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Ca-dependent Alteration in Basal Tone, Basal ^{45}Ca Uptake and ^{3}H-nitrendipine Binding in the Aorta of Spontaneously Hypertensive Rats

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= ABSTRACT =

We investigated the alterations in basal tone of aortic strips by changing the Ca concentration, basal ^{45}Ca uptake and ^{3}H-nitrendipine binding of the single cells of aortic smooth muscles in the spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats.

While the basal tone of the aortic strips in WKY rats was not affected by alteration of Ca concentration, that in SHR was decreased by the removal of Ca from the bath solution and was recovered by the restoration of Ca to normal levels. This contraction increased in a Ca concentration-dependent manner and reached a maximum at 2 mM Ca. The basal tone of aorta in SHR was suppressed by verapamil (10^{-6}M). The basal tone of aorta in SHR increased about 50% in the strips of endothelial rubbing, compared with that of intact endothelium. Basal ^{45}Ca uptake in the aortic single smooth muscle cells of SHR was greater than that of WKY ($p < 0.01$). Specific bindings of [^{3}H]nitrendipine in the aortic single smooth muscles of SHR and WKY were saturable. The dissociation constant (K_d) was 0.71 ± 0.15 and 1.18 ± 0.08nM in WKY and SHR, respectively, and the difference in K_d between two strains was statistically significant ($p<0.03$). The maximal binding capacity (B_{max}) was 34.6 ± 3.2 and 47.4 ± 4.3 fmol/10^6 cells in WKY and SHR, respectively, and the difference of B_{max} between two strains was statistically significant ($p<0.05$).

From the above results, it is suggested that the increase of Ca influx via potential-operated Ca channels and the increase of the number of dihydropyridine-sensitive Ca channels contribute to high basal tone of the aortic strips in SHR.

Key Words : Basal tone, Calcium, ^{45}Ca uptake, 3H-nitrendipine binding, Spontaneously hypertensive rats

INTRODUCTION

Abnormal Ca handling at the plasma membrane of arterial smooth muscle cells has been thought to play a role in the development and maintenance of hypertension.

The vascular smooth muscle of normotensive rats, especially the smooth muscle of large arteries, are almost quiescent. In addition they do not exhibit any spontaneous tension development in the absence of stimulation. On the other hand, the vascular smooth muscle of spontaneously hypertensive rats (SHR) exhibit spontaneously developing active tone as has been reported (Noon et al. 1979; Lindner & Heinle, 1987; Sada et al, 1989). Noon et al (1979) reported that high basal tone in the aorta of SHR was due to abnormal Ca leakage. Lindner and Heinle (1987)

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reported that Ca repletion of aortic vascular smooth muscle of SHR in the absence of any vasoconstrictor results from the influx of Ca, mainly via potential-dependent calcium channels which are sensitive to calcium antagonists. It has been predicted that the potential-dependent Ca channels may be altered in hypertensive human and animal models compared with their normotensive controls (Kwan, 1985; Bohr & Webb, 1988). However, the mechanism of the increased basal tone in the vascular smooth muscle of SHR is still controversial.

It was suggested that high basal tone in the aorta of SHR was due to the increase of intracellular Ca concentration of vascular smooth muscle (Sada et al, 1990). However, the cause of increased intracellular Ca concentration is not clear. It may be due to the increase of basal Ca influx via the leak channel or Ca channels in SHR. Also intracellular Ca concentration can be affected by change in the number of Ca channels.

Recently, Orlov et al (1993) reported that basal Ca uptake was approximately 50% greater in aortic vascular smooth muscle cells of SHR than that of WKY. In the binding studies of dihydropyridine derivatives (PN200 -110 and nitrendipine) on membrane preparations from the aorta and tail artery of SHR and WKY rats, there is no difference in the dissociation constant (K_d) between the two strains (Ikeda et al, 1990; Galletti et al, 1991). However Godfraind et al (1990) reported that specific binding of PN 200-110 in aortic membrane of SHR was higher than that of WKY at normal potassium concentration.

