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Potent vasoconstrictor actions of cyclopiazonic acid and thapsigargin on femoral arteries from spontaneously hypertensive rats

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1 The Ca^{2+} buffering function of sarcoplasmic reticulum (SR) in the resting state of arteries from spontaneously hypertensive rats (SHR) was examined. Differences in the effects of cyclopiazonic acid (CPA) and thapsigargin, agents which inhibit the Ca^{2+}-ATPase of SR, on tension and cellular Ca^{2+} level were assessed in endothelium-denuded strips of femoral arteries from 13-week-old SHR and normotensive Wistar-Kyoto rats (WKY).

2 In resting strips preloaded with fura-PE3, the addition of CPA (10 μM) or thapsigargin (100 nM) caused an elevation of cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and a contraction. These responses were significantly greater in SHR than in WKY.

3 The addition of verapamil (3 μM) to the resting strips caused a decrease in resting $[Ca^{2+}]_i$, which was significantly greater in SHR than in WKY. In SHR, but not in WKY, this decrease was accompanied by a relaxation from the resting tone, suggesting the maintenance of myogenic tone in the SHR artery.

4 Verapamil (3 μM) abolished differences between SHR and WKY. The effects of verapamil were much greater on the contraction than on the $[Ca^{2+}]_i$.

5 The resting Ca^{2+} influx in arteries measured after a 5 min incubation of the artery with ^{45}Ca was not increased by CPA or thapsigargin in either SHR or WKY. The net Ca^{2+} entry measured after a 30 min incubation of the artery with ^{45}Ca was decreased by CPA or thapsigargin in both SHR and WKY. The resting Ca^{2+} influx was significantly higher in SHR than in WKY, and was decreased by nifedipine (100 nM) in the SHR artery, but was unchanged in the WKY artery.

6 The resting ^{45}Ca efflux from the artery was increased during the addition of CPA (10 μM). This increase was less in SHR than in WKY. The resting ^{45}Ca efflux was the same in SHR and WKY.

7 These results suggest that (1) the Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels (VDCCs) was increased in the resting state of the SHR femoral artery, (2) the greater part of the increased Ca^{2+} influx was buffered by Ca^{2+} uptake into the SR and some Ca^{2+} reached the myofilaments resulting in the maintenance of the myogenic tone, and (3) therefore the functional elimination of SR by CPA or thapsigargin caused a large elevation of $[Ca^{2+}]_i$ and a potent contraction in this artery. During this process, the contraction was mainly due to the basal Ca^{2+} influx via L-type VDCCs. The present study also showed the existence of a relatively large compartment of $[Ca^{2+}]_i$ which does not contribute to the contraction during the addition of CPA or thapsigargin.

Keywords: Spontaneously hypertensive rats (SHR); sarcoplasmic reticulum; cyclopiazonic acid; thapsigargin; cytosolic Ca^{2+} level; Ca^{2+} influx; voltage-dependent Ca^{2+} channels

Introduction

Spontaneously hypertensive rats (SHR) have long been used as an experimental model for human essential hypertension. It has been proposed that the primary defect in essential hypertension occurs in the Ca^{2+} regulatory system in arterial smooth muscle (Bohr & Webb, 1988). Even in the resting state of arteries from SHR, an increased Ca^{2+} influx (Bhalla *et al*., 1978; van Breemen *et al*., 1986; Asano *et al*., 1993a) and a high cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) (Spieker *et al*., 1988; Jelicks & Gupta, 1990; Sada *et al*., 1990) when compared with normotensive Wistar-Kyoto rats (WKY) have been found. The high resting $[Ca^{2+}]_i$ in SHR arteries has been shown to maintain a spontaneous active tone and activate Ca^{2+}-activated K^+ channels (Asano *et al*., 1986; 1993a, c; 1995). Thus, activation of the K^+ channels is likely to be acting as a negative feedback mechanism to regulate the level of resting tone in SHR arteries.

It is also likely that the high resting $[Ca^{2+}]_i$ could be extruded by Na^+-Ca^{2+} exchange and Ca^{2+} pumping across the plasmalemma, and buffered by Ca^{2+} uptake into the sarcoplasmic reticulum (SR). The Ca^{2+} extrusion across the plasmalemma has been repeatedly shown to be decreased in arterial smooth muscle from SHR (for review, see Kwan, 1985; Bohr & Webb, 1988). The Ca^{2+} buffering function of SR measured by using subcellular fractions has also been shown to be decreased in arterial smooth muscle from SHR (Shibata *et al*., 1975; Moore *et al*., 1975; Webb & Bhalla, 1976; Kwan, 1985). Therefore, it is likely that these decreased functions contribute to the high resting $[Ca^{2+}]_i$ in SHR arteries. However, Levitsky *et al*. (1993) have recently demonstrated the increased Ca^{2+} buffering function of SR in aortic smooth muscle from SHR. They have further shown that the increased SR function results from quantitative modulation of expression of the SR Ca^{2+}-ATPase gene without alteration of pre-mRNA splicing. Thus, in studies investigating the Ca^{2+} buffering function of SR in SHR arteries conflicting results have been obtained.

Recent studies have demonstrated that SHR arteries exhibit a large contraction during the inhibition of Ca^{2+}-ATPase of SR by cyclopiazonic acid (CPA) (Low *et al*., 1993) or thapsi-

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gargin (Kanagy *et al*., 1994). Although the precise mechanism responsible for these contractions was not evaluated in these studies, these observations suggest that the Ca^{2+} buffering function of SR is increased in the resting state of SHR arteries. The present study was designed to clarify whether the SR of SHR arteries can buffer the high resting $[Ca^{2+}]_i$. For this purpose, we assessed the possible mechanism of the CPA and thapsigargin actions by measuring the mechanical activity, $[Ca^{2+}]_i$ (by using a fluorescent Ca^{2+} indicator fura-PE3), ^{45}Ca influx and ^{45}Ca efflux in the resting state of the SHR femoral artery, and these data were compared with the data from the WKY femoral artery. The prediction is that if the SR of the SHR artery is an effective barrier to the high resting $[Ca^{2+}]_i$, the functional elimination of SR by CPA or thapsigargin will cause a large elevation of $[Ca^{2+}]_i$ which results in a smooth muscle contraction. Here, we show that CPA or thapsigargin causes a larger elevation of $[Ca^{2+}]_i$ and a larger contraction in the SHR artery than in the WKY artery by compromising the Ca^{2+} buffering function in the face of increased resting Ca^{2+} influx.

Methods

Male SHR and WKY were inbred in our laboratory. They were studied at 13 weeks of age. The systolic blood pressure at this age, measured by a tail-cuff plethysmography (KN-210, Natsume Seisakusho, Tokyo, Japan), was significantly higher in SHR (198 ± 1.5 mmHg, $n = 92$) than in WKY (138 ± 1.0 mmHg, $n = 89$). The body weight was significantly lower in SHR (256 ± 3 g, $n = 92$) than in WKY (272 ± 3 g, $n = 89$).

