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A Sustained Occupancy *in Vivo* of Cardiovascular Calcium Antagonist Receptors by Mepirodipine and its Relation to Pharmacodynamic Effect in Spontaneously Hypertensive Rats

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ABSTRACT

The occupancy *in vivo* of cardiovascular and cortical Ca^{++} antagonist receptors by mepirodipine in spontaneously hypertensive rats (SHR) was investigated. At 0.5, 3 and 6 hr after an oral administration of mepirodipine (3 mg/kg) in SHR, there was a significant (69, 51 and 41%, respectively) decrease in the number of cardiac (+)-[^{3}H]PN 200–110 binding sites (B_{max}) compared to control values. At 12 hr later, the B_{max} value returned to the control value. On the other hand, the mepirodipine administration had little effect on the dissociation constant (K_d) for cardiac (+)-[^{3}H]PN 200-110 binding except at 0.5 hr, when there was a significant increase in the value, suggesting a change in the density rather than affinity of Ca^{++} antagonist receptors. In the cerebral cortex of these rats, there was a significant (34%) decrease in B_{max} values for (+)-[^{3}H]PN 200-110 binding only at 0.5 hr after mepirodipine administration. In contrast, nifedipine administration had a significant increase in K_d values for cardiac (+)-[^{3}H]PN 200-110 binding without a change in B_{max} values. The occupancy of cardiac Ca^{++} antagonist receptors by mepirodipine correlated significantly with its hypotensive effect in SHR. There was approximately a 39 mm Hg reduction of blood pressure by occupying 50% of these receptors. After an i.v. injection of (+)-[^{3}H]PN 200-110 (15 μCi) to SHR, there was specific binding of the ligand in particulate fractions of heart, aorta, ileum and cerebral cortex, but not liver and kidney. The *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in cardiac, aortic and ileal tissues of SHR was significantly (34–90%) decreased at 0.5 and 6 hr after an oral administration of mepirodipine (3 mg/kg). The present study indicates that mepirodipine may produce a selective and sustained occupancy *in vivo* of cardiovascular Ca^{++} antagonist receptors in SHR. Thus, the *in vivo* measurement of receptor occupancy in SHR by Ca^{++} antagonists may become a useful method to evaluate their pharmacokinetics and pharmacodynamics.

Ca^{++} channel antagonists are used clinically in the treatment of angina pectoris and systemic hypertension, and they produce therapeutic effects by blocking the slow inward current in cardiac and vascular tissues. Thus, the voltage-dependent Ca^{++} channel in these tissues is the target for Ca^{++} antagonists. Mepirodipine hydrochloride [(+)3′S,4S)-3-(1′-benzyl-3′-pyrrolidinyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate hydrochloride) is a novel light-resistant 1,4 DHP Ca^{++} antagonist possessing potent antihypertensive and antianginal activities (Tamazawa *et al.*, 1986; Motomura and Hashimoto, 1990; Kawashima *et al.*, 1991), and this drug binds stereospecifically to binding sites of [^{3}H]nitrendipine and (+)-[^{3}H]PN 200-110 in cardiac, brain and vascular tissues with high affinity (Nakayama *et al.*, 1989; Yamada *et al.*, 1990). In anesthetized dogs, mepirodipine caused the coronary vasodilating effect with a long duration. In addition, the inhibitory effect of mepirodipine on the potassium-induced contraction of isolated porcine coronary artery was maintained for over 4 hr despite repeated washout.

By using radiolabeled 1,4-DHP such as [^{3}H]nitrendipine and (+)-[^{3}H]PN 200–110, DHP Ca^{++} antagonist binding sites were identified and characterized *in vitro* in brain, cardiac and smooth muscles (Bellemann *et al.*, 1981; Bolger *et al.*, 1982; Ehlert *et al.*, 1982; Glossmann *et al.*, 1982; Gould *et al.*, 1982; Godfraind *et al.*, 1986; Yamada *et al.*, 1990). Using the radioreceptor assay technique, binding properties of DHP Ca^{++} antagonists to the receptors have been previously investigated *in vitro*, but not *in vivo*. The *in vitro* receptor affinity of radioligands does not appear to correlate with the tissue specificity of DHP Ca^{++} antagonists inferred from pharmacological data (Glossmann *et al.*, 1982). The apparent lack of tissue specificity may be related to pharmacokinetic and *in vitro* experimental conditions. The *in vivo* binding experiment would allow for a direct measurement of receptor occupancy under physiological conditions. Thus, *in vivo* characterization of Ca^{++} antagonist binding to the receptors appears to be important for the analysis

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ABBREVIATIONS: DHP, dihydropyridine; SHR, spontaneously hypertensive rats.

of pharmacokinetics and pharmacodynamics of this type of drug. In order to clarify the occupancy of Ca^{++} antagonist receptors by mepirodipine, therefore, we have performed *ex vivo* receptor binding experiments using (+)-[^{3}H]PN 200-110 in cardiac and brain tissues of SHR, an experimental model of essential hypertension in humans pretreated with this drug. In addition, the present study was undertaken to characterize *in vivo* binding of (+)-[^{3}H]PN 200-110 to cardiovascular DHP receptors in SHR and then to examine the effect of mepirodipine on these receptors. The hypotensive effect of mepirodipine in SHR was also measured. (+)-[^{3}H]PN 200-110 has been utilized to label DHP receptors because this radioligand binds to these receptors with much higher affinity than other ligands, including [^{3}H]nitrendipine (Lee *et al.*, 1984; Yamada *et al.*, 1990). A part of this work was presented in an abstract form elsewhere (Matsuoka *et al.*, 1991).