Reuter (1983) mentioned that Ca channels, unlike Na channels, are not functional in isolated membrane vesicles, and binding studies should preferably be done in intact cells. However, there is no data for the binding studies of dihydropyridine Ca channels in the intact vascular smooth muscles of SHR and WKY.

Therefore in the present study, we investigated the mechanism of increased basal tone in the smooth muscle of SHR, by comparing contractile response to changes of Ca concentration on the basal tone in the aortic strips, basal ^{45}Ca uptake and ^{3}H-nitrendipine binding in single cells of aortic smooth muscles in SHR and WKY.

METHODS

Animals

All experiments were performed on 12 ~16week-old spontaneously hypertensive rats (SHR) (Okamoto, 1969) and age-matched normotensive Wistar-Kyoto (WKY) rats. The systolic blood pressure was determined in conscious restrained rats by the tail-cuff plethysmographic method (PE-300, Narco-Biosystems, Houston, Texas). Systolic blood pressure was 204 ± 5 mmHg for SHR (n=20) and 136 ± 8 mmHg for WKY (n=20).

Measurement of isometric tension

The rats were stunned and exsanguinated. The aorta was quickly dissected and adhering adventitia and remaining fat were removed under a stereoscopic microscope. The aorta was left to recover for 2 hours at room temperature. The aorta was then carefully cut into a helical strip (2mm wide and 10mm long).

The helical strips of aorta were mounted vertically between glass hooks in a thermostatically controlled organ bath containing 50 ml of the Tris-buffered Tyrode's solution contained (mM): NaCl 158, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 6, and Tris 5 (pH 7.4 at 37 ℃). Organ bath solution was maintained at 37 ℃ and continuously bubbled with 100% O_2. The hook anchoring the upper end of the strip was connected to the lever of a force transducer (F-60, Narco-Bio system) and the strips were suspended under a tension of 0.5g. Each preparation was allowed to equilibrate for at least one hour. Isometric tensions were recorded on a physiograph (MK-IV, Narco-Bio system) (Chang et al, 1990).

To avoid the possible influence of the

endothelium, endothelium was removed by gently rubbing the intimal surface with a cotton ball. Successful removal of the endothelium was confirmed later by the inability of acetylcholine (10^{-6}M) to induce relaxation (Furchgott & Zawadzki, 1980)

Preparation of dispersed single smooth muscle cells

Aorta were excised, and the adventitia were removed in HEPES-buffered Tyrode's solution containing (mM) 140 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 6 glucose, and pH was adjusted to 7.4 at 37℃ Endothelium was removed by gently rubbing the intimal surface with a cotton ball. The aorta was cut into small strips (2mm wide and 10mm long), and incubated in HEPES-buffered Tyrode's solution for one hour. Then small strips were incubated in Ca-free HEPES-buffered Tyrode's solution containing 2mg/ml collagenase (Wako), 2mg/ml papain (Sigma), 1mg/ml dithiotreitol (Sigma), and 5mg/ml bovine serum albumin (Sigma) for 40 minutes. After digestion, the single cells were separated by gently pipetting the muscle strips through a wide-pore pasteur pipette. The suspension of single cells was filtered through double layers of nylon mesh (pore size: 0.5mm). The suspension was centrifuged at 1000 rpm for 5 minutes to eliminate the debris from the connective tissue.

Viability of the single cells was assessed by trypan blue exclusion test (Bagby et al, 1971; Johns & Riehl, 1982). The total number of stained and viable single smooth muscle cells was then determined. All experiments were carried out within 4 hours of cell suspension.