Preparation of arterial smooth muscle strips

Rats were stunned by a blow to the head and then exsanguinated. Femoral arteries (0.6–0.8 mm outside diameter) were excised and placed in a Krebs solution of the following composition (in mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and dextrose 10.0. Arteries were cut into helical strips (0.8 mm in width) as described previously (Asano *et al*., 1988). To avoid the possible influences of the endothelium-derived factors (e.g. relaxing, hyperpolarizing and contracting factors), the endothelium of the strip was removed by gently rubbing the endothelial surface with a cotton swab. Successful removal of the endothelium was confirmed later by the inability of acetylcholine (1 μM) to induce relaxation in prostaglandin $F_{2\alpha}$-contracted strips.

Measurement of $[Ca^{2+}]_i$ and isometric tension

$[Ca^{2+}]_i$ and isometric tension were measured simultaneously as described previously (Uyama *et al*., 1993; Asano *et al*., 1995; 1996). Briefly, arterial smooth muscle strips (0.8×8 mm) were loaded with 10 μM acetoxymethyl ester of fura-PE3 (fura-PE3/AM) in the presence of 0.03% cremophor EL, a non-cytotoxic detergent, under protection from light at 37°C. After loading for 2.5–3 h, each strip was mounted horizontally in a temperature-controlled perfusion chamber (approximately 1.2 ml volume) attached to a fluorimeter (CAF-100, Japan Spectroscopic, Tokyo, Japan). One end of the strip was connected to the force-displacement transducer for isometric tension recordings with a resting tension of 0.6 g. The optimal resting tension was determined by a length-passive tension study (Asano *et al*., 1988). The strips were perfused at a rate of 2.5 ml min^{-1} with the Krebs solution. Krebs solutions were maintained at 37°C and aerated with 95% O_2 and 5% CO_2. Strips were perfused for the next 50 min before addition of the test drugs. A part of the strip was excited by light obtained from a xenon high-pressure lamp (75 w) equipped with a rotating filter wheel (48 Hz) that contained 340 and 380 nm interference filters. The amounts of fluorescence measured at 500 nm induced by excitation at 340 nm (F340) and at 380 nm (F380) were determined. The time constant of the optimal channels was set to 1 s. The ratio of F340 to F380 (F340/F380) was automatically recorded and used as an indicator of $[Ca^{2+}]_i$ (Ozaki *et al*., 1987; Sato *et al*., 1988). After determination of the effects of the Krebs solution containing 65.9 mM KCl (K^+) (equimolar substitution of Na^+ with K^+) on $[Ca^{2+}]_i$ and isometric tension, the effects of CPA, thapsigargin and caffeine were determined. Since higher concentrations of caffeine (>20 mM) succeeded in depleting the SR Ca^{2+}, a transient contraction induced by caffeine can be taken as a semi-quantitative index of the amount of Ca^{2+} in the SR, as shown in other studies (Leijten & van Breemen, 1986; Naganobu *et al*., 1994). To characterize the action of CPA or thapsigargin, effects of verapamil (a blocker of L-type voltage-dependent Ca^{2+} channels, VDCCs) or Ca^{2+}-free solution were determined. The Ca^{2+}-free solution was prepared by omission of Ca^{2+} from the Krebs solution and by addition of 1 mM EGTA. Since fura-PE3 was designed to give fura-2 the capacity to resist leakage and compartmentation, $[Ca^{2+}]_i$ can be measured for at least 3 h. For a quantitative comparison of the $[Ca^{2+}]_i$, the resting and 65.9 mM K^+-induced F340/F380 were taken as 0 and 100%, respectively. Changes in isometric tension were expressed as % of the contraction induced by 65.9 mM K^+.

Measurement of resting Ca^{2+} influx and net Ca^{2+} entry

Resting Ca^{2+} influx was measured by using a cold La^{3+} wash procedure as described previously (Asano *et al*., 1993a). Briefly, isolated arteries were opened longitudinally and the endothelium was removed. They were equilibrated in Tris-buffered solution of the following composition (in mM): NaCl 154.0, KCl 5.4, $CaCl_2$ 2.5, dextrose 11.0 and Tris 6.0 (pH 7.4). Tris-buffered solutions were maintained at 37°C and aerated with 100% O_2. Arteries were then transferred to the Tris-buffered solution to which 1 μCi ml^{-1} ^{45}Ca had been added. After a 5 min incubation, the arteries were transferred to test tubes containing 80.8 mM La^{3+}-substituted solution (0.5°C) and washed for 45 min to remove extracellular ^{45}Ca. The amount of ^{45}Ca taken up by the tissue during the 5 min incubation can be assumed to be primarily due to Ca^{2+} influx with some efflux components (Asano *et al*., 1993a; 1995). Arteries were then transferred to a glass scintillation vial containing 0.1 ml NCS tissue solubilizer (Amersham International, Buckinghamshire). Solubilized tissues were mixed with 5 ml Amersham ACS II scintillant and counted for radioactivity in an Aloka liquid scintillation counter. Net Ca^{2+} entry was measured for incubating the arteries in the same ^{45}Ca solution for 30 min before the La^{3+} wash (Asano *et al*., 1993b; 1995). The amount of ^{45}Ca taken up by the tissue during the 30 min incubation time can be assumed to be determined by a net balance of Ca^{2+} influx and efflux components. Other experimental conditions were the same as in the resting Ca^{2+} influx measurements. Changes in the resting Ca^{2+} influx and the net Ca^{2+} entry during the addition of CPA, thapsigargin, nifedipine or 160 mM K^+ solution were determined. Values for resting Ca^{2+} influx and net Ca^{2+} entry were then calculated and expressed as μmol kg^{-1} tissue wet weight, as described previously (Asano *et al*., 1993a, b; 1995).

Measurement of resting ^{45}Ca efflux

Resting ^{45}Ca efflux was measured according to the method of Hwang & van Breemen (1987) with a modification that was used in our ^{86}Rb efflux studies (Masuzawa *et al*., 1990). Arterial strips were mounted vertically on stainless-steel rods and allowed to equilibrate in the Tris-buffered solution. Tris-buffered solutions were maintained at 37°C and aerated with 100% O_2. Larger strips were used for efflux studies. For loading with ^{45}Ca, the strips were incubated for 2 h in the Tris-buffered solution to which 2 μCi ml^{-1} ^{45}Ca had been added. Each strip was then dipped three times (a total of 15 s) into nonradioactive Tris-buffered solution to remove excess radio-

activity, and then mounted in a thermostatic superfusion chamber. A resting tension of 2.1 g was applied, and the strips were superfused at a rate of 1 ml min^{-1} with the Tris-buffered solution. The superfusates were collected at 2 min intervals. At the end of the efflux sequence, the strips were dissolved in 0.1 ml NCS tissue solubilizer followed by an adjustment of the sample volume with the Tris-buffered solution to 2.0 ml. The radioactivity in the collected superfusates and the solubilized tissue were counted in an Aloka liquid scintillation counter. All efflux data are expressed as fractional loss as a function of time (^{45}Ca fractional loss min^{-1}, Hwang & van Breemen, 1987).