Materials and Methods

Drug administration. Male SHR at 15 to 22 weeks of age (Charles River Japan Inc., Kanagawa, Japan) 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-hr dark/light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). They were fasted for 16 hr before the administration of drugs, and administered orally with mepirodipine hydrochloride (3 mg/kg) and nifedipine hydrochloride (10 mg/kg) dissolved in water containing ethanol (10%) and polyethylene glycol 400 (10%) as solvents. Control animals were administered with the vehicle. At 0.5 to 12 hr after the drug administration, SHR were sacrificed by taking the blood from descending aorta under light anesthesia with ethyl ether, and the heart and brain were perfused with 0.9% saline from the aorta. Then, both tissues were removed, and fat and blood vessels were trimmed.

The blood pressure in conscious SHR was measured indirectly by plethysmographic tail cuff method (Pfeffer *et al.*, 1971) using a rat tail manometer system (Tohiden, Model DSR-8014, Tokyo, Japan).

Tissue preparation. The cardiac tissue from SHR was minced with scissors and homogenized by a Kinematica Polytron homogenizer (type PT 10/35) in 20 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4). The cardiac homogenate was centrifuged at 500 × *g* for 10 min, and the supernatant fraction, after filtration through three layers of cheesecloth, 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 to utilize 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 utilized 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.

(+)-[^{3}H]PN 200-110 binding assay. The binding assay of (+)-[^{3}H]PN 200-110 was performed according to the methods by Lee *et al.* (1984) and Yamada *et al.* (1990). Briefly, the membranes (400–600 μg of protein) prepared from rat heart and brain were incubated with different concentrations of (+)-[^{3}H]PN 200-110 in 50 mM Tris-HCl buffer. Incubation was carried out in the dark with a sodium lamp for 60 min at 37°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD) through Whatman GF/B glass fiber 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 (2 l of toluene, 1 l 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 by a liquid scintillation counter. Specific (+)-[^{3}H]PN 200-110 binding was determined experimentally from the difference between counts in the absence and presence of 3 μM nifedipine. All assays were conducted in duplicate.

***In vivo* labeling of Ca^{++} antagonist receptors.** *In vivo* labeling of Ca^{++} antagonist receptors of (+)-[^{3}H]PN 200-110 was performed by a modification of *in vivo* binding of [^{3}H]nitrendipine to DHP receptors described previously by Schoemaker *et al.* (1983). Mepirodipine (3 mg/kg) was orally administered at 0.5 and 6 hr before an i.v. injection of 15 μCi 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 the peripheral tissues (heart, aorta, ileum, liver and kidney) and brain (cerebral cortex) were removed. After the dissection on ice, the tissues were homogenized in ice-cold 50 mM Tris-HCl buffer to a final tissue concentration of 10 mg/ml (peripheral tissues) or 20 mg/ml (cerebral cortex) using a Kinematica Polytron homogenizer. The particulate bound radioactivity was determined by rapid filtration of 1 ml of the homogenate over Whatman GF/C filters, which were washed subsequently with 3 × 3 ml of ice-cold buffer. Also, aliquots (1 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, (+)-[^{3}H]PN 200-110 (15 μCi) was injected i.v. to control and nifedipine (40 mg/kg, i.p., 0.5 hr pretreatment)-administered SHR, to determine total and nonspecific binding in each tissue.

Analysis of data. The analysis of binding data was performed as described previously (Yamada *et al.*, 1980). The apparent K_d and B_{max} for (+)-[^{3}H]PN 200-110 were estimated by Rosenthal analysis of the saturation data over concentration range of 0.02 to 2.4 nM (Rosenthal, 1967). The ability of antagonists to inhibit specific (+)-[^{3}H]PN 200-110 binding was estimated by IC_{50} values, which are the molar concentrations of unlabeled drugs necessary for displacing 50% of the specific binding (estimated by log probit analysis). The Hill coefficients for saturation data of (+)-[^{3}H]PN 200-110 binding and for inhibition by antagonists were obtained by the Hill plot analysis.

