



The receptor occupation and plasma concentration of NKY-722, a water-soluble dihydropyridine-type calcium antagonist, in spontaneously hypertensive rats

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1 The occupation *in vivo* by NKY-722 of 1,4-dihydropyridine (DHP) calcium antagonist receptors in myocardium, aorta and cerebral cortex was investigated. At 1 and 3 h after oral administration of NKY-722 (3 mg kg⁻¹) in spontaneously hypertensive rats (SHR), there was a significant (44 and 41%, respectively) decrease in the number of myocardial (+)-[³H]-PN 200–110 binding sites (B_{max}) compared to control values. A greater reduction of B_{max} values was observed at 1 (86%), 3 (88%), 6 (63%) and 12 (46%) h later by a higher dose (10 mg kg⁻¹) of this drug. The occupation of myocardial 1,4-DHP calcium antagonist receptors after oral administration of NKY-722 correlated significantly with its plasma concentration. There was a significant decrease in cerebral cortical (+)-[³H]-PN 200–110 binding (B_{max}) at 1 and 3 h after oral administration of NKY-722 (10 mg kg⁻¹).

2 Oral administration of nifedipine (10 mg kg⁻¹) in SHR caused a significant reduction of B_{max} values for (+)-[³H]-PN 200–110 binding in myocardium at 1 and 3 h later and in cerebral cortex at 1 h later.

3 The *in vivo* specific binding of (+)-[³H]-PN 200–110 in particulate fractions of aorta of SHR was significantly (79 and 83%, respectively) reduced at 1 and 6 h after oral administration of NKY-722 (3 mg kg⁻¹), while myocardial (+)-[³H]-PN 200–110 binding was decreased by 52% only at 1 h later. Also, nifedipine administration reduced *in vivo* (+)-[³H]-PN 200–110 binding in aorta at 1 and 6 h later and in myocardium at 1 h later. On the other hand, the administration of both NKY-722 and nifedipine had no significant effect on *in vivo* (+)-[³H]-PN 200–110 binding in cerebral cortex.

4 It is concluded that NKY-722 may exert more selective and sustained occupation *in vivo* of 1,4-DHP calcium antagonist receptors in vascular tissues of SHR than in myocardial and brain tissues.

Keywords: NKY-722; calcium antagonist receptor; receptor occupation *in vivo*; aorta; myocardium

Introduction

Calcium antagonists have been widely used for the treatment of angina pectoris, systemic hypertension and other cardiovascular diseases. Nifedipine, the prototype of the 1,4-dihydropyridine (DHP) calcium antagonist, has a short duration of cardiovascular effects. Thus, currently, novel 1,4-DHP derivatives possessing long-lasting antihypertensive effects and tissue selectivity have been introduced as 'second- and third-generation' agents (Kelly & O'Malley, 1992; Meredith & Elliott, 1992). The pharmacological effects of 1,4-DHP calcium antagonists are due to an interaction with specific receptors in the cardiovascular system. The binding properties of calcium antagonists to these receptors were characterized extensively *in vitro* in brain, myocardial, and smooth muscles (Bellemann *et al.*, 1981; Bolger *et al.*, 1982; Ehlert *et al.*, 1982; Gould *et al.*, 1982; Godfraind *et al.*, 1986). However, the *in vitro* receptor affinities of calcium antagonists may not reflect the pharmacological activity and the tissue selectivity *in vivo* of these drugs because the *in vitro* binding technique does not take various pharmacokinetic (absorption, distribution, elimination) and pharmacodynamic factors into account. Thus, *in vivo* characterization of calcium antagonist binding to the receptors in different tissues is important for the analysis of pharmacodynamics and pharmacokinetics of these drugs. However, little information is available regarding the comparison of receptor occupation *in vivo* by the 1,4-DHP derivatives in different tissues and its relationship to the drug plasma concentration.

NKY-722 [3-(4-allyl-1-piperazinyl)-2,2-dimethylpropyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine

dicarboxylate dihydrochloride], a novel water-soluble 1,4-DHP, exerts a potent and long-lasting antihypertensive effect in spontaneously hypertensive rats (SHR) and it has a vasoselective effect in isolated, blood-perfused heart preparations of the dog (Imagawa *et al.*, 1989; Osumi *et al.*, 1988; 1990; Wada *et al.*, 1994). However, the receptor binding properties of this drug have not been characterized previously. The purpose of the present study was to characterize the *in vivo* binding properties of NKY-722 to 1,4-DHP receptors in myocardial, vascular and brain tissues of SHR, particularly with respect to tissue selectivity, and to the relationship between its receptor occupation and plasma concentrations. The 1,4-DHP calcium antagonist receptors in these tissues have been measured by *in vitro* and *in vivo* binding techniques using (+)-[³H]-PN 200–110 as a radio ligand (Lee *et al.*, 1984; Yamada *et al.*, 1990; 1992). As SHR have been most commonly utilized to study an antihypertensive effect of calcium antagonists, we have used this model of experimental hypertension in the present study.

