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Possible mechanism of the potent vasoconstrictor actions of ryanodine 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 ryanodine that removes the function 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 The addition of ryanodine to the resting strips caused a concentration-dependent contraction in SHR. This contraction was extremely small in WKY. In the presence of 10^{-5} M ryanodine, caffeine (20 mM) failed to cause a further contraction in SHR, but it caused a small contraction in WKY. After washout of the strips with a Krebs solution, the resting tone was greatly elevated in SHR when compared with WKY.

3 The elevated resting tone in SHR strips was abolished by 10^{-7} M nifedipine. The ryanodine-induced contraction was also abolished by 10^{-7} M nifedipine. Nifedipine itself caused a relaxation from the resting tone of SHR strips, suggesting the maintenance of myogenic tone.

4 In strips preloaded with fura-PE3, the addition of 10^{-5} M ryanodine caused a large and moderate elevation of cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) in SHR and WKY, respectively. After washout, the resting $[Ca^{2+}]_i$ was greatly elevated in SHR. The ryanodine-induced elevation of $[Ca^{2+}]_i$ was decreased by 5×10^{-6} M verapamil in SHR. Verapamil itself caused a decrease in resting $[Ca^{2+}]_i$ which was significantly greater in SHR than in WKY, and caused a relaxation only in SHR.

5 The resting Ca^{2+} influx in arteries measured by a 5 min incubation with ^{45}Ca was significantly increased in SHR when compared with WKY. The resting Ca^{2+} influx was not increased by 10^{-5} M ryanodine in both SHR and WKY. The net cellular Ca^{2+} uptake in arteries measured by a 30 min incubation with ^{45}Ca was decreased by 10^{-5} M ryanodine in both strains.

6 The resting Ca^{2+} influx was decreased by 10^{-7} M nifedipine in the SHR artery, but it was unchanged in the WKY artery.

7 These results suggest that (1) the Ca^{2+} influx via L-type voltage-dependent Ca^{2+} channels 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 removal of SR by ryanodine caused a potent contraction in this artery.

Keywords: Spontaneously hypertensive rats (SHR); arterial myogenic tone; ryanodine; sarcoplasmic reticulum; cytosolic Ca^{2+} level; voltage-dependent Ca^{2+} channels; Ca^{2+} influx; 'superficial buffer barrier' hypothesis

Introduction

The total peripheral resistance is increased in patients and experimental animals with 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). Particularly, the cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) is elevated in various tissues and cells isolated from patients with established hypertension and SHR. Because the $[Ca^{2+}]_i$ plays a key role in arterial smooth muscle contraction, the alteration in $[Ca^{2+}]_i$ may explain some of the abnormal functions associated with hypertension including a maintenance of myogenic tone, a hyper-reactivity to vasoconstrictors and a decreased relaxation to vasodilators.

Arteries isolated from SHR have been shown to maintain a spontaneous active tone in the resting state (Noon *et al.*, 1978; Fitzpatrick & Szentivanyi, 1980; Winquist & Bohr, 1983; Asano *et al.*, 1986). The spontaneous active tone was independent of regional innervation and circulating hormones and has been called 'myogenic'. The myogenic tone is abolished by the removal of external Ca^{2+} (Noon *et al.*, 1978; Fitzpatrick & Szentivanyi, 1980; Winquist & Bohr, 1983) and by blockers of L-type voltage-dependent Ca^{2+} channels (VDCCs) (Asano *et al.*, 1986, 1993a), suggesting that an increase in Ca^{2+} influx via this channel is responsible for the myogenic tone in SHR arteries. The elevated $[Ca^{2+}]_i$ could be extruded by Na^+-Ca^{2+} exchange, Ca^{2+} pumping across the plasmalemma and Ca^{2+} uptake into the sarcoplasmic reticulum (SR). According to 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 SR is an effective barrier to Ca^{2+} influx by utilizing mechanisms of Ca^{2+} uptake and unloading to the extracellular space. Therefore, the maintenance of the myogenic tone in the resting state of SHR arteries may indicate two opposite possibilities: (1) the function of SR to buffer the influxed Ca^{2+} is impaired, or (2) this function is increased but more Ca^{2+} enters the smooth muscle than the Ca^{2+} buffering ability of SR.