Materials. (+)-[^{3}H]PN 200-110 (87.0 Ci/mmol) was purchased from Dupont-NEN Co. Ltd. (Boston, MA). The following drugs were kindly donated by the companies indicated: mepirodipine hydrochloride, Yamanouchi Pharmaceutical Company (Tokyo, Japan) and nifedipine hydrochloride, Bayer Pharmaceutical Company (Osaka, Japan). Drugs were dissolved in 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

Occupancy *ex vivo* of cardiac and brain Ca^{++} antagonist receptors. Specific binding of (+)-[^{3}H]PN 200-110 (0.02–0.5 nM) in cardiac 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 0.10 ± 0.01 (heart) and 0.07 ± 0.01 (cerebral cortex) nM (n = 6). The B_{max} values for (+)-[^{3}H]PN 200-110 in these tissues were 207 ± 7 (heart) and 119 ± 3 (cerebral cortex) fmol/mg of protein. The Hill coefficients of (+)-[^{3}H]PN 200-110 binding in both tissues were close to unity. Additionally, the specific (+)-[^{3}H]PN 200-110 binding in these tissues showed a pharmacological drug profile compatible with Ca^{++} antagonist receptors (Yamada *et al.*, 1990). Nifedipine (1–100 nM) and mepirodipine (0.1–10 nM) competed with (+)-[^{3}H]PN 200-110 for the cardiac and cerebral cortical binding sites. The IC_{50} values for the *in vitro* inhibition of specific (+)-[^{3}H]PN 200-110 binding by nifedipine in SHR heart and cerebral cortex were 3.36 ± 0.14 and 3.14 ± 0.29 nM (n = 4), respectively, and the values by mepirodipine were 0.61 ± 0.09 and 0.54 ± 0.03 nM (n = 4), respectively. The Hill coefficients for both drugs in these tissues were close to one (0.9–1.1).

At 0.5 to 6 hr after an oral administration of mepirodipine at a dose of 3 mg/kg in SHR, there was a significant decrease in specific (+)-[^{3}H]PN 200-110 binding to cardiac membranes. As shown in figure 1 and table 1, Rosenthal analysis revealed that there was a significant decrease in B_{max} values for cardiac (+)-[^{3}H]PN 200-110 binding at 0.5 to 6 hr after the mepirodipine administration compared to the control value. The decrease in B_{max} values at 0.5, 3 and 6 hr were 69, 51 and 41%, respectively. Thus, the reduction of B_{max} values by mepirodipine was more pronounced at 0.5 hr, and the effect was decreased significantly with time. The B_{max} value for cardiac (+)-[^{3}H]PN 200-110 binding at 12 hr after the oral administration of mepirodipine in SHR was almost identical to the control value, thereby suggesting the disappearance of mepirodipine effect. In contrast to the sustained decrease in B_{max} values, there was a significant increase in the K_d value for cardiac (+)-[^{3}H]PN 200-110 binding only at 0.5 hr after the mepirodipine administration.

At 0.5 and 3 hr after an oral administration of nifedipine (10 mg/kg) in SHR, on the other hand, there was a significant (90 and 120%, respectively) increase in K_d values for cardiac (+)-[^{3}H]PN 200-110 binding without a change in B_{max} values (fig. 2, table 1). The change in K_d values by nifedipine was not observed at 6 hr after the administration.

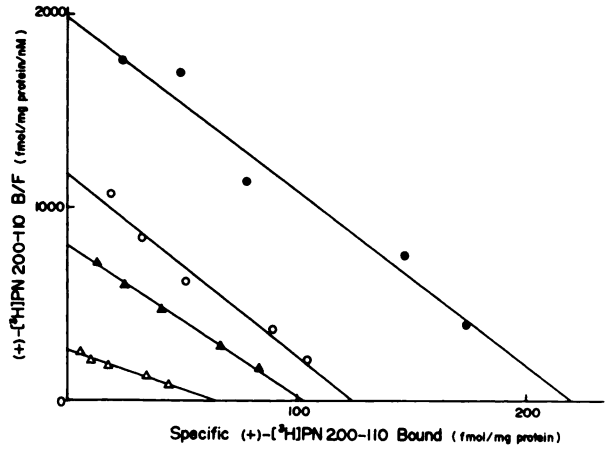

Fig. 1. Rosenthal plots of cardiac (+)-[^{3}H]PN 200-110 binding in control (●) and mepirodipine administered SHR. SHR received orally mepirodipine (3 mg/kg), and they were sacrificed 0.5 (△), 3 (▲) and 6 (○) hr after the administration. Each point represents the average of three to six animals. Ordinate, bound over free (B/F) (+)-[^{3}H]PN 200-110 (fmol/mg protein/nM). Abscissa, (+)-[^{3}H]PN 200-110 bound (fmol/mg protein).

TABLE 1

Effects of oral administration of mepirodipine and nifedipine on K_d and B_{max} values of specific (+)-[^{3}H]PN 200-110 binding to SHR hearts

SHR received orally mepirodipine (3 mg/kg) and nifedipine (10 mg/kg), and they were sacrificed 0.5 to 12 hr after the administration. Each value represents mean ± S.E. of three to six animals.

Drugs		Specific (+)-[^{3}H]PN 200-110 Binding	
		K_d	B_{max}
		nM	fmol/mg protein
Control		0.10 ± 0.01	207 ± 7
Mepirodipine (3 mg/kg)	0.5 hr	0.22 ± 0.04*	63.9 ± 1.7***
	3 hr	0.13 ± 0.003	101 ± 5***
	6 hr	0.11 ± 0.01	123 ± 4***
	12 hr	0.10 ± 0.01	217 ± 11
Nifedipine (10 mg/kg)	0.5 hr	0.19 ± 0.01***	199 ± 5
	3 hr	0.22 ± 0.02***	219 ± 20
	6 hr	0.12 ± 0.01	204 ± 7

* $P < .05$, significantly different from control.
*** $P < .001$, significantly different from control.