Methods

Drug administration

Male SHR at 14 to 18 weeks of age (Charles River Japan Inc.) were housed three or four per cage in the laboratory with free access to food (normal rat chow) and water, and maintained on a 12 h dark/light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). They were fasted for 16 h before the administration of drugs, and

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administered orally with NKY-722 (3,10 mg kg⁻¹) or nifedipine hydrochloride (10 mg kg⁻¹) dissolved in water and in water containing ethanol (10%) and polyethylene glycol 400 (10%), respectively, as solvents. Half of control animals examined were given the former vehicle and the rest had the latter vehicle. There was no significant difference in control values between these vehicles in each experiment of the present study, and thus, the pooled data are presented. At 1 to 24 h after the drug administration, SHR were killed by taking the blood from descending aorta under light anaesthesia 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. The plasma from rat blood was isolated by centrifugation, and stored at -20°C until concentration of NKY-722 was determined.

Tissue preparation

The myocardial tissues from SHR were minced with scissors and homogenized by a Kinematica Polytron homogenizer in 10 volumes of ice-cold 50 mM Tris HCl buffer (pH 7.4). The myocardial homogenate was centrifuged at 500 g for 10 min, and the supernatant fraction was centrifuged at 40,000 g for 15 min. The pellet was resuspended in the ice-cold buffer, and the suspension was centrifuged again at 40,000 g for 15 min. The resulting pellet was finally suspended in the buffer for use in the binding assay. The cerebral cortex was homogenized in 20 volumes of 50 mM Tris HCl buffer with a Polytron homogenizer, and the homogenate was centrifuged at 40,000 g for 15 min. The pellet was washed twice by centrifugation. The pellet was finally resuspended in the original volume of the buffer and used in the binding assay. All steps were performed at 4°C. Protein concentration was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

(+)-[³H]-PN 200-110 binding assay

The binding assay of (+)-[³H]-PN 200-110 was performed according to the methods by Lee *et al.* (1984) and Yamada *et al.* (1990, 1992). Briefly, the membranes (400-600 µg of protein) prepared from rat myocardium and brain were incubated with different concentrations (0.03-0.57 nM) of (+)-[³H]-PN 200-110 in 50 mM Tris HCl buffer. Incubation was carried out in the dark with a sodium lamp for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fibre filters, and filters were rinsed three times with 4 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid (21 of toluene, 11 of Triton X-100, 15 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene) and the radioactivity was determined in a liquid scintillation counter. Specific (+)-[³H]-PN 200-110 binding was determined experimentally from the difference between counts in the absence and presence of 1 µM nifedipine. All assays were conducted in duplicate.

In vivo labelling of calcium antagonist receptors

In vivo labelling of calcium antagonist receptors using (+)-[³H]-PN 200-110 was performed as described by Yamada *et al.* (1992). NKY-722 (3 mg kg⁻¹) and nifedipine (10 mg kg⁻¹) were administered orally to SHR at 1 and 6 h before an i.v. injection of 555 kBq of (+)-[³H]-PN 200-110 (66.0 ng) into the femoral vein under temporary anaesthesia with ethyl ether. The blood was taken from the descending aorta of rats 10 min after the administration of (+)-[³H]-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 to a final tissue concentration of 10 mg ml⁻¹ (myocardium and aorta) and 20 mg ml⁻¹ (cerebral cortex) with Kinematica Polytron homogenizer. The

particulate bound radioactivity was determined by rapid filtration of 1 ml (myocardium and cerebral cortex) and 1.5 ml (aorta) of homogenate over Whatman GF/C filters, which were washed subsequently with 3 ml of ice-cold buffer. Also, aliquots (1 or 1.5 ml) of the homogenate without filtering were measured as total radioactivity (bound + free). Total and particulate bound radioactivity were measured by liquid scintillation counter after the addition of scintillation fluids. Similarly, (+)-[³H]-PN 200-110 (555 kBq) was injected i.v. to control and nifedipine (40 mg kg⁻¹, i.p., 0.5 h pretreatment)-treated SHR, to determine total and non-specific binding in each tissue.

Determination of NKY-722 in plasma

The plasma concentration of NKY-722 was determined by high-performance liquid chromatography (h.p.l.c.). Six volumes of a mixture of *n*-hexane and diethyl ether (3:1) and 0.1 N NaOH were added to the plasma samples (1.0 ml) containing nifedipine hydrochloride as internal standard. After being stirred, the mixture was centrifuged at 15,000 g for 10 min. The organic phase was transferred to the glass tube, 0.1 N HCl (0.2 ml) was added, and the mixture was vortexed. After the centrifugation, a water phase (0.1 ml) was used as the h.p.l.c. sample. The h.p.l.c. system was constructed with a pump (PU-980, Jasco), UV-detector (UV-970, Jasco), injector (Model 7125, Rheodyne) and integrator (Chromatocorder 807-IT, Jasco). The analysis was performed on an Cosmosil C₁₈ (10 µM, 4.6 mm × 250 mm ID, Nacalai tesque Inc.). The mobile phase for assay consisted of a mixture of 0.05 M acetate buffer (pH 4.0) and acetonitrile (52:48) at a flow rate of 1.1 ml min⁻¹. The column eluate was monitored at a wavelength of 350 nm.

