. (Boston, MA, USA). MPC-1304 ((±)-methyl 2-oxopropyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate) was synthesized by Maruko Pharmaceutical Co. (Kasugai, Japan). Nifedipine (dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylate) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The drugs were dissolved in dimethyl sulfoxide and diluted in reaction buffer. For oral administration, the drugs were suspended in 0.25% carboxymethyl cellulose sodium solution, and given in a volume of 5 ml/kg. All steps were performed under sodium light to prevent decomposition of dihydropyridine derivatives.

3. Results

3.1. In vitro inhibition of specific [^{3}H](+)-PN 200-110 binding in myocardial and cerebral cortical membranes

Specific binding of [^{3}H](+)-PN 200-110 in myocardial and cerebral cortical membranes of SHR appeared to be saturable and Rosenthal analysis revealed a linear plot, suggesting the existence of a single population of binding sites with K_d values of 0.13 ± 0.01 (myocardium) and 0.13 ± 0.01 (cerebral cortex) nM, respectively (mean ± S.E., $n = 4$). The B_{max} values for [^{3}H](+)-PN 200-110 in these tissues were 231.0 ± 17.2 (myocardium) and 195.5 ± 18.5 (cerebral cortex) fmol/mg protein, respectively. The Hill coefficients of [^{3}H](+)-PN 200-110 binding in these tissues were 0.95 ± 0.01 and 0.83 ± 0.04, respectively. MPC-1304 (0.3–30 nM) competed with [^{3}H](+)-PN 200-110 for myocardial and cerebral cortical binding sites in vitro. The K_i values for inhibition of [^{3}H](+)-PN 200-110 binding by MPC-1304 in SHR myocardium and cerebral cortex were 2.95 ± 0.44 and 1.97 ± 0.09 nM, respectively (mean ± S.E., $n = 4$). The Hill coefficients for MPC-1304 in these tissues were 0.99 ± 0.04 and 1.05 ± 0.07, respectively.

Table 1
Effects of oral administration of MPC-1304 and nifedipine on K_d and B_{max} values of specific [^{3}H](+)-PN 200-110 binding to cardiac membranes of SHR

Condition	K_d (nM)	B_{max} (fmol/mg protein)	Receptor occupancy (%)
Control	0.13 ± 0.01	231.0 ± 17.2	
MPC-1304			
3 mg/kg			
1 h	0.12 ± 0.01	120.7 ± 9.1 [b]	47.7
6 h	0.17 ± 0.01	213.1 ± 20.6	7.7
10 mg/kg			
1 h	0.15 ± 0.01	118.0 ± 16.1 [b]	48.9
6 h	0.16 ± 0.02	171.4 ± 16.7 [a]	25.8
12 h	0.15 ± 0.01	201.7 ± 22.3	12.7
24 h	0.17 ± 0.01	186.5 ± 13.1	19.3
Nifedipine			
10 mg/kg			
0.5 h	0.19 ± 0.01 [b]	244.2 ± 22.1	
3 h	0.22 ± 0.01 [b]	245.7 ± 22.1	
6 h	0.12 ± 0.01	228.1 ± 7.4	

Values are means ± S.E. for 4–6 rats. Rats received MPC-1304 (3 and 10 mg/kg) and nifedipine (10 mg/kg) orally, and were killed at 0.5–24 h after the administration. Specific binding of [^{3}H](+)-PN 200-110 to myocardial membranes was measured. [a] $P < 0.05$ vs. control; [b] $P < 0.01$ vs. control.

3.2. Effects of oral administration of MPC-1304 on myocardial [^{3}H](+)-PN 200-110 binding

Following oral administration of MPC-1304 at doses of 3 and 10 mg/kg to SHR, there were significant decreases in B_{max} values for specific [^{3}H](+)-PN 200-110 binding to myocardial membranes compared to the control values, as shown in Table 1. The B_{max} values at 1 h (3 mg/kg), 1 and 6 h (10 mg/kg) were significantly decreased (47.7, 48.9 and 25.8%, respectively) compared to the control values. Therefore, the effect was greatest at 1 h and decreased with time. The B_{max} values at 6 h (3 mg/kg) and 12 or 24 h (10 mg/kg) after the oral administration of MPC-1304 were not significantly different from the control values, suggest-

ing the disappearance of the effect of MPC-1304. The K_d values for myocardial [^{3}H](+)-PN 200-110 binding were unaltered by oral administration of MPC-1304. At 0.5 and 3 h after oral administration of nifedipine (10 mg/kg) in SHR, the K_d values were significantly increased for myocardial [^{3}H](+)-PN 200-110 binding, with little change in B_{max} values (Table 1).