Ryanodine, a plant alkaloid, has been shown to accelerate

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Ca^{2+} release from SR having Ca^{2+}-induced Ca^{2+} release (CICR) channels and then finally abolish the Ca^{2+} release (Fleischer *et al.*, 1985; Rousseau *et al.*, 1987; Hwang & van Breemen, 1987). The mechanism of action of ryanodine is considered to be due to this alkaloid binding to CICR channels that are in an open state, and then locking them open (Fleischer *et al.*, 1985; Rousseau *et al.*, 1987; Iino *et al.*, 1988). Since the binding is practically irreversible (Fleischer *et al.*, 1985), CICR channels remain open even after the ryanodine has been washed out from the medium. Thus, ryanodine can be used as a tool to remove the function of SR in vascular smooth muscle cells (Iino *et al.*, 1988; Ito *et al.*, 1991; van Breemen *et al.*, 1995). Kojima *et al.* (1994) have recently demonstrated the dramatically larger ryanodine-induced contraction in the SHR femoral artery. They have previously shown that the ability of SR to buffer Ca^{2+} during high K^+-induced contraction is diminished in the SHR artery (Kojima *et al.*, 1991). They have also examined the mechanism of the ryanodine action by using functional experiments and concluded that ryanodine contracts the artery by promoting Ca^{2+} influx through VDCCs (Kojima *et al.*, 1994). To further clarify the mechanism of action of ryanodine in the SHR artery, we examined the effects of this alkaloid on the mechanical activity, $[Ca^{2+}]_i$ (by using a fluorescent Ca^{2+} indicator fura-PE3), resting ^{45}Ca influx and net cellular ^{45}Ca uptake in the resting state of the SHR artery, and data were compared with the findings in the WKY artery.

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 (197 ± 1.8 mmHg, $n=60$) than in WKY (139 ± 1.2 mmHg, $n=60$). The body weight was significantly lower in SHR (248 ± 4 g, $n=60$) than in WKY (270 ± 4 g, $n=60$).

Preparation of arterial strips

Rats were stunned by a blow to the head and then exsanguinated. Femoral arteries (0.7–0.9 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.

Measurement of isometric tension

Arterial strips (0.8×8 mm) were mounted vertically in water-jacketed muscle baths containing 10 ml Krebs solution. Krebs solutions were maintained at 37°C and aerated with 95% O_2 and 5% CO_2. The isometric tension was recorded with a force-displacement transducer (TB-612T, Nihon Kohden Kogyo, Tokyo, Japan). Strips were stretched passively to optimal length by imposing a resting tension of 0.6 g and a 90 min equilibration period preceded each experiment. The optimal resting tension was determined by a length-passive tension study (Asano *et al.*, 1988). All experiments were conducted in phenoxybenzamine-treated strips to eliminate possible α-adrenoceptor responses to endogenously released noradrenaline and 5×10^{-7} M timolol was added to the Krebs solution to eliminate possible β-adrenoceptor responses (Asano *et al.*, 1988).

After the equilibration, contractile responses of the strips to the Krebs solution containing 65.9 mM KCl (K^+) (equimolar substitution of Na^+ with K^+) were repeated two or three times until the responses were reproducible. After washout of the strips with a Krebs solution, contractile responses to caffeine or ryanodine were determined. To characterize the ryanodine-induced contraction, effects of nifedipine, verapamil, diltiazem (blockers of L-type VDCCs), cromakalim (an opener of ATP-sensitive K^+ channels) or Ca^{2+}-free solution on the contractile responses to ryanodine were determined. The Ca^{2+}-free solution was prepared by omission of Ca^{2+} from the Krebs solution and by addition of 0.1 mM EGTA. In some experiments, nifedipine was added to the strips precontracted with ryanodine or 65.9 mM K^+. In this experiment, cumulative concentration-response curves for the relaxant effects of nifedipine were constructed (Asano *et al.*, 1993a).

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

$[Ca^{2+}]_i$ was measured simultaneously with tension changes as described previously (Uyama *et al.*, 1993; Asano *et al.*, 1995). Briefly, arterial strips (0.8×8 mm) were loaded with 10^{-5} M 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. Fura-PE3 was designed to give fura-2 the capacity to resist leakage and compartmentation, so that $[Ca^{2+}]_i$ can be measured at least for 3 h. 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 strips were perfused at a rate of 2.5 ml min^{-1} with the oxygenated Krebs solution at 37°C. Strips were perfused for the next 50 min before application 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) which 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.*, 1988a). Changes in $[Ca^{2+}]_i$ during the addition of ryanodine were determined. Effects of verapamil were also determined. 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.