Data analysis

The analysis of binding data was performed as described previously (Yamada *et al.*, 1980). The apparent dissociation constant (*K*_d) and maximal number of binding sites (*B*_{max}) for (+)-[³H]-PN 200-110 binding were estimated by Rosenthal analysis of the saturation data (Rosenthal, 1967). The ability of NKY-722 and nifedipine to inhibit specific (+)-[³H]-PN 200-110 (0.24 nM) binding *in vitro* was estimated by IC₅₀ values, which are the molar concentrations of unlabelled 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 = IC₅₀ / (1 + L/*K*_d), where L equals the concentration of (+)-[³H]-PN 200-110. The Hill coefficients for saturation data of (+)-[³H]-PN 200-110 binding and for inhibition of NKY-722 and nifedipine were obtained by the Hill plot analysis.

The occupancy (%) of myocardial calcium antagonist receptors by NKY-722 was calculated by the equation: $\{[B_{\max}(\text{control}) - B_{\max}(\text{NKY-722 or nifedipine})] / B_{\max}(\text{control})\} \times 100$. The receptor occupancy by NKY-722 was plotted against its plasma drug concentrations (*C*_p) in SHR received orally the drug. The plot was found to obey Hill's equation:

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

Statistical analysis of data was performed by Student's two-tailed *t* test.

Drugs

(+)-[5-methyl-³H]-PN 200-110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate) (2605 GBq mmol⁻¹) was purchased from DuPont-NEN Co. Ltd. (Boston, MA). NKY-722 and nifedipine hydrochloride were donated by Kyoto Pharmaceutical Company (Kyoto, Japan) and Yamanouchi Pharmaceutical Company (Tokyo, Japan), respectively. Drugs were dissolved in distilled water or ethanol and diluted in 50 mM Tris HCl buffer for *in vitro* experiments. All solutions were

prepared daily. All other chemicals were obtained from commercial sources.

Results

In vitro inhibition of specific (+)-[³H]-PN 200-110 binding in myocardial and brain membranes

Specific binding of (+)-[³H]-PN 200-110 (0.03–0.57 nM) in myocardial and cerebral cortical membranes of SHR was saturable and Rosenthal analysis revealed a linear plot (data not shown), suggesting a single population of binding sites with K_d values of 0.19 ± 0.01 (myocardium) and 0.11 ± 0.01 (cerebral cortex) nM (mean \pm s.e.mean, $n = 8$). The B_{max} values for (+)-[³H]-PN 200-110 in these tissues were 266 ± 14 (myocardium) and 171 ± 13 (cerebral cortex) fmol mg⁻¹ of protein. The Hill coefficients of (+)-[³H]-PN 200-110 binding in both tissues were close to unity. NKY-722 (0.3–30 nM) and nicardipine (0.3–30 nM) competed with (+)-[³H]-PN 200-110 for myocardial and cerebral cortical binding sites *in vitro*. The K_i values for inhibition of (+)-[³H]-PN 200-110 binding by NKY-722 in SHR myocardium and cerebral cortex were 6.00 ± 1.44 and 1.68 ± 0.05 nM (mean \pm s.e.mean, $n = 4$), respectively, and the values by nicardipine were 1.86 ± 0.24 and 0.53 ± 0.06 nM (mean \pm s.e.mean, $n = 4$), respectively. The Hill coefficients for both drugs in these tissues were close to one (1.03–1.17).

Effects of oral administration of NKY-722 and nicardipine on myocardial and brain (+)-[³H]-PN 200-110 binding

The effect of oral administration of NKY-722 and nicardipine on 1,4-DHP calcium antagonist receptors in myocardial and brain tissues of SHR was investigated. Following oral administration of NKY-722 at doses of 3 and 10 mg kg⁻¹ in SHR, there was a significant decrease in B_{max} values for specific (+)-[³H]-PN 200-110 binding to myocardial membranes compared to the control values, as shown in Figure 1 and Table 1. The decreases in B_{max} values at 1 and 3 h were 44 and 41%, respectively. Similarly, the decreases in B_{max} values at 1, 3, 6 and 12 h after oral administration of higher dose (10 mg kg⁻¹) were 86, 88, 63 and 46%, respectively. Therefore, the reduction by NKY-722 was most pronounced at 1 to 3 h, and the effect decreased with time. The B_{max} values at 6 (3 mg kg⁻¹) and 24 (10 mg kg⁻¹) h after the oral administration of NKY-722 were not significantly different from the control value, suggesting the disappearance of the effect of NKY-722. The K_d values for myocardial

(+)-[³H]-PN 200-110 binding were unchanged by oral administration of NKY-722 at a dose of 3 mg kg⁻¹, and they were significantly increased at 1 and 3 h after the administration at 10 mg kg⁻¹. Compared to the control values, the K_d and B_{max} values for (+)-[³H]-PN 200-110 binding in cerebral cortex of SHR were unchanged by oral administration of NKY-722 at the dose of 3 mg kg⁻¹, except an increase in the K_d value at 6 h later, but there was a significant (41 and 43%, respectively) decrease in the B_{max} values at 1 and 3 h after the drug administration at the dose of 10 mg kg⁻¹ (Table 2).