3.3. Occupation of in vivo Ca^{2+} channel antagonist receptors

At 10 min after an i.v. injection of [^{3}H](+)-PN 200-110 (555 kBq) to control SHR, total amounts of radioactivity were 3052 ± 106 (myocardium), 2614 ± 570 (aorta) and 1923 ± 168 (cerebral cortex) Bq/g tissue, respectively. The particulate bound radioactivity in these tissues, expressed as a percentage of the total amount of radioactivity in each tissue, was 63.2% (myocardium), 57.9% (aorta) and 56.6% (cerebral cortex), respectively. To measure the in vivo non-specific binding of [^{3}H](+)-PN 200-110 to these tissues, nifedipine (40 mg/kg i.p.) was administered at 0.5 h before an i.v. injection of [^{3}H](+)-PN 200-110 in SHR. The total radioactivity in each tissue of nifedipine-pretreated SHR was similar to that in control rats, but the particulate bound radioactivity was markedly reduced. A significant amount of specific [^{3}H](+)-PN 200-110 binding, defined as the difference in the particulate bound radioactivity between control and nifedipine-pretreated SHR, was thus demonstrated in the myocardium, aorta and cerebral cortex, as previously reported (Yamada et al., 1992; Uchida et al., 1995). The in vivo specific binding of [^{3}H](+)-PN 200-110 in particulate fractions of myocardium and aorta of SHR was significantly decreased (61.6 and 74.8%, respectively) at 1 h after oral administration of MPC-1304 (3 mg/kg), compared to each control value (Fig. 1). A significant reduction (37.9%) in aortic [^{3}H](+)-PN 200-110 binding was also observed 6 h after oral administration of MPC-1304 while myocardial [^{3}H](+)-PN 200-110 binding had returned to the control value at this time. On the other hand, the administration of MPC-1304 had no significant effect on the in vivo specific binding of [^{3}H](+)-PN 200-110 in particulate fractions of cerebral cortex (Fig. 1).

3.4. Plasma concentration of MPC-1304

The plasma concentrations of MPC-1304 in SHR used to measure myocardial [^{3}H](+)-PN 200-110 binding at 1–24 h after oral administration (3, 10 mg/kg), were determined; they were (in ng/ml): 3 mg/kg, 161.1 ± 5.2 (1 h), 89.2 ± 14.1 (6 h); 10 mg/kg, 862.0 ± 128.1 (1 h), 307.4 ± 46.1 (6 h), 53.4 ± 26.8 (12 h) (mean ± S.E., $n = 2$–5). The plasma concentration of MPC-1304 in SHR correlated significantly ($P < 0.05$) with the occupation of myocardial Ca^{2+} channel antagonist receptors by this drug (Table 1).

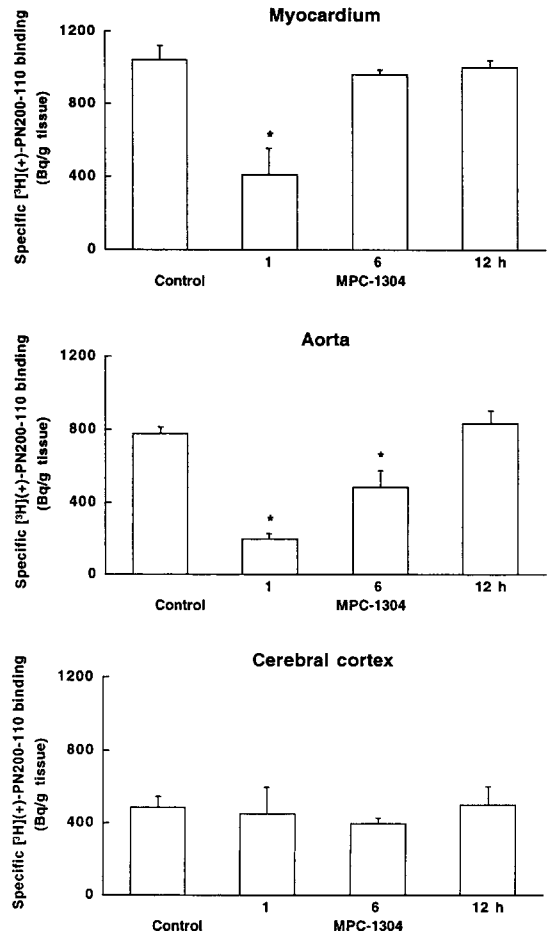

Fig. 1. In vivo inhibition of specific [^{3}H](+)-PN 200-110 binding in particulate fractions from myocardium, aorta and cerebral cortex of SHR at 1, 6 and 12 h after oral administration of MPC-1304. [^{3}H](+)-PN 200-110 (555 kBq) was injected into the femoral vein in control and MPC-1304 (3 mg/kg)-pretreated SHR, and they were killed 10 min later. The asterisk indicates a significant difference from the control value (*$P < 0.01$).

4. Discussion

The major findings of this study were that: (1) MPC-1304 produced a selective occupancy of cardiovascular 1,4-dihydropyridine Ca^{2+} channel antagonist receptors in SHR and its occupation of myocardial receptors correlated significantly with the plasma concentration of this drug, and (2) MPC-1304 reduced more effectively the in vivo specific binding of [^{3}H](+)-PN 200-110 in aortic tissue than in myocardial and brain tissues.