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

Resting ^{45}Ca influx was measured by using a substituted-La^{3+} solution at 0.5°C as described previously (Asano *et al.*, 1993a). Briefly, isolated arteries were opened longitudinally and equilibrated in the 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 the 5 min incubation time, arteries were transferred to test tubes containing 80.8 mM La^{3+}-substituted solution (0.5°C) for 45 min to remove extracellular ^{45}Ca. The amount of ^{45}Ca taken up by the tissue during the 5 min incubation time can be assumed to be primarily due to Ca^{2+} influx with minimal efflux components (Meisheri *et al.*, 1981; Meisheri & van Breemen, 1982). 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 cellular ^{45}Ca uptake was measured by incubating the arteries in the same ^{45}Ca solution for 30 min before the La^{3+} wash (Asano *et al.*, 1993b). 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 ^{45}Ca influx measurements. Changes in resting ^{45}Ca influx and net cellular ^{45}Ca uptake during the addition of ryanodine, 160 mM K^+ solution or nifedipine were determined. Values for resting Ca^{2+} influx and net cellular Ca^{2+} uptake were then calculated and expressed as nmol g^{-1} tissue wet weight, as described previously (Asano *et al.*, 1993 a,b).

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 <0.05 was taken as significant.

Drugs and isotope

The drugs used were ryanodine (lot 704RWP-1, S.B. Penick, Lyndhurst, NJ, U.S.A.), caffeine (Wako Pure Chemical Industries, Osaka, Japan), nifedipine (Bayer Yakuhin Ltd., Osaka, Japan), verapamil hydrochloride (Eisai Co. Ltd., Tokyo, Japan), diltiazem hydrochloride (Tanabe Pharmaceutical Co., Osaka, Japan), cromakalim (Beecham Pharmaceuticals Research Division, Harlow, Essex), fura-PE3/AM (Texas Fluorescence Lab. Inc., Austin, TX, U.S.A.), cremophor EL (Nacalai Tesque Inc., Kyoto, Japan), papaverine hydrochloride (Wako), phenoxybenzamine hydrochloride (Nacalai) and timolol maleate (Banyu Pharmaceuticals, Tokyo, Japan). $^{45}CaCl_2$ (specific activity initially 14.6–28.5 mCi mg^{-1}) was obtained from Amersham International (Buckinghamshire).

Nifedipine (1 mM) and cromakalim (10 mM) were dissolved in 50 and 60% ethanol, respectively, with further dilution in distilled water before use. Fura-PE3/AM (0.5 mM) was dissolved in 100% dimethyl sulphoxide. Phenoxybenzamine (2 mM) was dissolved in 99.5% ethanol. Caffeine (20 mM) was dissolved in the Krebs solution. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations.

Results

Ryanodine-induced contractions

After the determination of the maximum contraction induced by 65.9 mM K^+, the addition of 20 mM caffeine caused a transient contraction in strips of femoral arteries from WKY and SHR (Figure 1). The caffeine-induced contraction was significantly larger in SHR than in WKY (Table 1). Since 20 mM caffeine succeeded in depleting the stored Ca^{2+}, a transient contraction induced by caffeine could be a rough index of the Ca^{2+} amount in the SR, as shown in other studies (Leijten & van Breemen, 1986; Naganobu *et al.*, 1994). The addition of 10^{-5} M ryanodine to the resting tone of the strips caused a relatively sustained contraction, which was significantly larger in SHR than in WKY (Figure 1, Table 1). The difference in the ryanodine-induced contractions between SHR and WKY was much larger than the difference in the caffeine-induced contractions. When caffeine was then added in the presence of ryanodine, it caused a small contraction in WKY, but caused a relaxation without causing a contraction in SHR (Figure 1). After washout of the strips with a Krebs solution, the resting tone was significantly elevated in SHR when compared with WKY (Figure 1). When caffeine was added after a 45 min washout, it caused a relaxation in both strains (Figure 1). Lower concentrations of ryanodine ($\sim 3 \times 10^{-7}$ M) were found to act in a similar manner, but it took longer for the response to occur (data not shown). Concentration-response curves for the contractile effects of ryanodine are shown in Figure 2. Above the concentration of 10^{-7} M, ryanodine caused a larger contraction in SHR than in WKY (Figure 2).