As shown in Table 3, there was a significant (57 and 48%, respectively) decrease in B_{max} values for specific (+)-[³H]-PN 200-110 binding to myocardial membranes at 1 and 3 h after oral administration of nicardipine at the dose of 10 mg kg⁻¹ in SHR and a marked increase in the K_d values at 1 h later. In addition, the administration of nicardipine showed a

Table 1 Effects of oral administration of NKY-722 on K_d and B_{max} values of specific (+)-[³H]-PN 200-110 binding to myocardial membranes of SHR

	K_d (nM)	B_{max} (fmol mg ⁻¹ protein)	Receptor occupancy (%)
Control	0.19 ± 0.01	266 ± 14	
NKY-722 3 mg kg ⁻¹			
1 h	0.22 ± 0.03	$150 \pm 15^{***}$	43.6
3 h	0.21 ± 0.02	$158 \pm 20^{***}$	40.6
6 h	0.16 ± 0.01	218 ± 13	18.0
10 mg kg ⁻¹			
1 h	$0.34 \pm 0.05^{***}$	$38.6 \pm 0.7^{***}$	85.5
3 h	$0.37 \pm 0.07^{**}$	$31.4 \pm 3.3^{***}$	88.2
6 h	0.19 ± 0.02	$97.3 \pm 8.9^{***}$	63.4
12 h	$0.15 \pm 0.01^*$	$144 \pm 15^{***}$	45.9
24 h	0.25 ± 0.04	271 ± 14	

Values are mean \pm s.e.mean of four to eight rats. Rats received 3 and 10 mg kg⁻¹ of NKY-722 orally, and they were killed at 1 to 24 h after the administration. Specific binding of (+)-[³H]-PN 200-110 (0.03–0.57 nM) to myocardial membranes was measured. The receptor occupancy (%) was calculated by the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{NKY-722})]/B_{max}(\text{control})\} \times 100$. Asterisks show a significant difference from control values; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2 Effects of oral administration of NKY-722 on K_d and B_{max} values of specific (+)-[³H]-PN 200-110 binding to cerebral cortical membranes of SHR

	K_d (nM)	B_{max} (fmol mg ⁻¹ protein)	Receptor occupancy (%)
Control	0.11 ± 0.01	171 ± 13	
NKY-722 3 mg kg ⁻¹			
1 h	0.15 ± 0.01	198 ± 17	
3 h	0.15 ± 0.01	168 ± 17	
6 h	$0.22 \pm 0.01^*$	212 ± 7	
10 mg kg ⁻¹			
1 h	0.07 ± 0.01	$100 \pm 4^*$	41.5
3 h	0.07 ± 0.001	$97.7 \pm 4.6^*$	42.9
6 h	0.09 ± 0.001	145 ± 6	15.2

Values are mean \pm s.e.mean of four to eight rats. Rats received 3 and 10 mg kg⁻¹ of NKY-722 orally, and they were killed at 1 to 6 h after the administration. Specific binding of (+)-[³H]-PN 200-110 (0.03–0.57 nM) to cerebral cortical membranes was measured. The receptor occupancy (%) was calculated by the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{NKY-722})]/B_{max}(\text{control})\} \times 100$. Asterisks show a significant difference from control values; * $P < 0.01$.

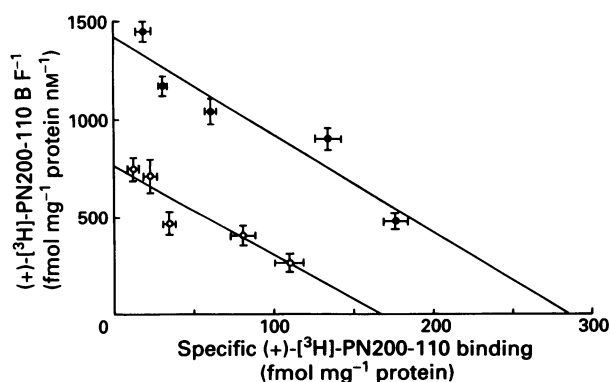


Figure 1 Rosenthal plots of myocardial (+)-[³H]-PN 200-110 binding in control (●) and NKY-722 administered (○) SHR. SHR received orally NKY-722 (3 mg kg⁻¹), and were killed 3 h after the administration. Each point represents mean \pm s.e.mean of 8 (control) and 6 (NKY-722) rats. Ordinate scale: bound over free (B F⁻¹) (+)-[³H]-PN 200-110 (fmol mg⁻¹ protein nM⁻¹). Abscissa scale: (+)-[³H]-PN 200-110 binding (fmol mg⁻¹ protein).

significant (35%) decrease in B_{max} value for cerebral cortical (+)-[³H]-PN 200–110 binding 1 h later.