Statistical analysis

The results are expressed as means ± s.e.mean (n = number of preparations). Student's t test for unpaired data or variance analysis was used to determine the significance of differences between means, and a P value of <0.05 was taken as significant.

Drugs and isotope

The drugs used were CPA (Sigma Chemical Co., St. Louis, U.S.A.), thapsigargin (Sigma), caffeine (Wako Pure Chemical Industries, Osaka, Japan), verapamil hydrochloride (Eisai Co. Ltd., Tokyo, Japan), nifedipine (Bayer Yakuhin Ltd., Osaka, Japan), ethylene glycol bis (β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA, Sigma), acetylcholine chloride (Sigma), prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co. Ltd., Osaka, Japan), fura-PE3/AM (Texas Fluorescence Lab. Inc., Austin, U.S.A.) and cremophor EL (Nacalai Tesque Inc., Kyoto, Japan). $^{45}CaCl_2$ (specific activity initially 14.6–28.5 mCi mg^{-1}) was obtained from Amersham International (Buckinghamshire, U.K.).

CPA (3 mM), thapsigargin (100 μM) and fura-PE3/AM (0.5 mM) were dissolved in 100% dimethyl sulphoxide. Nifedipine (1 mM) was dissolved in 50% ethanol, with further dilution in distilled water before use. Caffeine was dissolved in the Krebs (or Tris-buffered) solution. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations.

Results

CPA-induced elevation of $[Ca^{2+}]_i$ and contractions

After determination of the elevation of $[Ca^{2+}]_i$ and contraction induced by 65.9 mM K^+, the addition of 10 μM CPA to the strips caused an elevation of $[Ca^{2+}]_i$ that was accompanied by a contraction in both WKY and SHR (Figure 1). The peak of the elevation of $[Ca^{2+}]_i$ induced by CPA was significantly greater in SHR than in WKY (Figure 1, Table 1A). The CPA-induced contraction was also significantly greater in SHR than in WKY (Figure 1, Table 1A). As shown in Figure 1, these responses decreased gradually with time. At 30 min after the addition of CPA, the elevation of $[Ca^{2+}]_i$ and contraction induced by CPA were still significantly greater in SHR than in WKY (Figure 1, Table 1A). Since an elevation of $[Ca^{2+}]_i$ induced by a high concentration of caffeine is a semi-quantitative index of the amount of Ca^{2+} in the SR, the effects of caffeine were then determined in the presence of CPA (Figure 1). In the presence of CPA, caffeine (40 mM) caused a small transient increase in $[Ca^{2+}]_i$ and tension followed by a decrease in $[Ca^{2+}]_i$ and tension in WKY (Figure 1). The initial peak responses to caffeine (24.9 ± 5.4% and 7.5 ± 2.0% of the response to 65.9 mM K^+ for $[Ca^{2+}]_i$ and tension, respectively, $n = 7$) in the presence of CPA were significantly smaller than the control responses to caffeine which are shown in Table 1A. On the other hand, in SHR, the addition of caffeine (40 mM) caused a decrease in $[Ca^{2+}]_i$ and tension (Figure 1).

Concentration-dependent effects of CPA on $[Ca^{2+}]_i$ and tension in strips from WKY and SHR are shown in Figure 2.

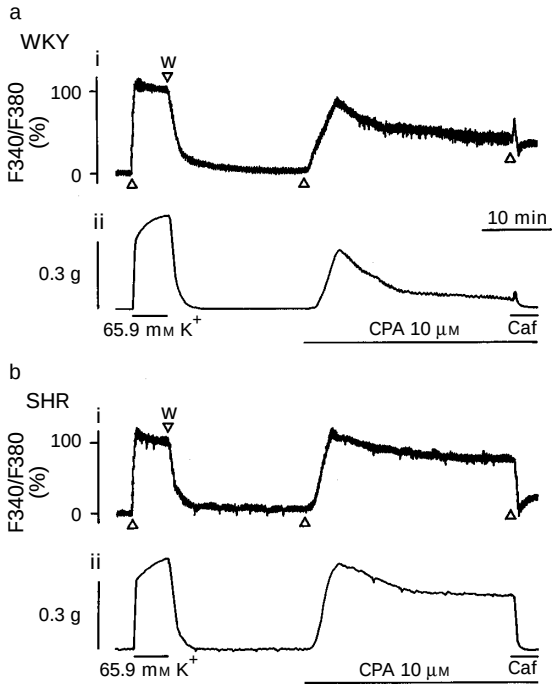

Figure 1 Typical recordings of the effects of cyclopiazonic acid (CPA) and caffeine (Caf) on (i) $[Ca^{2+}]_i$ (indicated by F340/F380) and (ii) tension in strips of femoral arteries from 13-week-old (a) WKY and (b) SHR. After being loaded with fura-PE3, the strips were exposed to 65.9 mM K^+ for 5 min to determine the maximum elevation of $[Ca^{2+}]_i$ and contraction. Following washout with a Krebs solution, 10 μM CPA was added for 30 min and then 40 mM Caf was added for 4 min, as indicated. Triangles in each upper panel (F340/F380) denote the addition of each agent and the washout (w) with Krebs solution.

At all the concentrations of CPA used (0.1–10 μM), the elevation of $[Ca^{2+}]_i$ was significantly greater in SHR than in WKY (Figure 2). The CPA-induced contraction was also significantly greater in SHR than in WKY (Figure 2). In both WKY and SHR, the elevation of $[Ca^{2+}]_i$ always exceeded the contraction. At lower concentrations of CPA, there was an elevation of $[Ca^{2+}]_i$ that was not accompanied by a contraction (Figure 2).

Thapsigargin-induced elevation of $[Ca^{2+}]_i$ and contractions

Effects of thapsigargin on $[Ca^{2+}]_i$ and tension are shown in Figure 3 and Table 1A. Since micromolar concentrations of thapsigargin have been shown to inhibit the function of L-type voltage-dependent Ca^{2+} channels (VDCCs) (Rossier *et al.*, 1993; Nelson *et al.*, 1994; Buryi *et al.*, 1995), we employed 100 nM thapsigargin, a concentration which abolished the function of SR without inhibiting the activity of L-type VDCCs. The addition of 100 nM thapsigargin to the strips caused a slow elevation of $[Ca^{2+}]_i$ that was accompanied by a slow contraction in both WKY and SHR (Figure 3). The elevation of $[Ca^{2+}]_i$ induced by thapsigargin was significantly greater in SHR than in WKY (Figure 3, Table 1A). The thapsigargin-induced contraction was also significantly greater in SHR than in WKY (Figure 3, Table 1A). When caffeine (40 mM) was added in the presence of thapsigargin, caffeine caused a decrease in $[Ca^{2+}]_i$ and tension in both WKY and SHR (Figure 3). Similar to the effect of CPA, the responses to thapsigargin also decreased gradually with time (data not shown).