The present study was undertaken to characterize ex vivo and in vivo occupancy by MPC-1304 of Ca^{2+} channel antagonist receptors in myocardium, aorta and

cerebral cortex of SHR. MPC-1304 competed with [^{3}H](+)-PN 200-110 for the binding sites in myocardium and cerebral cortex of SHR in vitro. There was a similarity in the inhibitory effect of [^{3}H](+)-PN 200-110 binding by this drug between myocardium and cerebral cortex of SHR in vitro, as shown by K_i values of 2.95 and 1.97 nM, respectively. Compared to the control value, there was a significant reduction (47.7, 48.9 and 25.8%, respectively) in myocardial [^{3}H](+)-PN 200-110 binding sites (B_{max}) at 1 h (3 mg/kg), 1 and 6 h (10 mg/kg) following the oral administration of MPC-1304 in SHR. It was recognized that the reduction of the B_{max} value in myocardium was more sustained after the administration of MPC-1304 10 mg/kg than 3 mg/kg. The B_{max} value in myocardium returned to the control level at 6 h (3 mg/kg) and 12 h (10 mg/kg) after MPC-1304 administration. The maximal occupancy by MPC-1304 of Ca^{2+} channel antagonist receptors occurred 1 h after administration and then declined with time. At 3 and 10 mg/kg, MPC-1304 had little effect on the K_d values for myocardial [^{3}H](+)-PN 200-110 binding in SHR. The predominant alteration in the density rather than in the apparent affinity of Ca^{2+} channel antagonist receptors by MPC-1304 may be ascribable to its slowly dissociating blockade of the receptors, as shown previously for the non-equilibrium blockade by other long-lasting Ca^{2+} channel antagonists (Yamada et al., 1992, 1994; Uchida et al., 1995). In contrast, oral administration of nifedipine produced only a change in K_d values for cardiac [^{3}H](+)-PN 200-110 binding, suggesting an easily reversible blockade of the receptor.

In the present study, the in vivo specific binding of [^{3}H](+)-PN 200-110 in particulate fractions from aortic tissues in SHR was significantly reduced at 1 and 6 h after oral administration of MPC-1304 (3 mg/kg), while myocardial [^{3}H](+)-PN 200-110 binding was significantly decreased only at 1 h later. The time course of in vivo occupation of myocardial Ca^{2+} channel antagonist receptors corresponded to that in the ex vivo receptor occupation experiment. In contrast to the observation in myocardium, it is noteworthy that a significant decrease in in vivo aortic [^{3}H](+)-PN 200-110 binding lasted at least for 6 h after MPC-1304 administration. These data suggest that MPC-1304 exerts a more selective and sustained occupation in vivo of Ca^{2+} channel antagonist receptors in vascular tissues than in myocardial tissues of SHR. The in vivo specific binding of [^{3}H](+)-PN 200-110 to the cerebral cortex, rather than to cardiovascular tissues in SHR, was unchanged by MPC-1304 administration. Thus, MPC-1304 may show more selective occupation in vivo of Ca^{2+} channel antagonist receptors in cardiovascular tissues of SHR rather than cerebral cortex. In addition, in our preliminary experiment, it was shown that, at 0.5, 8 and 24 h after the oral administration of [^{14}C]MPC-1304 in rats, the radioactivities in the brain were only 0.03, 0.18 and 0.10% of plasma levels, respectively. In the in vitro experiment, MPC-1304 competed with [^{3}H](+)-PN 200-110 for the binding sites in myocardium and cerebral cortex of SHR, and there was a similarity in the inhibitory effect (K_i) between both tissues. Taking these observations into account, it is possible that the failure of MPC-1304 to occupy brain receptors in SHR is mainly ascribable to poor transport of this drug through the blood-brain barrier. Thus, the in vivo measurement of receptor occupation by Ca^{2+} channel antagonists in different tissues may be of great value for evaluating the extent and duration of receptor occupation by, as well as tissue selectivity of, these drugs in consideration of their pharmacokinetics and pharmacodynamics.

The plasma concentration of MPC-1304 in SHR was maximal at 1 h after oral administration of this drug at doses of 3 and 10 mg/kg, and then declined with time. We have found a significant ($P < 0.05$) relationship between the plasma concentration and the occupation of myocardial Ca^{2+} channel antagonist receptors after the oral administration of MPC-1304. Kanda et al. (1992) have previously shown that the hypotensive effect following the oral administration of MPC-1304 at the dose of 3 mg/kg in SHR was maximal at 1 h later and lasted for 8 h after the administration. Despite this fact, there was no significant occupancy of myocardial Ca^{2+} channel antagonist receptors at 6 h after oral administration of MPC-1304 (3 mg/kg), whereas significant occupancy in aorta was also observed at 6 h later. Thus, the time course of the hypotensive effect of MPC-1304 was parallel to that of the in vivo occupation by this drug of Ca^{2+} channel antagonist receptors in vascular tissues rather than those in myocardial tissues of SHR. We propose a possible mechanism to explain the difference in receptor occupancy manner between myocardial and vascular tissues: slower dissociation of MPC-1304 from Ca^{2+} channel antagonist receptors in vascular tissues than in myocardial tissues of SHR might result in potent and long-lasting vasodilating and antihypertensive activities (Kanda et al., 1992).

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