Occupation *in vivo* of calcium antagonist receptors

The *in vivo* specific binding of (+)-[³H]-PN 200–110 in tissues of SHR was measured. At 10 min after an i.v. injection of (+)-[³H]-PN 200–110 (555 kBq) to control SHR, total amounts of radioactivity were 2596 ± 209 , 2423 ± 125 , and 1566 ± 116 Bq g⁻¹ tissue (mean \pm s.e.mean, $n = 5$) in myocardium, aorta and cerebral cortex, respectively (Figure 2). The particulate bound radioactivity in these tissues, expressed as a percentage of total amount of radioactivity in each tissue, were 64 (myocardium), 48 (aorta) and 55 (cerebral cortex) %, respectively. To measure the *in vivo* nonspecific binding of (+)-[³H]-PN 200–110 to these tissues, nifedipine (40 mg kg⁻¹, i.p.) was administered at 0.5 h before an i.v. injection of (+)-[³H]-PN 200–110 (555 kBq) in SHR. As shown in Figure 2, 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. The significant amount of specific (+)-[³H]-PN 200–110 binding, therefore, defined as the difference in the particulate bound radioactivity between control and nifedipine-pretreated SHR, was demonstrated in the myocardium, aorta and cerebral cortex, as previously reported (Yamada *et al.*, 1992).

The *in vivo* specific binding of (+)-[³H]-PN 200–110 in particulate fractions of myocardium and aorta of SHR was significantly (52 and 79%, respectively) decreased at 1 h after oral administration of NKY-722 (3 mg kg⁻¹), compared to each control value (Figure 3). A similar degree (83%) of reduction in aortic (+)-[³H]-PN 200–110 binding was also observed at 6 h after oral administration of NKY-722; however, the myocardial (+)-[³H]-PN 200–110 binding was almost identical to the control value at this time. Also, oral administration of nicardipine (10 mg kg⁻¹) showed a marked (83%) decrease in the *in vivo* specific binding of (+)-[³H]-PN 200–110 in particulate fractions of SHR myocardium at 1 h later but not at 6 h later. At 1 and 6 h after the nicardipine administration, there was a significant (71 and 53%, respectively) decrease in aortic (+)-[³H]-PN 200–110 binding, and

thus, the extent of the reduction at 6 h was smaller than that at 1 h. On the other hand, the administration of both NKY-722 and nicardipine had little significant effect on the *in vivo* specific binding of (+)-[³H]-PN 200–110 in particulate fractions of cerebral cortex (Figure 3).

Plasma concentration of NKY-722

Plasma concentrations of NKY-722 in SHR (Table 1), used to measure myocardial (+)-[³H]-PN 200–110 binding at 1–24 h after the oral drug administration (3, 10 mg kg⁻¹), were determined; they were (in ng ml⁻¹, mean \pm s.e.mean, $n = 4-6$): 3 mg kg⁻¹, 60.1 ± 11.6 (1 h), 80.3 ± 4.4 (3 h),

Table 3 Effects of oral administration of nicardipine on K_d and B_{max} values of specific (+)-[³H]-PN 200–110 binding to myocardial and cerebral cortical membranes of SHR

	K_d (nM)	B_{max} (fmol mg ⁻¹ protein)	Receptor occupancy (%)
Myocardium			
Control	0.19 ± 0.01	266 ± 14	
Nicardipine (10 mg kg ⁻¹)			
1 h	$0.44 \pm 0.04^{**}$	$115 \pm 14^{**}$	56.8
3 h	0.35 ± 0.04	$138 \pm 18^{**}$	48.1
6 h	0.16 ± 0.01	221 ± 5	16.9
Cerebral cortex			
Control	0.11 ± 0.02	171 ± 13	
Nicardipine (10 mg kg ⁻¹)			
1 h	0.07 ± 0.01	$111 \pm 16^*$	35.1
3 h	0.08 ± 0.01	$132 \pm 4^*$	22.8
6 h	0.07 ± 0.01	160 ± 13	6.4

Values are mean \pm s.e.mean of four to eight rats. Rats received 10 mg kg⁻¹ of nicardipine orally, and they were killed at 1 to 6 h after the administration. Specific binding of (+)-[³H]-PN 200–110 (0.03–0.57 nM) to myocardial and cerebral cortical membranes was measured. The receptor occupancy (%) was calculated by the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{nicardipine})] / B_{max}(\text{control})\} \times 100$. Asterisks show a significant difference from control values; * $P < 0.05$; ** $P < 0.001$.