To determine whether CPA and thapsigargin caused a contraction by the functional elimination of SR, the contractile effects of thapsigargin (100 nM) in the presence of a maximally-effective concentration of CPA (10 μM) were determined in

Table 1 (A) The elevation of $[Ca^{2+}]_i$ and the contraction induced by cyclopiazonic acid (CPA), thapsigargin and caffeine and (B) the effects of verapamil on these responses in strips of femoral arteries from 13-week-old WKY and SHR

Condition		Parameter	WKY (% of 65.9 mM K^+)	SHR (% of 65.9 mM K^+)
A CPA 10 μM[a]	Peak	F340/F380	81.7±7.7 (7)	102.3±4.0* (5)
		Tension	62.3±8.4 (7)	90.7±4.6* (5)
	30 min	F340/F380	42.5±3.4 (7)	63.5±4.3* (5)
		Tension	10.7±2.9 (7)	59.5±7.4* (5)
TG 100 nM[b]	Peak	F340/F380	53.9±7.7 (6)	89.4±7.8* (5)
		Tension	19.7±7.4 (6)	68.9±6.9* (5)
Caf 40 mM	Peak	F340/F380	85.0±5.4 (9)	88.2±8.9 (6)
		Tension	30.0±3.4 (9)	30.3±1.6 (6)
B Verap + CPA[c]	Peak	F340/F380	54.2±6.7# (7)	51.1±4.2# (7)
		Tension	11.2±3.0# (7)	10.1±2.3# (7)
	30 min	F340/F380	32.0±6.7 (7)	34.9±3.1# (7)
		Tension	1.4±1.1# (7)	2.4±0.9# (7)
Verap + TG[d]	Peak	F340/F380	35.8±2.1# (5)	32.9±5.1# (4)
		Tension	2.3±0.2# (5)	2.0±0.5# (4)
Verap + Caf	Peak	F340/F380	80.3±6.1 (3)	83.3±7.7 (3)
		Tension	29.6±3.1 (3)	31.3±1.0 (3)

[a,b,c,d]Results from experiments shown in Figures 1, 3 ,4 and 5, respectively. Cyclopiazonic acid (CPA) 10 μM, thapsigargin (TG) 100 nM, caffeine (Caf) 40 mM and verapamil (Verap) 3 μM were used. Data are % of the response to 65.9 mM K^+ and are expressed as means±s.e.mean; numbers in parentheses indicate the number of preparations used. *Significantly different from WKY ($P<0.05$). #Significantly different from the respective control value shown in (A) ($P<0.05$).

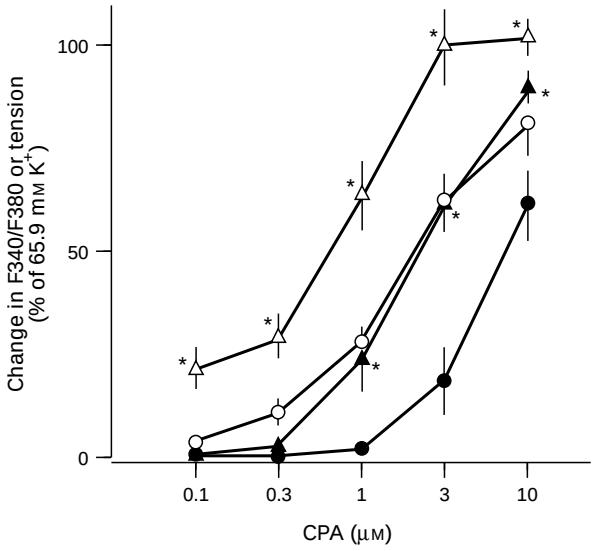

Figure 2 Concentration-dependent effects of cyclopiazonic acid (CPA) on $[Ca^{2+}]_i$ (indicated by F340/F380; ○, △) and tension (●, ▲) in strips of femoral arteries from 13-week-old WKY (○, ●) and SHR (△, ▲). After being loaded with fura-PE3, the strips were exposed to 65.9 mM K^+ as in Figure 1. Following washout with a Krebs solution, CPA was added in a cumulative fashion. Peak values of the change in $[Ca^{2+}]_i$ and tension induced by each concentration of CPA are expressed as % of the maximum response to 65.9 mM K^+. Data points are means of 4 preparations, and s.e.mean are shown by vertical lines. *Significantly different from WKY ($P<0.05$).

strips from WKY and SHR. Under these conditions, thapsigargin at 100 nM failed to cause an additional contraction in both WKY ($n=4$) and SHR ($n=4$).

Effects of verapamil and Ca^{2+}-free solution on the CPA and thapsigargin action

To characterize the CPA- and thapsigargin-induced elevation of $[Ca^{2+}]_i$ and contraction, effects of verapamil on these responses were determined (Figures 4 and 5, Table 1B). As shown in Figure 4, the addition of 3 μM verapamil to the strips decreased the resting $[Ca^{2+}]_i$ in both WKY and SHR. This decrease was significantly greater in SHR (40.5±4.8% of the 65.9 mM K^+-induced elevation of $[Ca^{2+}]_i$, $n=24$) than in WKY (11.9±1.5%, $n=24$). In SHR, but not in WKY, this decrease was accompanied by a relaxation from the resting tone.

Verapamil inhibited the elevation of $[Ca^{2+}]_i$ and contraction induced by 10 μM CPA in both WKY and SHR (Figure 4, Table 1B). This inhibitory effect was greater on the contraction (82.0±5.0% for WKY and 88.9±2.5% for SHR) than on the $[Ca^{2+}]_i$ (33.7±8.2% for WKY and 50.0±4.2% for SHR), suggesting that the CPA-induced contraction was mainly due to Ca^{2+} influx via L-type VDCCs in both WKY and SHR (Table 1B). As shown in Table 1B, the elevation of $[Ca^{2+}]_i$ or the contraction induced by 10 μM CPA in the presence of verapamil was not significantly different between WKY and SHR, suggesting that the verapamil-sensitive component of the CPA-induced elevation of $[Ca^{2+}]_i$ and contraction is larger in SHR than in WKY.