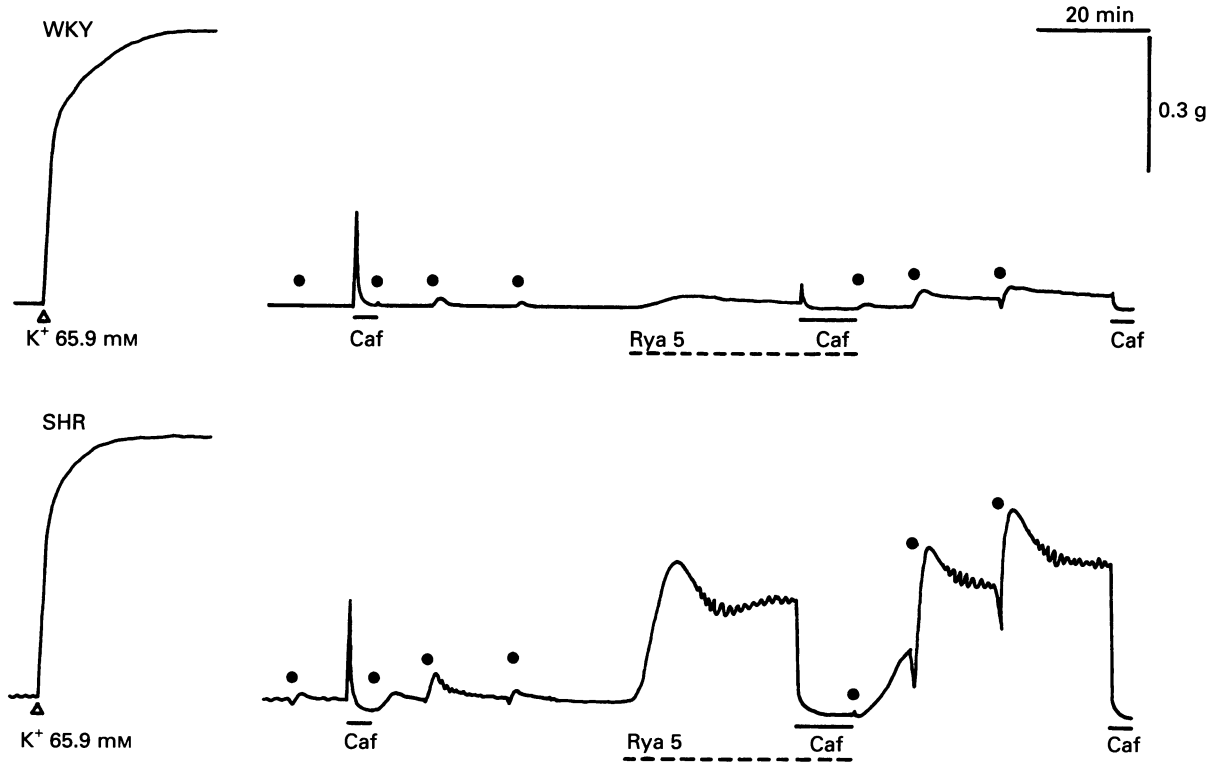

Figure 1 Typical recordings of the contractions induced by caffeine and ryanodine in strips of femoral arteries from 13-week-old WKY and SHR. After the determination of the maximum contraction induced by 65.9 mM KCl (K^+), 20 mM caffeine (Caf) was added for 4 min. After washout for 45 min, 10^{-5} M ryanodine (Rya 5) was added for 30 min. In the presence of ryanodine, Caf was added for 10 min. After washout for 45 min, Caf was added for 4 min. Dots denote the washing of the strips with a Krebs solution.

Table 1 Effects of nifedipine, verapamil, diltiazem, cromakalim and Ca^{2+}-free solution on ryanodine- and caffeine-induced contractions in strips of femoral arteries from 13-week-old SHR and WKY[a]

Condition	Contraction (% of 65.9 mM K^+)[b] SHR	WKY
Ryanodine 10^{-5} M (control)	58.0±4.9# (9)	4.5±1.7 (9)
+Nifedipine 10^{-8} M[c]	14.5±1.3* (5)	ND[e]
+Nifedipine 10^{-7} M[c]	2.2±0.4* (5)	1.7±0.4* (5)
+Verapamil 10^{-5} M[c]	1.5±0.3* (4)	ND[e]
+Diltiazem 10^{-5} M[c]	0.8±0.4* (3)	ND[e]
+Cromakalim 3×10^{-6} M[c]	1.3±0.5* (5)	1.5±0.6* (5)
+Ca^{2+}-free solution[d]	0.6±0.4* (5)	0.7±0.3* (5)
Caffeine 20 mM (control)	37.2±1.6# (9)	32.7±1.2 (9)
+Nifedipine 10^{-7} M[c]	33.9±1.7 (7)	31.8±1.2 (7)