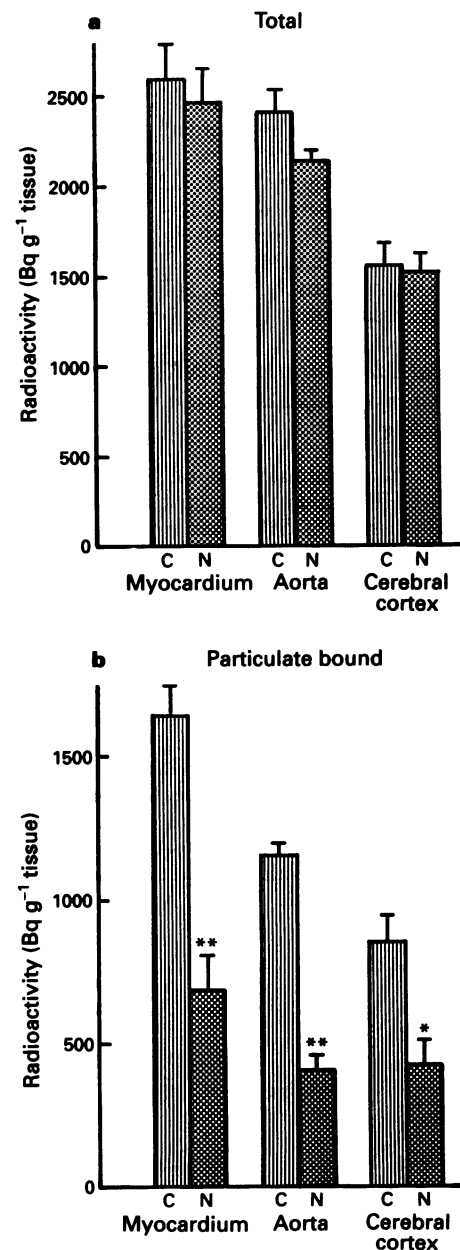


Figure 2 Total and particulate bound radioactivity in myocardium, aorta and cerebral cortex of SHR after the i.v. injection of (+)-[³H]-PN 200–110. (+)-[³H]-PN 200–110 (555 kBq) was injected into the femoral vein in control (C) and nifedipine (N, 40 mg kg⁻¹, i.p., 0.5 h)-pretreated SHR, and they were killed 10 min later: (a) and (b) represent the total tissue radioactivity and particulate bound radioactivity, respectively. Each column represents mean \pm s.e.mean of 5 control and 4 nifedipine-pretreated SHR. Asterisks show a significant difference from control value in each tissue; * $P < 0.05$; ** $P < 0.001$.

5.0 ± 1.6 (6 h); 10 mg kg^{-1} , 355 ± 19 (1 h), 405 ± 18 (3 h), 112 ± 31 (6 h), 44.2 ± 6.6 (12 h), 16.5 ± 6.7 (24 h). As illustrated in Figure 4, the plasma concentration of NKY-722 in each SHR correlated significantly with the occupation by this drug of myocardial calcium antagonist receptors (Table 1).

Discussion

The major findings of this study are that (1) NKY-722 occupied selectively cardiovascular 1,4-DHP calcium antagonist receptors in SHR and its occupation of myocardial receptors correlated significantly with the plasma concentration of this drug; and (2) NKY-722 produced more sustained decrease in the *in vivo* specific binding of (+)-[³H]-PN 200-110 in aortic tissues than in myocardial and brain tissues.

The occupation by NKY-722 of 1,4-DHP calcium antagonist receptors in myocardium, aorta and cerebral cortex of SHR has been investigated in comparison with that by nicardipine. NKY-722 competed with (+)-[³H]-PN 200-110 for the binding sites in myocardium and cerebral cortex of SHR *in vitro*, and this drug showed approximately three times higher binding affinity to the (+)-[³H]-PN 200-110 binding sites in the cerebral cortex than in myocardium, as shown by K_i values of 1.68 and 6.00 nM, respectively. The inhibitory effect of NKY-722 in both tissues was three times less potent than that of nicardipine. At 1 and 3 h after oral administration of NKY-722 (3 mg kg^{-1}), there was a significant (41–44%) reduction in myocardial (+)-[³H]-PN 200-110 binding sites (B_{max}) in SHR compared with control values. The administration of a higher dose (10 mg kg^{-1}) of this drug produced a more sustained and greater (46–88%)

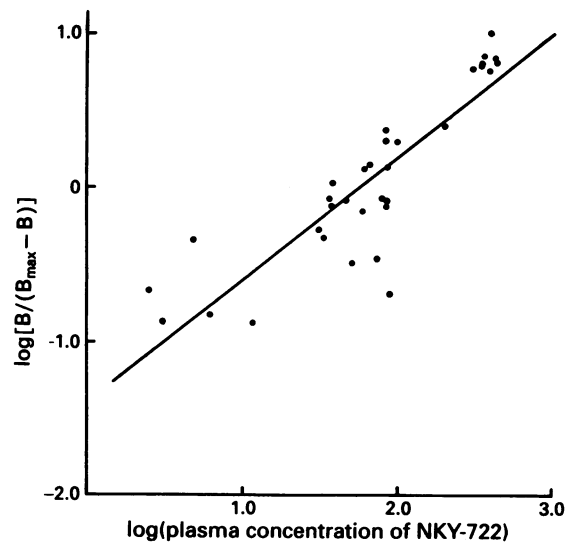


Figure 4 Hill plots for the occupancy of myocardial 1,4-dihydropyridine calcium antagonist receptors as a function of plasma concentration of NKY-722 in SHR. The time-dependent changes in the receptor occupancy in SHR were analysed by a Hill equation in relation to the plasma concentration of NKY-722. The values of B_{max} and B were derived from the maximal number (fmol mg^{-1} protein) of (+)-[³H]-PN 200-110 binding sites in myocardial membranes of control and NKY-722-administered SHR, respectively (Table 1). The plasma concentrations (ng ml^{-1}) of NKY-722 in these SHR were measured: each point represents the values from each SHR received 3 and 10 mg kg^{-1} of NKY-722. The points fit to the linear equation, $y = 0.79x - 1.40$, correlation coefficient $r = 0.87$, where y is the amount of receptor occupancy and x is the plasma concentration of NKY-722.