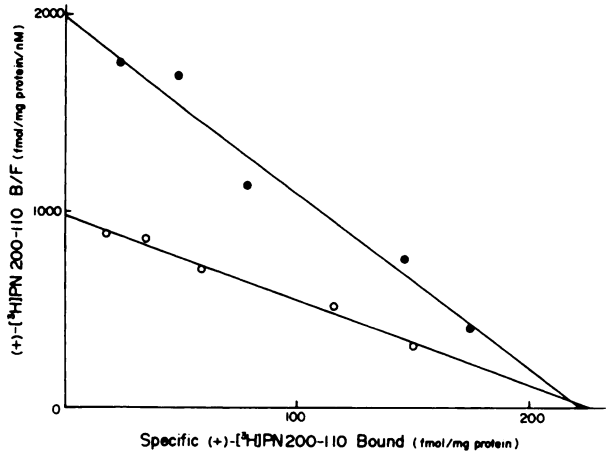

Fig. 2. Rosenthal plots of cardiac (+)-[^{3}H]PN 200-110 binding in control (●) and nifedipine (○) administered SHR. SHR received orally nifedipine (10 mg/kg), and they were sacrificed 3 hr after the administration. Each point represents the average of six control and three nifedipine-treated animals. Ordinate, bound over free (B/F) (+)-[^{3}H]PN 200-110 (fmol/mg protein/nM). Abscissa, (+)-[^{3}H]PN 200-110 bound (fmol/mg protein).

TABLE 2

Effects of oral administration of mepirodipine and nifedipine on K_d and B_{max} values of specific (+)-[^{3}H]PN 200-110 binding to SHR cerebral cortex

SHR received orally mepirodipine (3 mg/kg) and nifedipine (10 mg/kg), and they were sacrificed at 0.5 to 12 hr after the administration. Each value represents mean ± S.E. of three to six animals.

Drugs		Specific (+)-[^{3}H]PN 200-110 Binding	
		K_d	B_{max}
		nM	fmol/mg protein
Control		0.07 ± 0.01	119 ± 3
Mepirodipine (3 mg/kg)	0.5 hr	0.13 ± 0.06	78.0 ± 13.9**
	3 hr	0.08 ± 0.02	94.0 ± 17.5
	6 hr	0.06 ± 0.003	117 ± 4
	12 hr	0.07 ± 0.01	115 ± 18
Nifedipine (10 mg/kg)	0.5 hr	0.08 ± 0.01	122 ± 6
	3 hr	0.13 ± 0.01**	125 ± 7
	6 hr	0.07 ± 0.03	115 ± 5

** $P < .01$, significantly different from control.

The specific (+)-[^{3}H]PN 200-110 binding in cerebral cortical membranes of SHR after an oral administration of mepirodipine and nifedipine was simultaneously measured. Compared to the control value, the B_{max} value for cortical (+)-[^{3}H]PN 200-110 binding was significantly (34%) decreased at 0.5 hr after an oral administration of mepirodipine (3 mg/kg), but not at 3, 6 and 12 hr (table 2). The K_d values were unchanged by the mepirodipine administration. Thus, there was a definite tissue variation in the development of decreased (+)-[^{3}H]PN 200-110 binding between heart and cerebral cortex after an oral administration of mepirodipine in SHR.

The oral administration of nifedipine (10 mg/kg) in SHR produced a significant (86%) increase in the K_d value of cortical (+)-[^{3}H]PN 200-110 binding only 3 hr later (table 2).

Occupancy *in vivo* of Ca^{++} antagonist receptors. The *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in several tissues of SHR was measured. At 10 min after an i.v. injection of (+)-[^{3}H]PN 200-110 (15 μCi) to SHR, total amount of radioactivity varied from 1186 ± 95 (aorta) to 4678 ± 152 (liver) dpm/10 mg tissue in peripheral tissues (n = 4) (fig. 3, upper panel). The particulate bound radioactivity in control SHR, expressed as a