Effects of verapamil on the thapsigargin-induced elevation of $[Ca^{2+}]_i$ and contraction were then determined (Figure 5, Table 1B). Verapamil also inhibited both the elevation of $[Ca^{2+}]_i$ and contraction induced by 100 nM thapsigargin in both WKY and SHR. This inhibitory effect was also greater on the contraction than on the $[Ca^{2+}]_i$ in both WKY and SHR (Figure 5, Table 1B). As shown in Table 1B, the elevation of $[Ca^{2+}]_i$ or the contraction induced by 100 nM thapsigargin in the presence of verapamil was not significantly different between WKY and SHR, suggesting that the verapamil-sensitive component in the thapsigargin-induced elevation of $[Ca^{2+}]_i$ and contraction is larger in SHR than in WKY. The caffeine-induced elevation of $[Ca^{2+}]_i$ and contraction were not affected by verapamil (Table 1A,B).

Effects of Ca^{2+}-free solution on the CPA-induced elevation of $[Ca^{2+}]_i$ and contraction were then determined in both WKY and SHR. When the Krebs solution was replaced with a Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ decreased gradually, as already shown elsewhere (Asano *et al.*, 1993c). When 10 μM CPA was added after the 2 min exposure to Ca^{2+}-free solution, CPA failed to cause a contraction but caused an elevation of $[Ca^{2+}]_i$ in both WKY and SHR. The elevation of $[Ca^{2+}]_i$ induced by 10 μM CPA in the Ca^{2+}-free solution was not significantly different between WKY (27.4±3.6% of the 65.9 mM K^+-induced elevation of $[Ca^{2+}]_i$, $n=4$) and SHR (22.1±4.9%, $n=3$), suggesting that this $[Ca^{2+}]_i$ did not contribute to the CPA-induced contraction in either WKY or SHR.

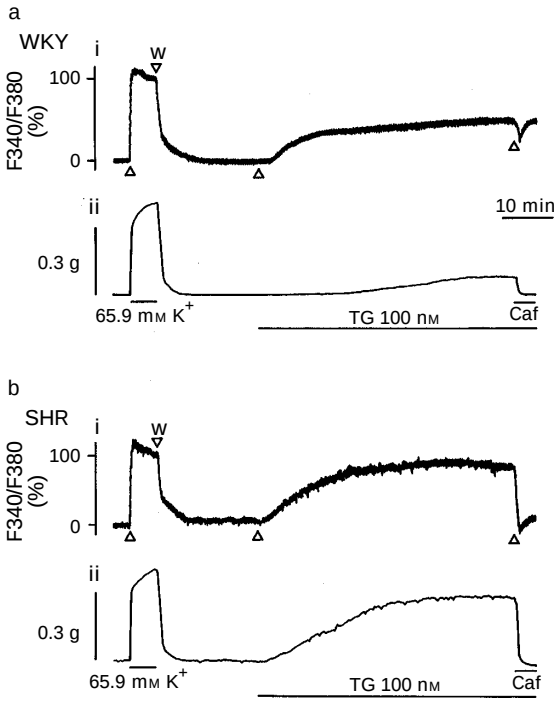

Figure 3 Typical recordings of the effects of thapsigargin (TG) and caffeine (Caf) on (i) $[Ca^{2+}]_i$ (indicated by F340/F380) and (ii) tension in strips of femoral arteries from 13-week-old (a) WKY and (b) SHR. After being loaded with fura-PE3, the strips were exposed to 65.9 mM K^+ as in Figure 1. Following washout with a Krebs solution, 100 nM TG was added for 50 min and then 40 mM Caf was added for 4 min, as indicated. Triangles in each upper panel (F340/F380) denote the addition of each agent and the washout (w) with Krebs solution.

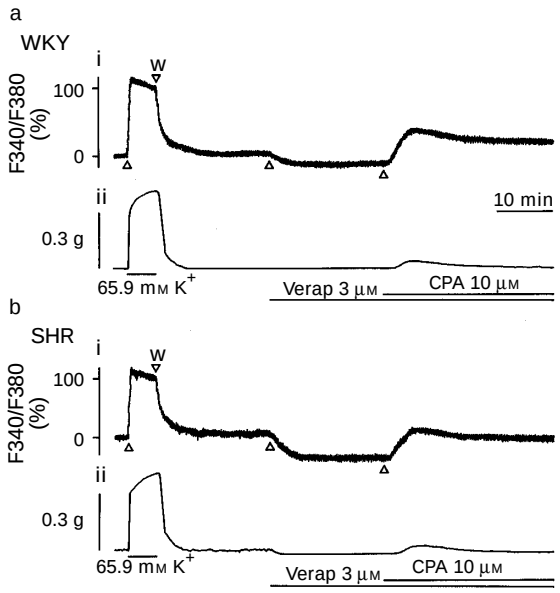

Figure 4 Typical recordings of the effects of verapamil (Verap) on cyclopiazonic acid (CPA)-induced changes in (i) $[Ca^{2+}]_i$ (indicated by F340/F380) and (ii) tension in strips of femoral arteries from 13-week-old (a) WKY and (b) SHR. After being loaded with fura-PE3, the strips were exposed to 65.9 mM K^+ as in Figure 1. Following washout with a Krebs solution, 3 μM verapamil was added for 20 min and then 10 μM CPA was added for 30 min, as indicated. Triangles in each upper panel (F340/F380) denote the addition of each agent and the washout (w) with Krebs solution.

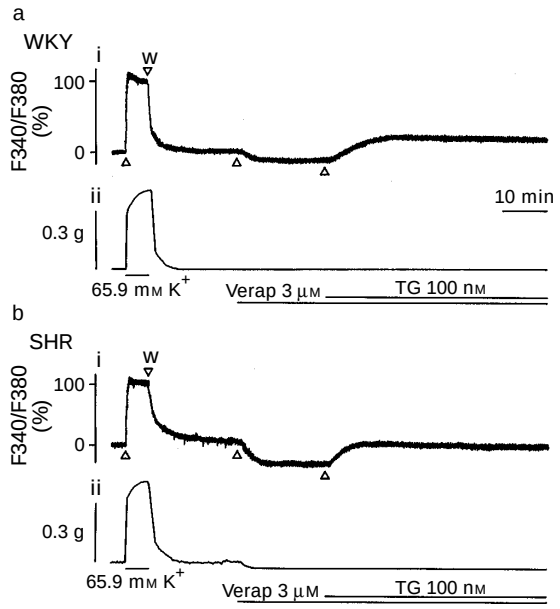

Figure 5 Typical recordings of the effects of verapamil (Verap) on thapsigargin (TG)-induced changes in (i) $[Ca^{2+}]_i$ (indicated by F340/F380) and (ii) tension in strips of femoral arteries from 13-week-old (a) WKY and (b) SHR. After being loaded with fura-PE3, the strips were exposed to 65.9 mM K^+ as in Figure 1. Following washout with a Krebs solution, 3 μM verapamil was added for 20 min and then 100 nM TG was added for 50 min, as indicated. Triangles in each upper panel (F340/F380) denote the addition of each agent and the washout (w) with Krebs solution.