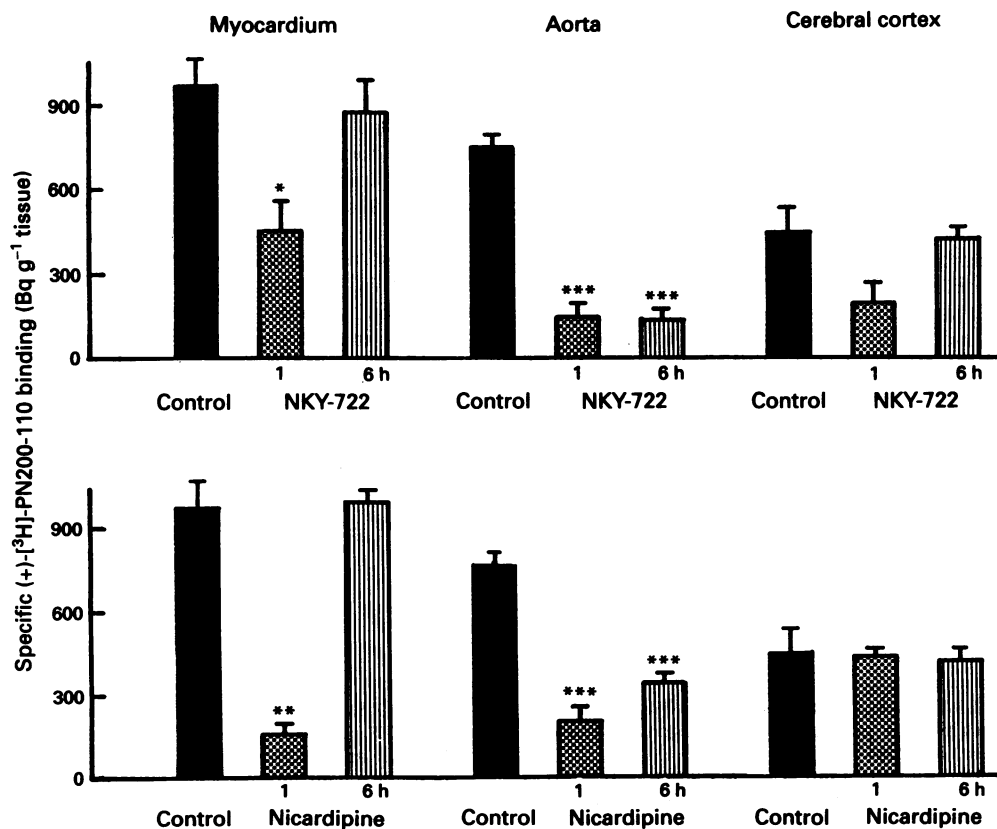


Figure 3 *In vivo* inhibition of specific (+)-[³H]-PN 200-110 binding in particulate fractions from myocardium, aorta and cerebral cortex of SHR at 1 and 6 h after oral administration of NKY-722 and nicardipine. (+)-[³H]-PN 200-110 (555 kBq) was injected into the femoral vein in control, NKY-722 (3 mg kg^{-1} , p.o.) and nicardipine (10 mg kg^{-1} , p.o.)-pretreated SHR, and they were killed 10 min later. Each column represents mean \pm s.e.mean of 3–5 SHR. Asterisks show significant difference from control values; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

decrease in B_{\max} values for myocardial (+)-[³H]-PN 200–110 binding at 1, 3, 6 and 12 h later. The extent of loss of the receptor was greatest at 1 and 3 h later and then decreased with time. The myocardial receptor density returned to the control level at 6 h (3 mg kg⁻¹) and 24 h (10 mg kg⁻¹) after NKY-722 administration. The K_d values of myocardial (+)-[³H]-PN 200–110 binding in SHR were unaltered by NKY-722 at the dose of 3 mg kg⁻¹, but they were significantly increased at 1 and 3 h later by this drug of 10 mg kg⁻¹. Thus, NKY-722 appears to produce mainly a change in the receptor density rather than affinity of 1,4-DHP calcium antagonist receptors.

Oral administration of nicardipine (10 mg kg⁻¹) in SHR also produced a significant (48–57%) reduction in B_{\max} value for myocardial (+)-[³H]-PN 200–110 binding at 1 and 3 h later but not at 6 h later. This inhibitory effect by nicardipine was similar to or slightly greater than the effect by NKY-722 at a dose of 3 mg kg⁻¹ in terms of extent and duration, but it was much weaker than that at a dose of 10 mg kg⁻¹. Such a difference between NKY-722 and nicardipine in the *in vivo* binding potency to myocardial 1,4-DHP calcium antagonist receptors may be responsible for the difference in both potency and duration of their pharmacological effects. In other words, NKY-722 (3 mg kg⁻¹, p.o.) produced a similar extent and duration of hypotensive effect in SHR as observed by nicardipine (10 mg kg⁻¹, p.o.) (Wada *et al.*, 1994). Thus, *in vitro* affinity of NKY-722 for myocardial 1,4-DHP binding sites was three times lower than that of nicardipine as described above, but the hypotensive effect of NKY-722 was approximately three times more potent. This observation might relate to the classical receptor theory by Stephenson (1956) that a drug with lower affinity *in vitro* could appear more potent *in vivo* due to its slower dissociation from the receptor site. In the cerebral cortex, the NKY-722 administration at 3 mg kg⁻¹ had little effect on (+)-[³H]-PN 200–110 binding, and at 10 mg kg⁻¹, it produced a significant reduction of B_{\max} values for (+)-[³H]-PN 200–110 binding at 1 and 3 h later. Thus, the *ex vivo* experiment with NKY-722 has indicated that this drug produced a selective occupation of myocardial, rather than cerebral cortical, 1,4-DHP calcium antagonist receptors in SHR.