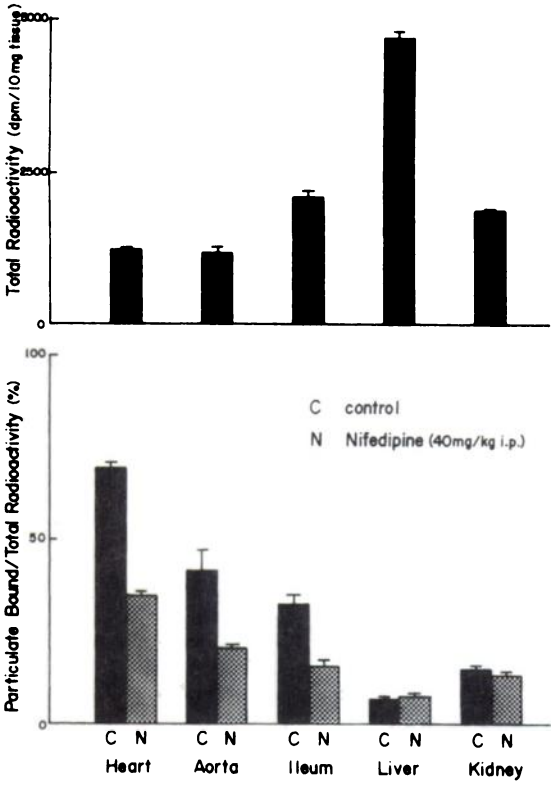

Fig. 3. Total (upper panel) and particulate (lower panel) bound radioactivity in heart, aorta, ileum, liver and kidney of SHR after the i.v. injection of (+)-[^{3}H]PN 200-110. (+)-[^{3}H]PN 200-110 (15 μCi) was injected into the femoral vein in control (C) and nifedipine (N, 40 mg/kg, i.p., 0.5 hr)-pretreated SHR, and they were sacrificed 10 min later. The upper panel represents the total tissue radioactivity in control SHR. Each column represents mean ± S.E. of four control and three nifedipine-pretreated animals.

percentage of total amount of radioactivity in each tissue, ranged from 7.1 ± 0.6 (liver) to 68.2 ± 9.9% (heart) (fig. 3, lower panel, closed column). In the liver, there was the lowest amount of particulate bound radioactivity despite the highest total tissue radioactivity, indicating that this tissue includes a considerably large amount of free ligand derived from bloods. To measure the *in vivo* nonspecific binding of (+)-[^{3}H]PN 200-110 to these tissues, nifedipine (40 mg/kg, i.p.) was administered at 0.5 hr before an i.v. injection of (+)-[^{3}H]PN 200-110 (15 μCi) in SHR. As shown in figure 3 (lower panel), the particulate bound radioactivity was markedly decreased in the heart, aorta and ileum, but not in the liver and kidney by an i.p. administration of nifedipine. The specific binding of (+)-[^{3}H]PN 200-110 (dpm/10 mg tissue or percentage of total radioactivity), therefore, defined as the difference in the particulate bound radioactivity between control and nifedipine-pretreated SHR, could be demonstrated in the heart, aorta and ileum, but not in the liver and kidney (fig. 4).

The *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions of heart, aorta and ileum of SHR was significantly (34–90%) decreased at 0.5 and 6 hr after an oral administration of mepirodipine (3 mg/kg) compared to each control value (fig. 4). The extent of decrease in the *in vivo* cardiac (+)-[^{3}H]PN 200-110 binding was greater at 0.5 hr than 6 hr. The reduction by mepirodipine at 6 hr tended to be more pronounced in the aorta than in the heart and ileum.

The total amount of radioactivity in cerebral cortex of SHR injected i.v. (+)-[^{3}H]PN 200-110 was 1332 ± 16 dpm/20 mg tissue (n = 4), and the *in vivo* specific binding in the particulate fraction was 277 ± 26 dpm/20 mg tissue (18.0 ± 2.1% as a percentage of total radioactivity). The oral administration of mepirodipine had little significant effect on the *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions of cerebral cortex at both 0.5 and 6 hr (214 ± 17 and 255 ± 17 dpm/20 mg tissue, respectively, n = 4).

Hypotensive effect by mepirodipine. The systolic blood pressure of conscious SHR at 15 to 22 weeks of age was 173 ± 3 mmHg (n = 17), indicating an established hypertensive stage. At 0.5, 3 and 6 hr after an oral administration of mepirodipine (3 mg/kg) in SHR, there were 35, 23 and 15% decreases in the systolic blood pressure. At 12 hr, the blood pressure in SHR tended to return to the pretreated level.

Discussion

The major findings of this study are that: 1) mepirodipine produced a selective and sustained occupancy *in vivo* of cardiovascular Ca^{++} antagonist receptors in SHR and its receptor occupancy correlated significantly with the hypotensive effect; and 2) the measurement of *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions of tissues after a systemic administration of Ca^{++} antagonists may be useful for the analysis of their pharmacodynamic effects.

Since the discovery of specific binding sites for DHP Ca^{++} antagonists in central and peripheral tissues (Belleman *et al.*, 1981; Bolger *et al.*, 1982; Ehlert *et al.*, 1982; Glossman *et al.*, 1982; Gould *et al.*, 1982), the affinities of a number of compounds with DHP structures for the binding sites have been screened by *in vitro* binding techniques. One of the objectives of such *in vitro* screening is the selection of drugs which will act in the cardiovascular system *in vivo*. However, *in vitro* binding techniques do not take various pharmacokinetic and pharmacodynamic factors (absorption, distribution, elimination) into account so that high affinity for the DHP binding sites *in vitro* is by no means a guarantee of pharmacological activity *in vivo*. Ideally, then, a simple *in vivo* screening technique for potential Ca^{++} antagonist receptor ligands would be more advantageous. Measurements of receptor sites in animals *in vivo* are currently receiving considerable interest. This technique has been applied to several receptor sites mainly in the central nervous system, such as muscarinic (Yamamura *et al.*, 1975), dopaminergic (Köhler *et al.*, 1985) and benzodiazepine (Goeders and Kuhar, 1985; Miller *et al.*, 1987) receptors, but not to Ca^{++} antagonist receptors. The *in vivo* binding studies confer a measure of the actual occupancy of central benzodiazepine receptors and show very good correlation between receptor occupancy and pharmacological effects (Igari *et al.*, 1985; Miller *et al.*, 1987). In the present study, we have investigated the *ex vivo* effect of mepirodipine and nifedipine on cardiac and cortical Ca^{++} antagonist receptors in SHR by radioreceptor assay technique using (+)-[^{3}H]PN 200-110, and then attempted to label these receptors in several tissues of SHR *in vivo*.