Effects of CPA and thapsigargin on resting Ca^{2+} influx and net Ca^{2+} entry

Because the elevation of $[Ca^{2+}]_i$ and contraction induced by CPA or thapsigargin was inhibited by verapamil in both WKY and SHR, it is likely that the Ca^{2+} influx via L-type VDCCs is involved in these effects of CPA and thapsigargin. Therefore, the effects of CPA and thapsigargin on resting Ca^{2+} influx and net Ca^{2+} entry were examined, and data are shown in Table 2. The elevation of $[Ca^{2+}]_i$ induced by CPA reached a peak level at approximately 5 min and was sustained for 30 min, and the elevation of $[Ca^{2+}]_i$ induced by thapsigargin reached a plateau at approximately 30 min; these periods are in good agreement with the ^{45}Ca incubation periods of resting Ca^{2+} influx and net Ca^{2+} entry. The resting Ca^{2+} influx in the arteries measured after a 5 min incubation with ^{45}Ca was not increased by either 10 μM CPA or 100 nM thapsigargin in either WKY or SHR (Table 2A). After a 30 min incubation with ^{45}Ca, the net Ca^{2+} entry was decreased by 10 μM CPA or 100 nM thapsigargin in both WKY and SHR (Table 2B). However, 160 mM K^+ solution increased both the resting Ca^{2+} influx and the net Ca^{2+} entry (Table 2A, B).

As shown in Table 2A, the resting Ca^{2+} influx was higher in SHR than in WKY. The resting Ca^{2+} influx was decreased by 100 nM nifedipine in SHR but not in WKY. The resting Ca^{2+} influx in the presence of nifedipine was still higher in SHR than in WKY (Table 2A). The resting Ca^{2+} influx in the presence of CPA or thapsigargin was also higher in SHR than in WKY (Table 2A).

CPA-induced ^{45}Ca efflux

Measurements of the net Ca^{2+} entry during the addition of CPA or thapsigargin indicate that the elevated $[Ca^{2+}]_i$ induced by these inhibitors could be extruded across the plasmalemma by a number of compensatory mechanisms which aim overall to reduce $[Ca^{2+}]_i$ back to its resting levels. To assess this possibility, the effects of CPA on resting ^{45}Ca efflux were examined in both WKY and SHR (Figure 6). After being loaded with ^{45}Ca for 2 h, the strips were washed with a Tris-buffered solution for 46 min. The resting ^{45}Ca efflux was not sig-

Table 2 Effects of cyclopiazonic acid (CPA), thapsigargin (TG), nifedipine (Nif) and high K^+ solution on (A) resting Ca^{2+} influx and (B) net Ca^{2+} entry in femoral arteries from 13-week-old WKY and SHR

	^{45}Ca incubation[a]	Ca^{2+} taken up by the tissue (μmol kg^{-1} tissue wet wt)	
Time	*Condition*	*WKY*	*SHR*
A 5 min	Control	86.4±2.2 (13)	103.8±2.4* (14)
	+CPA 10 μM	77.3±2.4$^{\#}$ (13)	98.9±1.7* (14)
	+TG 100 nM	82.4±1.5 (14)	99.2±3.0* (15)
	+Nif 100 nM	84.3±2.8 (15)	94.1±2.0*$^{\#}$ (14)
	160 mM K^+	166.2±4.9$^{\#}$ (12)	190.3±6.8*$^{\#}$ (12)
B 30 min	Control	163.3±6.6 (15)	178.7±6.5 (15)
	+CPA 10μM	138.3±3.6$^{\#}$ (13)	154.0±4.7*$^{\#}$ (13)
	+TG 100 nM	138.2±5.1$^{\#}$ (15)	154.7±1.8*$^{\#}$ (15)
	160 mM K^+	283.6±6.8$^{\#}$ (21)	328.1±6.5*$^{\#}$ (21)

[a]Arteries were incubated for 5 min (resting Ca^{2+} influx) or 30 min (net Ca^{2+} entry) in each solution to which ^{45}Ca had been added. Data are expressed as means±s.e.mean, and numbers in parentheses indicate the number of measurements. *Significantly different from WKY ($P<0.05$). $^{\#}$Significantly different from the respective 'Control' ($P<0.05$).

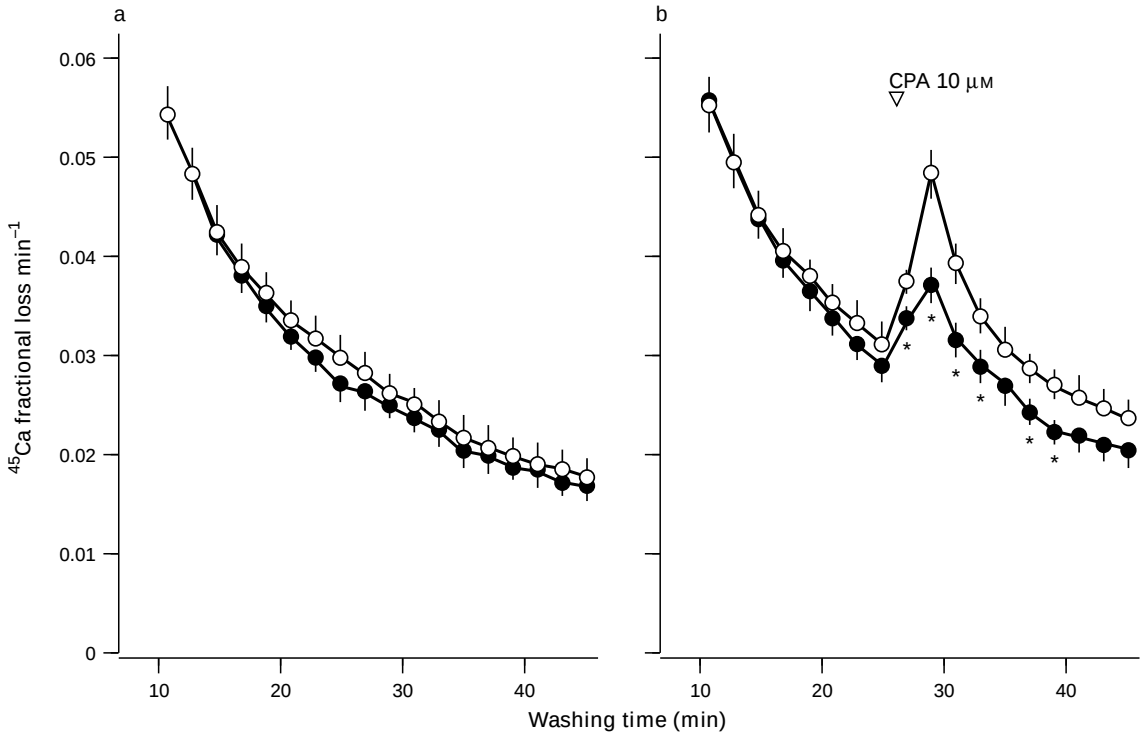

Figure 6 Effects of cyclopiazonic acid (CPA) on the resting ^{45}Ca efflux (fractional loss min^{-1}) in strips of femoral arteries from 13-week-old WKY (○) and SHR (●). (b) After being loaded with ^{45}Ca, the strips were washed with a Tris-buffered solution for 26 min and then 10 μM CPA was added for 20 min. (a) The resting ^{45}Ca efflux without addition of CPA. Data points are means of 6 preparations, and s.e.mean are shown by vertical lines. *Significantly different from WKY ($P<0.05$).

nificantly different between WKY and SHR. When 10 μM CPA was added during the efflux period, the resting ^{45}Ca efflux was increased in both WKY and SHR. This increase was significantly less in SHR than in WKY (Figure 6).