The specific binding of (+)-[³H]-PN 200–110 in particulate fractions from myocardial, aortic and brain tissues after an i.v. injection of (+)-[³H]-PN 200–110 to SHR reflects *in vivo* labelling of 1,4-DHP calcium antagonist receptors in tissues (Yamada *et al.*, 1992). The *in vivo* specific binding of (+)-[³H]-PN 200–110 in particulate fractions from myocardial and aortic tissues in SHR reduced significantly (52 and 79%, respectively) at 1 h after oral administration of NKY-722 (3 mg kg⁻¹), and the myocardial (+)-[³H]-PN 200–110 binding restored to the control level at 6 h later. The time course of occupation *in vivo* of myocardial 1,4-DHP calcium antagonist receptors by NKY-722 was consistent with that in the *ex vivo* receptor binding experiment. Interestingly, a marked (83%) decrease in aortic (+)-[³H]-PN 200–110 binding *in vivo* was observed even at 6 h after NKY-722 administration. Therefore, NKY-722 may produce a selective and sustained occupation *in vivo* of vas-

cular 1,4-DHP calcium antagonist receptors in SHR. This finding agrees with the pharmacological data that NKY-722 was highly vasoselective. The dose of NKY-722 that produced 50% decrease in the force of contraction of the paced canine papillary muscle was about 100 times the dose that doubled coronary blood flow (Imagawa *et al.*, 1989). In contrast to a significant occupation of cardiovascular receptors, the *in vivo* specific binding of (+)-[³H]-PN 200–110 in the cerebral cortex of SHR was not significantly reduced by NKY-722 administration. Inasmuch as NKY-722 competed with (+)-[³H]-PN 200–110 for the binding sites in the cerebral cortex of SHR with higher affinity than in the myocardium *in vitro*, this observation, with the *ex vivo* binding data described above, suggests poor transport of this drug through the blood-brain barrier. The nicardipine administration also produced a sustained decrease in aortic (+)-[³H]-PN 200–110 binding *in vivo*. In conclusion, the present study provides the first evidence demonstrating a significant selectivity of 1,4-DHP calcium antagonists in vascular tissues at the receptor level *in vivo*. Thus, the *in vivo* measurement of calcium antagonist receptor occupation may offer a better insight into the interaction at receptor level by calcium antagonists and into the elucidation of tissue selectivity of these drugs under physiological conditions.

Some 1,4-DHP derivatives are subject to extensive and variable first pass metabolism by the liver (Higuchi & Shiobara, 1980; Foster *et al.*, 1983) which reduces systemic bioavailability and drug efficacy after oral administration. The relationship between the cardiovascular effects of some 1,4-DHP derivative calcium antagonists and their plasma concentrations in experimental animals and in man has been investigated (Takata & Kato, 1986; Graefe *et al.*, 1988; Wu *et al.*, 1988). In the present study, the plasma concentration of NKY-722 in SHR was greatest at 1 and 3 h after the oral administration of this drug at doses of 3 and 10 mg kg⁻¹, and at 6 and 12 h later, it decreased markedly to approximately one-tenth of the maximum level. The plasma concentration of NKY-722 in SHR correlated significantly with the occupation of myocardial 1,4-DHP calcium antagonist receptors in the same rats (Figure 4). Recently, Wada *et al.* (1994) have shown that oral administration of NKY-722 (3 mg kg⁻¹) in SHR produced a potent hypotension which was pronounced at 1 to 6 h later and lasted until 8 h. However, at 6 h after oral administration of this dose of NKY-722, there was no significant occupation of myocardial 1,4-DHP calcium antagonist receptors in *ex vivo* and *in vivo* experiments, and the *in vivo* binding data demonstrated more sustained occupation by this drug of 1,4-DHP calcium antagonist receptors in vascular tissues of SHR than in myocardial tissues. Taken together, it is possible that the dissociation of NKY-722 from vascular 1,4-DHP receptors in SHR is significantly slower than the dissociation from myocardial receptors, depending heavily on the plasma concentration of this drug. Thus, a slowly dissociating blockade of vascular 1,4-DHP calcium antagonist receptors by NKY-722 may contribute to the potent and long-lasting hypotensive effect in SHR.

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(Received June 6, 1994
Revised September 1, 1994
Accepted September 2, 1994)