Measurement of ^{45}Ca uptake

^{45}Ca uptake was measured in 0.5ml of HEPES-buffered Tyrode's solution. Then 1~2 $\times 10^5$ cells were incubated with ^{45}Ca (4 μCi /ml) at 37℃ for 15 minutes. Incubation was terminated at a selected time by addition of 1 ml ice-cold HEPES solution containing La^{3+} (30 mM) and followed by centrifugation at 1000 rpm for 5 minutes. The supernants was removed and cell pellets washed 2 times with ice-cold HEPES solution containing La^{3+} (30mM). Cell pellets were lysed with 0.5ml of 0.5N NaOH, and radioactivity was determined in a scintillation cocktail (Luma-Gel) with a liquid scintillation counter (Tri-Carb 350C).

^{3}H-nitrendipine binding assay

Single cells were incubated with various concentrations of ^{3}H-nitrendipine in a total volume of 0.5ml of incubation medium at 37 ℃ for 30 minutes. The incubation mixture was then rapidly filtered through a Whatman GF/C glass fiber which was coated with 0.1% bovine serum albumin and the filters were washed 5 times with 3 ml of ice-cold HEPES buffered salt solution (pH 7.4). A Whatman GF/C glass fiber which was coated with 0.1% bovine serum albumin was used to reduce the total and nonspecific binding, without changing the specific binding. After filtration of the incubation mixture, the GF/C filters were dried and radioactivity was determined in a scintillation cocktail (Luma-Gel) with a liquid scintillation counter (Tri-Carb 350C).

Nonspecific binding was determined by the radioactivity bound to the single cells in the presence of 20 μM nitrendipine (Bayer). Specific binding was determined as the difference between total binding and nonspecific binding. The binding to the single cells was analyzed using Scatchard (1949) analysis to determine the affinity and the number of binding sites

The results are expressed as means and S.E. Student's t-tests were used for statistical analysis. P values of less than 0.05 were considered to be statistically significant.

Drugs used were norepinephrine, verapamil, papain, dithiotreitol, bovine serum albumin (Sigma); collagenase (Wako); nitrendipine (Bayer); ^{45}Ca (specific acitivity, 18.7 mCi/mg) and ^{3}H-nitrendipine (specific activity 73 Ci /mmol) were obtained from New England Nuclear.

RESULTS

Effect of Ca concentration on the basal tone

The removal of Ca from the bath solution induced a clear relaxation and the restoration of Ca returned the contraction to the original level in the aortic strips of SHR. On the other hand, the basal tone in aorta of WKY was not affected by alteration of Ca concentration (Fig. 1). The aortic strip from SHR, relaxed in Ca-free Tyrode's solution, contracted in a dose-dependent manner by stepwise addition of Ca from 0 to 10mM (Fig. 2). This Ca-induced contraction in the Ca-free Tyrode's solution in SHR (basal tone in SHR) began at 0.3mM Ca and reached the maximal value at 2mM Ca.

Effects of verapamil on the basal tone

It is well known that 10^{-6}M of verapamil can inhibit only the potential-operated Ca channels in vascular smooth muscles. When verapamil (10^{-6}M) was added on Ca-induced contraction after exposure to Ca-free Tyrode's solution in endothelium-rubbed aortic strips in SHR, this contraction was markedly reduced to 5.4 ± 1.6% (n=6) of that of the control (Fig. 3).

Effects of endothelium on the basal tone

In order to investigate the effect of endothelium on the basal tone in the aorta of SHR, basal tones in the strips with intact endothelium were compared with those in the strips with rubbed endothelium (Fig. 4). As shown in Fig. 4, basal tone was 162±5 mg in aortic strips with rubbed endothelium and 105±9 mg in that with intact endothelium. It was significantly increased by endothelial rubbing ($p < 0.01$).