Discussion

CPA, a mycotoxin from *Aspergillus* and *Penicillium*, has been shown to inhibit Ca^{2+} uptake into the SR by inhibiting SR Ca^{2+}-ATPase (Seidler *et al*., 1989; Deng & Kwan, 1991; Uyama *et al*., 1992). Thapsigargin, a non-phorbol ester-type tumour-promoting sesquiterpene lactone, has also been found to inhibit Ca^{2+} uptake into the SR by irreversibly inhibiting SR Ca^{2+}-ATPase (Thastrup *et al*., 1990). To clarify whether the SR of SHR arteries can buffer the high resting $[Ca^{2+}]_i$, the effects of CPA and thapsigargin were determined in the present study (Figures 1, 2 and 3). When CPA or thapsigargin was added to the resting tone of the strip, an elevation of resting $[Ca^{2+}]_i$ occurred that was accompanied by an arterial contraction in both SHR and WKY. Because these responses were greater in SHR than in WKY, it is likely that the Ca^{2+} buffering function of SR was increased in the resting state of the SHR artery. We considered that the CPA- and thapsigargin-induced elevation of $[Ca^{2+}]_i$ and contraction can be explained by the 'superficial buffer barrier' hypothesis proposed by van Breemen and his colleagues (van Breemen & Saida, 1989; Chen *et al*., 1992; van Breemen *et al*., 1995). The greater effects of CPA and thapsigargin on the SHR artery may be due to the increased resting Ca^{2+} influx via L-type VDCCs, which would be expected to cause a greater effect if the Ca^{2+} buffering function of SR is eliminated. Thus, we propose that the SR of the SHR artery serves as an effective barrier to Ca^{2+} entry by utilizing the Ca^{2+} uptake mechanism in the face of high resting $[Ca^{2+}]_i$.

A similar conclusion was proposed in our previous study in which the possible mechanism of the potent vasoconstrictor actions of ryanodine on femoral arteries from SHR was ex-

amined (Asano *et al*., 1996). Ryanodine has been used as a tool to eliminate the function of SR through the different mechanisms from CPA and thapsigargin. Ryanodine depletes SR Ca^{2+} by opening the Ca^{2+}-induced Ca^{2+}-release channels in SR (for details, see Asano *et al*., 1996). Thus, the potent Ca^{2+} buffering function of SR in the SHR artery was proposed from the studies with ryanodine (Asano *et al*., 1996) and SR Ca^{2+}-ATPase inhibitors (present study).

Since the steady state $[Ca^{2+}]_i$ in the resting state of arterial smooth muscle is considered to be determined by an equilibrium among various processes of Ca^{2+} mobilization, such as Ca^{2+} entry across the plasmalemma, Ca^{2+} release from the SR, Ca^{2+} extrusion across the plasmalemma and Ca^{2+} uptake into the SR (van Breemen & Saida, 1989; Chen *et al*., 1992; van Breemen *et al*., 1995), several other mechanisms may be considered to account for the actions of CPA and thapsigargin. Because the CPA- and thapsigargin-induced contractions were mainly due to Ca^{2+} influx via L-type VDCCs, Ca^{2+} in the SR did not contribute to these contractions. This conclusion arises from the following observations: (1) the CPA- and thapsigargin-induced contractions were greatly inhibited by verapamil or a Ca^{2+}-free solution; (2) the caffeine-induced contractions were not affected by verapamil, and (3) in a Ca^{2+}-free solution, CPA caused an elevation of $[Ca^{2+}]_i$ but failed to cause a contraction. The involvement of Ca^{2+} influx via L-type VDCCs in the CPA- and thapsigargin-induced contractions may suggest the direct action of these inhibitors on L-type VDCCs. Moreover, it has been demonstrated that CPA 10 μM depolarized the smooth muscle cells (Uyama *et al*., 1993; Maggi *et al*., 1995; Sekiguchi *et al*., 1996). However, these effects were not detected in the present study; with a 5 min incubation in ^{45}Ca CPA and thapsigargin failed to increase the resting Ca^{2+} influx. Moreover, the net Ca^{2+} entry measured by using a 30 min incubation with ^{45}Ca was decreased during the addition of CPA or thapsigargin. If CPA and thapsigargin have inhibitory effects on the Ca^{2+} extrusion across the plasmalemma, these effects may contribute to the elevation of $[Ca^{2+}]_i$ and result in a contraction. However, this is unlikely, because the ^{45}Ca efflux studies showed that CPA increased the Ca^{2+} extrusion across the plasmalemma.