At 0.5, 3 and 6 hr after an oral administration of mepirodipine (3 mg/kg) in SHR, there was a significant (41–69%) decrease in the number of cardiac (+)-[^{3}H]PN 200-110 binding sites compared to control values. The effect by mepirodipine was more pronounced at 0.5 hr, and decreased with time. The B_{max} value for cardiac (+)-[^{3}H]PN 200-110 binding restored to the control value at 12 hr after the mepirodipine administration.

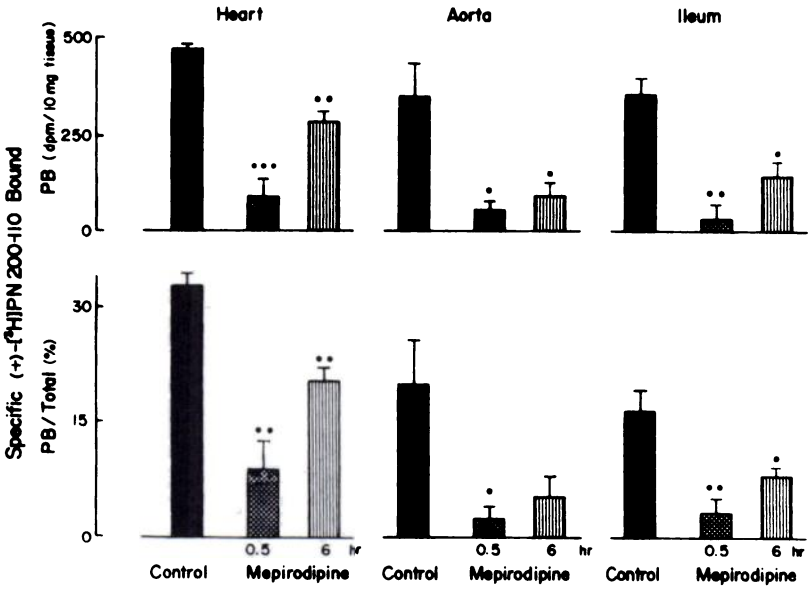

Fig. 4. *In vivo* inhibition of specific (+)-[^{3}H]PN 200-110 binding in particulate fractions from heart, aorta and ileum of SHR at 0.5 and 6 hr after an oral administration of mepirodipine. (+)-[^{3}H]PN 200-110 (15 μCi) was injected into the femoral vein in control and mepirodipine (3 mg/kg, p.o.)-pretreated SHR, and they were sacrificed 10 min later. Each column represents mean ± S.E. of four animals. Asterisks show a significant difference from control values (* $P < .05$, ** $P < .01$, *** $P < .001$).

On the other hand, the K_d value was unchanged by mepirodipine administration except 0.5 hr, when there was a significant increase in the value, suggesting a change in the density rather than affinity of Ca^{++} antagonist receptors. By contrast, the nifedipine administration had a significant increase in K_d values for cardiac (+)-[^{3}H]PN 200-110 binding without a change in B_{max} values at 0.5 and 3 hr later, suggesting a reduction only in the apparent affinity for Ca^{++} antagonist receptors. Such a difference in the *ex vivo* binding characteristics of mepirodipine and nifedipine to DHP receptors may be responsible for the difference in both potency and duration of their pharmacological effects (Tamazawa *et al.*, 1986; Nakayama *et al.*, 1989; Motomura and Hashimoto, 1990). In the cerebral cortex, the mepirodipine administration produced a significant (34%) decrease in specific (+)-[^{3}H]PN 200-110 binding sites at 0.5 hr, but not at 3, 6 and 12 hr. Thus, the *ex vivo* experiment with mepirodipine has indicated that this drug produced a sustained occupation of cardiac, but not cortical, Ca^{++} antagonist receptors in SHR.

The mechanism responsible for a sustained blockade of (+)-[^{3}H]PN 200-110 biding sites by mepirodipine is not clarified. In our recent study, the *in vitro* blockade of cardiac (+)-[^{3}H] PN 200-110 binding sites induced by mepirodipine was not reversed by repeated washout procedure (suspension-centrifugation) with Tris-HCl buffer, whereas the blockade of binding sites by nifedipine was easily reversible under these conditions (unpublished observation). These results indicate that unlike nifedipine, mepirodipine may bind persistently to Ca^{++} antagonist receptors. Thus, the sustained occupation *in vivo* of cardiac (+)-[^{3}H]PN 200-110 binding sites by mepirodipine appears to be due to a slowly dissociating blockade of Ca^{++} antagonist receptor sites, which may be closely associated with an observed higher affinity of the drug for the receptors compared to nifedipine.

The specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions from cardiac, aortic and ileal tissues after an i.v. injection of (+)-[^{3}H]PN 200-110 to SHR was observed. In these animals, on the other hand, there was little specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions from liver and kidney despite a relatively large amount of total radioactivity. The observed regional distribution of specific (+)-[^{3}H]PN 200-110 binding sites *in vivo* agrees well with *in vitro* distribution of DHP Ca^{++} antagonist receptors in rats previously reported (Glossman *et al.*, 1982; Gould *et al.*, 1982). Accordingly, specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions from cardiac, aortic and ileal tissues of SHR reflects *in vivo* labeling of Ca^{++} antagonist receptors in tissues. The *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions from cardiac, aortic and ileal tissues of SHR was significantly reduced at 0.5 and 6 hr after an oral administration of mepirodipine. The time course and extent of occupancy *in vivo* of cardiac Ca^{++} antagonist receptors by mepirodipine appear to be consistent with those obtained in the *ex vivo* receptor binding experiment. Also, the *in vivo* specific binding of (+)-[^{3}H]PN 200-110 in particulate fractions of cerebral cortex of SHR was

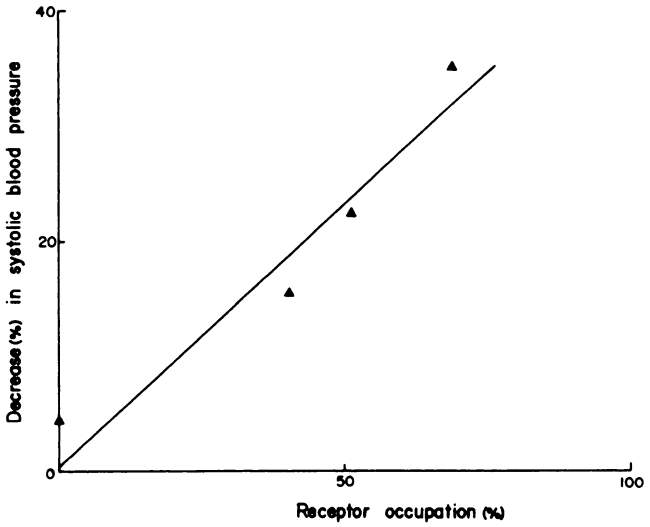

Fig. 5. Correlation between occupancy of cardiac Ca^{++} antagonist receptors and hypotensive effect by mepirodipine in SHR. The receptor occupancy by mepirodipine was calculated from B_{max} values for cardiac (+)-[^{3}H]PN 200-110 binding in mepirodipine administered SHR (table 1). The hypotensive effect by mepirodipine was expressed as the percentage of decrease in systolic blood pressure of conscious SHR at 0.5, 3, 6 and 12 hr after an oral administration of this drug (3 mg/kg). The data of receptor occupancy by mepirodipine were significantly ($P < .05$) correlated with its hypotensive effect. The correlation coefficient for this relationship was $r = 0.98$.

unaffected by an oral administration of mepirodipine. These data strongly suggest that mepirodipine produced a selective and sustained occupation *in vivo* of cardiovascular Ca^{++} antagonist receptors in SHR. This finding may provide the first *in vivo* evidence that DHP Ca^{++} antagonist occupies significantly Ca^{++} antagonist receptors in cardiovascular tissues rather than brain. The present experiment also indicates that highly potent Ca^{++} antagonist ligand (+)-[^{3}H]PN 200-110 can be utilized successfully to label DHP Ca^{++} antagonist receptors *in vivo*. This technique, which measures the receptor occupancy under physiological conditions, may offer a better insight into the interaction in receptor level by Ca^{++} antagonists *in vivo* and into the mechanism of tissue specificity of these drugs than that obtained from *in vitro* experiments, and it may be also useful as a screening method for potential Ca^{++} antagonists.

The oral administration of mepirodipine (3 mg/kg) in SHR produced a prolonged (10–35%) decrease in the blood pressure as previously reported in stroke-prone SHR by Kawashima *et al.* (1991). As illustrated in figure 5, the occupancy of cardiac Ca^{++} antagonist receptors by mepirodipine correlated significantly ($P < .05$) with its hypotensive effect. There was an approximately 39 mm Hg reduction of blood pressure by occupying 50% of Ca^{++} antagonist receptors in SHR heart. Accordingly, the *in vivo* measurement of receptor occupation in SHR by Ca^{++} antagonists may become a useful method to evaluate their pharmacokinetics and pharmacodynamics.

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References

BELLEMANN, P., FERRY, D. R., LÜBBECKE, F. AND GLOSSMAN, H.: [^{3}H]Nitrendipine, a potent calcium antagonist, binds with high affinity to cardiac membranes. Arzneim.-Forsch./Drug Res. **31:** 2064–2067, 1981.

BOLGER, C. T., GENGO, P. T., LUCKOWSKI, E. M., SIEGEL, H., TRIGGLE, D. J. AND JANIS, R. A.: High affinity binding to a calcium channel antagonist to smooth and cardiac muscle. Biochem. Biophys. Res. Commun. **104:** 1604–1609, 1982.

EHLERT, F. J., ROESKE, W. R., ITOGA, E. AND YAMAMURA, H. I.: The binding of [^{3}H]-nitrendipine to receptors for calcium channel antagonists in the heart, cerebral cortex and ileum of rats. Life Sci. **30:** 2191–2202, 1982.