Basal ^{45}Ca uptake in the aortic single smooth muscle cells

When 1~2 × 10^5 cells/ml were incubated with ^{45}Ca (4 μCi/ml) at 37℃ for 15 minutes, basal ^{45}Ca uptakes in the extracellular solution contaning 1mM Ca in SHR and WKY are 2050.37 ± 149.94, 1419.57 ± 124.38 (fmol/10^6 cells/15minutes), respectively. It was greater in SHR than in WKY ($p < 0.01$). This difference of ^{45}Ca uptake between the two strains was

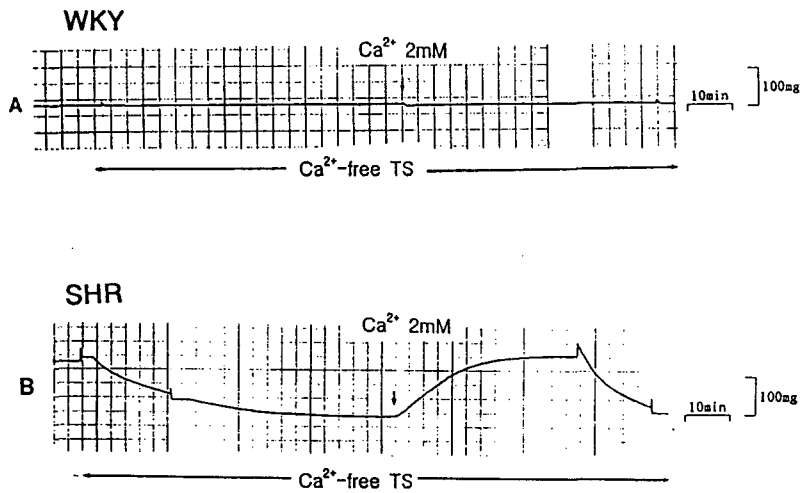

Fig. 1. Effects of the removal and the restoration of extracellular Ca on the basal tone in the aortic strips of Wistar-Kyoto(WKY) rats(A) and spontaneously hypertensive rats (SHR)(B).

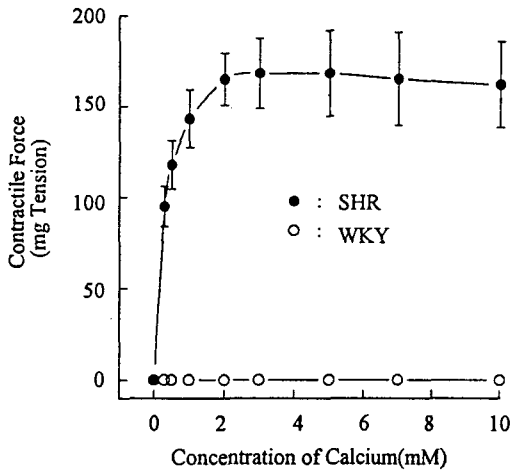

Fig. 2. Dependence of basal tone on the extracellular Ca concentration. The basal tone in the aortic strips of spontaneously hypertensive rats (SHR) depends on extracellular Ca concentration. Ordinate : Contractile force as mg of tension. Each point shows mean (n=5) and each vertical bar is S.E.M..

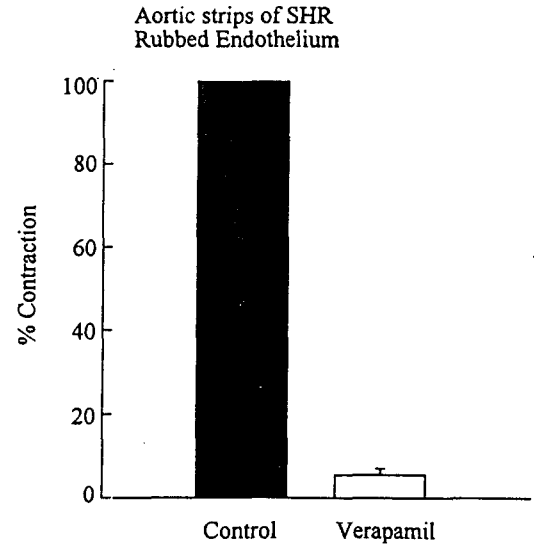

Fig. 3. Effect of verapamil $10^{-6}M$ on the basal tone in the aortic strips (rubbed endothelium) of spontaneously hypertensive rats (SHR). Each bar shows mean (n=5) and each vertical bar is S.E.M..