As estimated by the CPA- or thapsigargin-induced elevation of $[Ca^{2+}]_i$ (Table 1A), it is likely that a large amount of Ca^{2+} enters the cell and is taken up into the SR in the resting state of the SHR artery. The data that CPA elevated the $[Ca^{2+}]_i$ by 102% of the response to 65.9 mM K^+ and caused a 91% contraction clearly indicate that this amount of Ca^{2+} was always taken up into the SR in the resting state of the SHR artery. When the source of $[Ca^{2+}]_i$ was analysed from the data shown in Table 1, Ca^{2+} influx via L-type VDCCs was calculated as 50% in the SHR artery, whereas in the WKY artery, it was calculated as 34%. Interestingly, the greater part of the CPA-induced contraction was due to the Ca^{2+} influx via L-type VDCCs in both SHR (89%) and WKY (82%). The rest of the CPA-induced contraction may be due to Ca^{2+} influx via leak pathway, since the contraction was abolished in the Ca^{2+}-free solution. A similar conclusion can be drawn from the effects of thapsigargin shown in Table 1. This result is in good agreement with the observation that Ca^{2+} influx via L-type VDCCs is mainly involved in the thapsigargin-induced contraction of rat aorta (Mikkelsen *et al*., 1988) and dog mesenteric artery (Low *et al*, 1991), and in the CPA-induced contraction of SHR aorta (Low *et al*., 1993). It has been shown that the magnitude of the contraction in vascular smooth muscle depends on the rate of Ca^{2+} influx rather than on the net amount of Ca^{2+} influx (van Breemen, 1977; van Breemen & Saida, 1989; van Breemen *et al*., 1995). Thus, the effectiveness of the SR buffering depends on the nature of Ca^{2+} influx. For instance, if the Ca^{2+} influx is large but slow, such as the resting influx via leak pathway, the SR buffering would be effective in blunting the contraction. On the other hand, if the Ca^{2+} influx is small but fast, such as the resting influx via L-type VDCCs, then the Ca^{2+} uptake by the SR is less able to compete with the Ca^{2+} influx resulting in the delivery of Ca^{2+} to the myofilaments being more effective in initiating contraction. Similarly, with elimination of SR Ca^{2+} uptake, the plasmalemmal Ca^{2+} extrusion systems (e.g. Na^+-Ca^{2+} exchanger and Ca^{2+}-ATPase) would be effective in blunting the contraction against the Ca^{2+} influx via leak pathway. On the other hand, with elimination of SR Ca^{2+} uptake, the Ca^{2+} extrusion across the plasmalemma is less able to compete with the Ca^{2+} influx via L-type VDCCs resulting in the delivery of Ca^{2+} to the myofilaments being more effective in initiating contraction.

The time course of responses to CPA and thapsigargin was quite different, as shown in Figures 1 and 3. This suggests that drug access to, or interaction with the SR Ca^{2+}-ATPase is different between the two inhibitors. This may be due to the difference in the concentration of inhibitors used (10 μM CPA vs. 100 nM thapsigargin) and the difference in the membrane permeability of the inhibitors (many studies have shown that the onset of thapsigargin action is slower than that of CPA, Mikkelsen *et al*., 1988; Low *et al*., 1991; Chen *et al*., 1992).

The resting ^{45}Ca influx was increased in SHR, and this influx was reduced by nifedipine. In the resting state of SHR arteries, an increased Ca^{2+} influx (Bhalla *et al*., 1978; van Breemen *et al*., 1986; Asano *et al*., 1993a) and a high resting $[Ca^{2+}]_i$ (Spieker *et al*., 1988; Jelicks & Gupta, 1990; Sada *et al*., 1990) have been found. Although the precise mechanism responsible for the increased resting Ca^{2+} influx in the SHR artery is not clear, a possible explanation for this change appears to be that the SHR artery is more depolarized in the resting state than the WKY artery (for details, see Asano *et al*., 1993a; 1995). Indeed, membrane depolarization has been observed in the resting state of several arteries from SHR (Hermsmeyer, 1976; Cheung, 1984; Stekiel *et al*., 1986; Sekiguchi *et al*., 1996). Therefore, the possible mechanism of the CPA- or thapsigargin-induced potent contraction in the SHR artery is that although the Ca^{2+} influx was increased in the resting state of the SHR artery, the greater part of the increased Ca^{2+} influx was buffered by Ca^{2+} uptake into the SR, so that the functional elimination of SR by CPA or thapsigargin caused a potent contraction in this artery.

As shown in Figures 4 and 5, the addition of verapamil during the resting tone of the SHR artery decreased the resting $[Ca^{2+}]_i$ which was accompanied by a relaxation, suggesting that the resting $[Ca^{2+}]_i$ was already elevated in the SHR artery. Because the verapamil-induced decrease in the resting $[Ca^{2+}]_i$ was also observed in the WKY artery, it is likely that the L-type VDCCs are also activated in this artery. However, such an activation was not detected by measuring resting ^{45}Ca influx. If the activation of L-type VDCCs in the WKY artery is small, such a discrepancy may be explained. It has been demonstrated that the opening of L-type VDCCs can occur at the resting membrane potential of smooth muscle cells from guinea-pig coronary artery and most probably serve as a pathway for Ca^{2+} entry into the resting cell (Ganitkevich & Isenberg, 1990). Therefore, despite the fact that the opening of L-type VDCCs is rare in the resting state of the WKY artery, it is probably that some Ca^{2+} enter the cell and are taken up into the SR.

The present study also demonstrated the existence of a relatively large compartment of $[Ca^{2+}]_i$ which does not contribute to the contraction during the addition of CPA or thapsigargin, suggesting the cytosolic localization of Ca^{2+} in both SHR and WKY. This conclusion arises from the following observations: (1) CPA at low concentrations failed to cause a contraction but caused an elevation of $[Ca^{2+}]_i$, (2) in the presence of verapamil, thapsigargin failed to cause a contraction but caused an elevation of $[Ca^{2+}]_i$, and (3) verapamil or Ca^{2+}-free solution inhibited the CPA-induced contraction more potently than the CPA-induced elevation of $[Ca^{2+}]_i$. These contraction-independent $[Ca^{2+}]_i$ compartments probably reflect the Ca^{2+} gradient created around the outer surface of the SR and the inner surface of the plasmalemma during the addition CPA or thapsigargin. After the blockade of Ca^{2+} influx via L-type VDCCs, CPA or thapsigargin exhibited similar actions on SHR and WKY, suggesting that these com-

partments were the same in the arteries from SHR and WKY. Similar compartments were also observed in the ryanodine-induced contractions of our previous study (Asano *et al*., 1996). These Ca^{2+} may then be extruded by the Na^{+}-Ca^{2+} exchanger and the Ca^{2+}-ATPase of the plasmalemma, as proposed in the 'superficial buffer barrier' hypothesis. This assumption was supported by the observation that the net Ca^{2+} entry was decreased during the addition of CPA or thapsigargin, suggesting that the ^{45}Ca efflux was secondarily increased during this period. Actually, as shown in Figure 6, ^{45}Ca efflux from the plasmalemma was increased during the addition of CPA in both SHR and WKY. However, this increase was less in SHR than in WKY, suggesting that the ability of Ca^{2+} extrusion across the plasmalemma is decreased in the SHR artery, as has been repeatedly demonstrated (for review, see Kwan, 1985; Bohr & Webb, 1988). This decreased Ca^{2+} extrusion may contribute to the CPA-induced large elevation of $[Ca^{2+}]_i$ and potent contraction in the SHR artery.

From the present study, we conclude that CPA and thapsigargin cause an elevation of $[Ca^{2+}]_i$ and contraction in arterial smooth muscle by compromising the function of a superficial buffer barrier in the face of resting Ca^{2+} influx via L-type VDCCs and other pathways. When the SR Ca^{2+} uptake is inhibited by these agents, the resting Ca^{2+} influx via L-type VDCCs is the main contributor to the arterial contraction. Since the resting Ca^{2+} influx was increased in the SHR artery, CPA and thapsigargin caused greater effects in this artery than in the artery WKY artery.

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