[a] Experimental conditions were the same as in Figure 1. [b] Contractions induced by ryanodine and caffeine are expressed as % of the maximum contraction induced by 65.9 mM K^+. [c] Nifedipine, verapamil, diltiazem or cromakalim was added 20 min before the application of ryanodine or caffeine. [d] After a 2 min exposure of the strip to a Ca^{2+}-free solution, ryanodine was added. [e] ND, not determined. Data are expressed as means±s.e.mean, and numbers in parentheses indicate the number of preparations used. #Significantly different from WKY ($P<0.05$). *Significantly different from the respective 'control' ($P<0.05$).

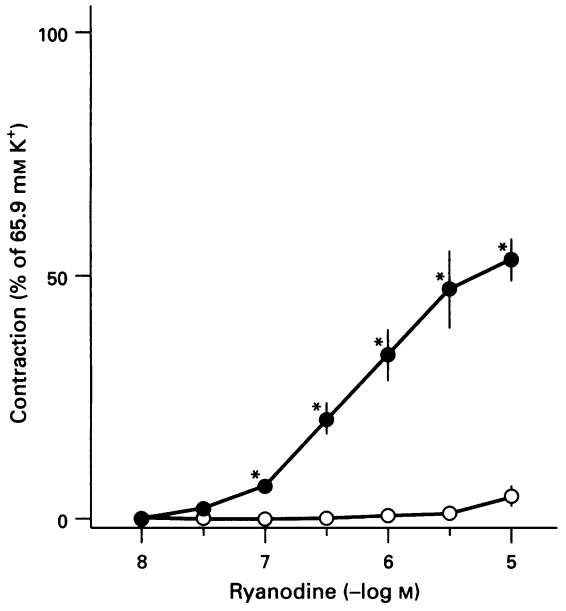

Figure 2 Concentration-response curves for the contractile effects of ryanodine in strips of femoral arteries from 13-week-old WKY (○) and SHR (●). The contraction induced by each concentration of ryanodine was determined in one strip, as shown in Figure 1. Peak contractions induced by each concentration of ryanodine are expressed as % of the maximum contraction induced by 65.9 mM K^+. Data points are means of 5–16 preparations, and s.e.mean are shown by vertical bars. *Significantly different from WKY.

Characteristics of ryanodine-induced contraction

The ryanodine-induced contraction in SHR strips was attenuated by nifedipine in a concentration-dependent manner (Table 1). In the presence of 10^{-7} M nifedipine, the ryanodine-induced contraction was almost abolished. Nifedipine itself caused a relaxation from the resting tone of the strip. The relaxation induced by 10^{-7} M nifedipine was 6.9±1.4% ($n=5$) of the 65.9 mM K^+-induced maximum contraction. The addition of 10^{-5} M verapamil or 10^{-5} M diltiazem also caused a relaxation from the resting tone and abolished the ryanodine-induced contraction in SHR (Table 1). Similar effects were obtained with 3×10^{-6} M cromakalim (Table 1). The ryanodine-induced contraction in SHR strips was also abolished by a 2 min exposure of the strip to a Ca^{2+}-free solution (Table 1). The ryanodine-induced contraction in WKY strips was also attenuated by nifedipine, cromakalim or a Ca^{2+}-free solution (Table 1). However, in WKY, these blockers themselves did not cause a relaxation from the resting tone. On the other hand, the caffeine-induced contraction was not attenuated by 10^{-7} M nifedipine in both SHR and WKY strips (Table 1).

Effects of nifedipine were then determined against the elevated resting tone that was observed in SHR strips after treatment with ryanodine plus caffeine (Figure 3). The addition of nifedipine to the elevated resting tone caused a concentration-dependent relaxation. Nifedipine also relaxed the SHR strips precontracted with 65.9 mM K^+. The relaxant effects of nifedipine were similar in the strips with the elevated resting tone and those precontracted with 65.9 mM K^+ (Figure 3).