Control : Ca (2mM)-induced contraction after the exposure to Ca-free Tyrode's solution, Verapamil : Percentage to control of the suppressed Ca-induced contraction by verapamil.

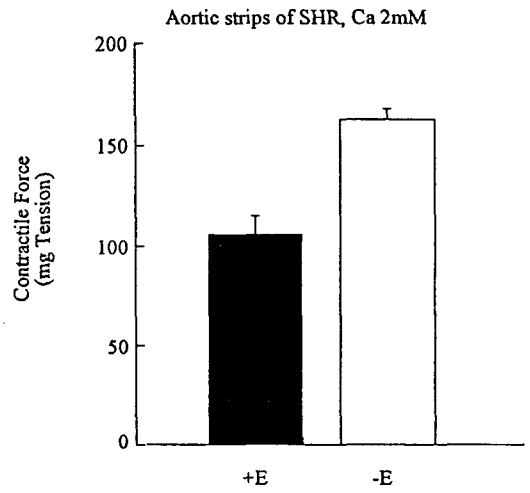

Fig. 4. Effects of endothelium on Ca-induced contraction after the exposure to Ca-free Tyrode's solution in the aortic strips of spontaneously hypertensive rats (SHR). Ordinate : Contractile force as mg of tension. Each bar shows mean (n =5) and each vertical bar is S.E.M.. +E : Intact endothelium, -E: Rubbed endothelium.

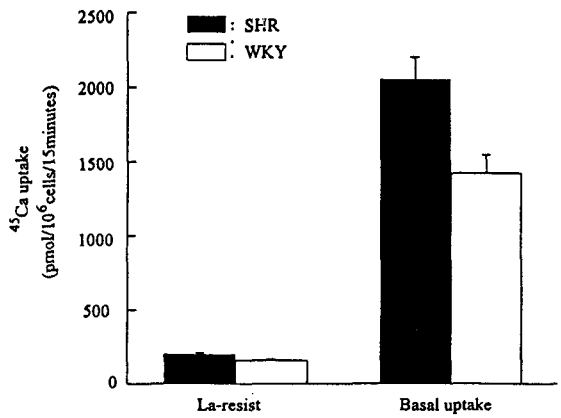

Fig. 5. ^{45}Ca uptake in the aortic single smooth muscle cells of spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rats. ^{45}Ca (4μCi /ml) uptake was measured in the extracellular Ca 1mM-containg Tyrode's solution for 15 minutes. La-resist : ^{45}Ca uptake for 15 minutes in the presence of La^{3+} 5mM. Basal uptake : ^{45}Ca uptake for 15 minutes in the absence of La^{3+} 5mM.

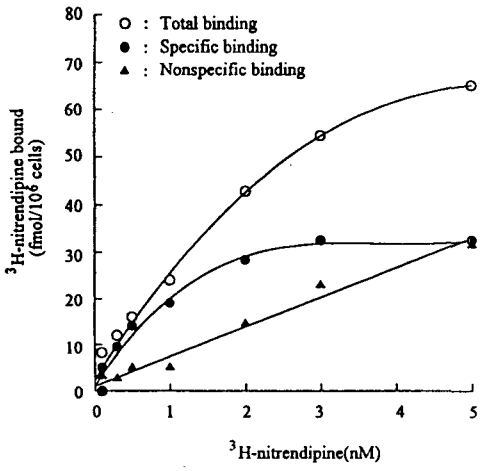

Fig. 6. Equilibrium binding of ^{3}H-nitrendipine to single aortic smooth muscle cells of Wistar-Kyoto (WKY) rats. Total and nonspecific binding was measured in the absence or presence of 2 μM unlabeled nitrendipine. Specific binding was determined by subtracting nonspecific binding from totol binding.