GLOSSMAN, H., FERRY, D. R., LÜBBECKE, F., MEWES, R. AND HOFMANN, F.: Calcium channels: Direct identification with radioligand binding studies. Trends Pharmacol. Sci. **3:** 431–437, 1982.

GODFRAIND, T., MILLER, R. AND WIBO, M.: Calcium antagonism and calcium entry blockade. Pharmacol. Rev. **38:** 321–416, 1986.

GOEDERS, N. E. AND KUHAR, M. J.: Benzodiazepine receptor binding in vivo with ^{3}H-Ro 15-1788. Life Sci. **37:** 345–355, 1985.

GOULD, R. J., MURPHY, K. M. M. AND SNYDER, S. H.: [^{3}H]Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. Proc. Natl. Acad. Sci. USA **79:** 3656–3660, 1982.

IGARI, Y., SUGIYAMA, Y., SAWADA, Y., IGA, T. AND HANANO, M.: Kinetics of receptor occupation and anticonvulsive effects of diazepam in rats. Drug Metab. Dispos. **13:** 102–106, 1985.

KAWASHIMA, K., TODA, H., OOHATA, H., FUJIMOTO, K., SUZUKI, T. AND INAGAKI, O.: Antihypertensive and diuretic effects of YM-09730-5, a new calcium antagonist, in stroke-prone spontaneously hypertensive rats. Gen. Pharmacol. **22:** 263–266, 1991.

KÖHLER, C., HALL, H., ÖRGEN, S. O. AND GAWELL, L.: Specific in vitro and in vivo binding of ^{3}H-raclopride. A potency substituted benzamide drug with high affinity for dopamine D_2 receptors in the rat brain. Biochem. Pharmacol. **34:** 2251–2259, 1985.

LEE, H. R., ROESKE, W. R. AND YAMAMURA, H.I.: High affinity specific (+)-[^{3}H]PN 200-110 binding to dihydropyridine receptors associated with channels in rat cerebral cortex and heart. Life Sci. **35:** 721–732, 1984.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265–275, 1951.

MATSUOKA, Y., YAMADA, S., SUZUKI, N., KATO, Y. AND KIMURA, R.: Occupation of cardiac Ca^{++} antagonist receptors and hypotensive effect by mepirodipine in spontaneously hypertensive rats (SHR). Jpn. J. Pharmacol. **55:** suppl., 179P, 1991.

MILLER, L. G., GREENBLATT, D. J., PAUL, S. M. AND SHADER, R. I.: Benzodiazepine receptor occupancy *in vivo*: Correlation with brain concentrations and pharmacodynamic actions. J. Pharmacol. Exp. Ther. **240:** 516–522, 1987.

MOTOMURA, S. AND HASHIMOTO, K.: Reconsideration of vascular selectivity of dihydropyridine calcium antagonists: Comparison of cardiovascular profile of mepirodipine, a novel dihydropyridine consisting of a single stereoisomer with (+)-(S)-(S) conformation, with those of nifedipine and nicardipine. Jpn. J. Pharmacol. **52:** 319–330, 1990.

NAKAYAMA, K., KASHIWABARA, T., YAMADA, S. AND TANAKA, Y.: Assessment in pig coronary artery of long-lasting and potent calcium antagonistic actions of the novel dihydropyridine derivative mepirodipine hydrochloride. Arzneim.-Forsch./Drug Res. **39:** 50–55, 1989.

PFEFFER, J. M., PFEFFER, M. A., FROHLICH, E. D.: Validity of an indirect tail cuff method for determining systolic arterial pressure in unanesthetized normotensive and spontaneously hypertensive rats. J. Lab. Clin. med. **78:** 957–962, 1971.

ROSENTHAL, H. E.: Graphic method for the determination and presentation of binding parameters in a complex system. Anal. Biochem. **20:** 525–532, 1967.

SCHOEMAKER, H., LEE, H. R., ROESKE, W. R. AND YAMAMURA, H. I.: In vivo identification of calcium antagonist binding sites using [^{3}H]nitrendipine. Eur. J. Pharmacol. **88:** 275–276, 1983.

TAMAZAWA, K., ARIMA, H., KAJIMA, T., ISOMURA, Y., OKADA, M., FUJITA, S., FURUYA, T., TAKENAKA, T., INAGAKI, O. AND TERAI, M.: Stereoselectivity of a potent calcium antagonist, 1-benzyl-3-pyrrolidinyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate. J. Med. Chem. **29:** 2504–2511, 1986.

YAMADA, S., KIMURA, R., HARADA, Y. AND NAKAYAMA, K.: Calcium channel receptor sites for (+)-[^{3}H]PN 200-110 in coronary artery. J. Pharmacol. Exp. Ther. **252:** 327–332, 1990.

YAMADA, S., YAMAMURA, H. I. AND ROESKE, W. R.: Characterization of *alpha*-1 adrenergic receptors in the heart using [^{3}H]WB4101: Effect of 6-hydroxydopamine treatment. J. Pharmacol. Exp. Ther. **215:** 176–185, 1980.

YAMAMURA, H. I., KUHAR, M. J. AND SNYDER, S. H.: In vivo identification of muscarinic cholinergic receptor binding in rat brain. Brain Res. **80:** 170–176, 1975.

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