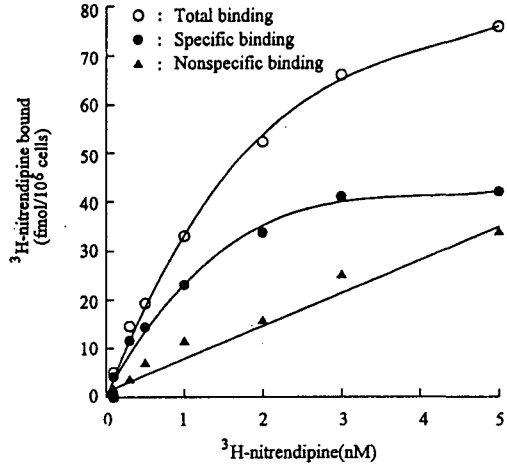

Fig. 7. Equilibrium binding of ^{3}H-nitrendipine to single aortic smooth muscle cells of spontaneously hypertensive rat (SHR). Total and nonspecific binding was measured in the absence or presence of 2 μM unlabeled nitrendipine. Specific binding was determined by subtracting nonspecific binding from totol binding.

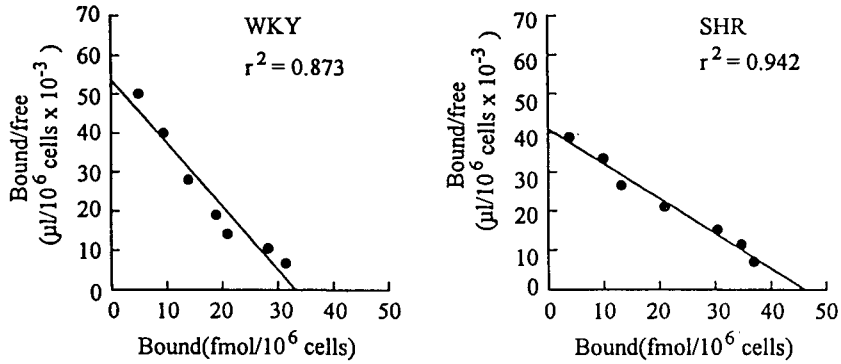

Fig. 8. Scatchard plots of the specific binding of ^{3}H-nitrendipine in single aortic smooth muscle cells of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rat (SHR). Data are from same experiment as in Fig. 6&7.

abolished in the presence of 0.5mM $LaCl_3$ (Fig. 5).

[^{3}H]nitrendipine in the aortic single smooth muscle cells

Specific binding of [^{3}H]nitrendipine in the single cells of aortic smooth muscles of SHR and WKY was saturable (Fig. 6, 7). Scatchard analysis of specific binding of ^{3}H-nitrendipine in the single cells of aortic smooth muscles of WKY (A) and SHR (B) represented in a linear plot (Fig. 8). The dissociation constants

Table 1. Maximum binding (B_{max}), dissociation constant (K_d) and correlation coefficient (r^2) obtained from specific binding of ^{3}H-nitrendipine to single cells from aortic smooth muscle of WKY and SHR

Strains	N	B_{max}(fmol/10^6 cells)	K_d (nM)	Correlation coefficient (r^2)
WKY	5	34.6±4.3	0.71±0.15	0.87±0.50
SHR	5	47.4±3.2*	1.18±0.08*	0.94±0.02

n represents the number of experiments. Each value is presented as a mean ± S.E.M. of experiments. *: significant difference from corresponding values by Student's t-test ($P<0.05$)

(K_d) in WKY and SHR were 0.71±0.15 and 1.18±0.08 nM respectively. The maximal binding capacities (B_{max}) in WKY and SHR were 34.6±3.2, 47.4±4.3 fmol/10^6 cells respectively. K_d was less in WKY than in SHR and the difference between the two strains was significant ($p<0.03$). Maximum binding capacity was larger in SHR than that in WKY, and the difference in maximum binding capacity between two strains was significant ($p<0.05$).

DISCUSSION

The vascular smooth muscle of normotensive rats does not exhibit any spontaneously active tone. On the other hand, the vascular smooth muscle of SHR exhibits high basal tone (Noon et al, 1979; Lindner & Heinle, 1987; Sada et al, 1989).

In the present study, the basal tone in SHR aorta was suppressed by the removal of extracellular Ca and was recovered by the addition of Ca. The basal tone in SHR was concentration-dependent to extracellular Ca and reached a maximal value at 2mM Ca. This contraction was suppressed by verapamil10^{-6}M, which was known to block only potential-operated Ca channels. This finding suggests that high basal tone in the aortic strips of SHR be mainly due to the Ca influx via potential-operated Ca channels. Recently, Ohya et al (1993) suggested that the increased activity of voltage-dependent L-type Ca channels of resistance arteries of SHR may be related to the development of hypertension.

Basal tone in the aortic strips of SHR was increased by the removal of endothelium (Fig. 4.). This finding is thought to be due to spontaneous release of endothelium-derived relaxing factor (EDRF). However, it is uncertain that EDRF inactivates potential-operated Ca channels in the aortic strips of SHR.

In the present study, we used single cells of vascular smooth muscle for measuring the basal ^{45}Ca uptake. Basal ^{45}Ca uptake of single vascular smooth muscle cells of SHR in the extracellular solution containing 1mM Ca was greater than that of WKY. This difference of ^{45}Ca uptake between WKY and SHR was abolished in the presence of 0.5mM $LaCl_3$ (Fig. 5). These indicate that the observed difference in basal ^{45}Ca uptake between SHR and WKY probably reflects an increased basal rate of ^{45}Ca influx in the SHR. An increased basal rate of Ca influx in SHR may contribute to the increase of intracellular Ca level in the vascular smooth muscle cells and then the higher basal tone of aorta in SHR. Intracellular Ca concentration in vascular smooth muscle cells from SHR and WKY rats has been studied using Ca indicators. Most studies showed that the cells from adult SHR after the development of hypertension had higher intracellular Ca concentration than those from adult WKY rats (Sugiyama et al, 1986; Bukoski, 1990; Sada et al, 1990; Storm et al, 1992)

Reuter (1983) mentioned that Ca channels, unlike Na channels, are not functional in isolated membrane vesicles, and binding

studies should preferably be done in intact cells. We investigated the binding of ^{3}H-nitrendipine in the dispersed intact smooth muscle cells. Specific bindings of ^{3}H-nitrendipine in the single cells of aortic smooth muscles of SHR and WKY were saturable. The dissociation constant (K_d) of ^{3}H-nitrendipine in WKY was smaller than that of SHR but the maximum binding capacity of ^{3}H-nitrendipine in SHR was greater than that of WKY. This finding suggested that the number of dihydropyridine-sensitive Ca channels in the single smooth muslce cells of SHR was more than that of WKY, while the affinity of ^{3}H-nitrendipine for dihydropyridine Ca channels in SHR was lower than that in WKY. Although the increased binding site of Ca channels observed in the present study could not be directly linked to the basal tone of SHR, increased number of Ca channels could be contributed to the high basal tone of SHR.

Ikeda et al (1990) and Galletti et al (1991) reported that there is no difference in K_d value between the two strains (SHR and WKY) in the binding studies of dihydropyridine derivatives. They used the membrane preparation from the aorta and tail arteries of SHR and WKY rats instead of single smooth muscle cells for binding studies. The discrepancy between our results and those in the binding studies may be due to the different methods, i.e., membrane preparation versus single smooth muscle cells.

From the above results, it is suggested that increased basal tone in aortic strips of SHR may be due to the increase of Ca influx via potential-operated Ca channels and the increase in the number of dihydropyridine-sensitive Ca channels.

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