Ryanodine-induced changes in $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ during the addition of caffeine and ryanodine were determined (Figure 4). After determination of the contraction and the elevation of $[Ca^{2+}]_i$ induced by 65.9 mM K^+, the addition of 20 mM caffeine caused a transient contraction that was accompanied by a rapid followed by a sustained elevation of $[Ca^{2+}]_i$ in both WKY and SHR strips. The addition of 10^{-5} M ryanodine to the WKY strips caused a small contraction that was accompanied by a moderate elevation of $[Ca^{2+}]_i$ (44.2±7.6% of the 65.9 mM K^+-induced elevation of $[Ca^{2+}]_i$, $n=6$). However, in SHR, ryanodine caused a potent contraction that was accompanied by a large elevation of $[Ca^{2+}]_i$ (88.7±5.4%, $n=6$, significantly greater than WKY). In the presence of ryanodine, the addition of caffeine caused a further elevation of $[Ca^{2+}]_i$ in WKY, but decreased the $[Ca^{2+}]_i$ in SHR. After washout of the strips, the resting $[Ca^{2+}]_i$ was significantly elevated in SHR when compared with WKY (Figure 4).

Effects of verapamil on resting $[Ca^{2+}]_i$ and ryanodine-induced elevation of $[Ca^{2+}]_i$

Effects of verapamil on resting $[Ca^{2+}]_i$ were determined (Figure 5). The addition of 5×10^{-6} M verapamil to the resting tone of the strips decreased the resting $[Ca^{2+}]_i$ in both WKY and SHR. This decrease was greater in SHR (44.5±9.5% of the 65.9 mM K^+-induced elevation of $[Ca^{2+}]_i$, $n=4$) than in WKY (20.2±5.4%, $n=4$), suggesting that the resting $[Ca^{2+}]_i$ was already elevated in SHR when compared with WKY. In SHR, but not in WKY, the decrease in resting $[Ca^{2+}]_i$ induced by verapamil was accompanied by a relaxation from the resting tone.

In the presence of verapamil, the addition of 10^{-5} M ryanodine caused an elevation of $[Ca^{2+}]_i$, but failed to cause a contraction in both WKY and SHR strips (Figure 5). This elevation of $[Ca^{2+}]_i$ was not significantly different between WKY (37.9±6.2% of the 65.9 mM K^+-induced elevation of

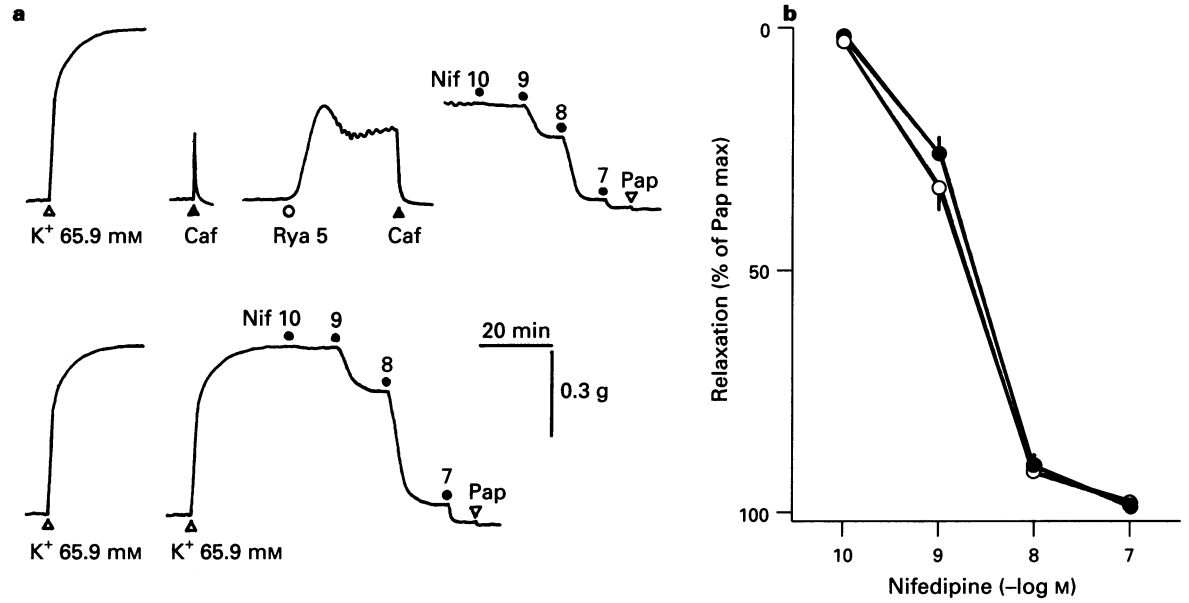

Figure 3 Effects of nifedipine (Nif) on the elevated resting tone after ryanodine-treatment and the 65.9 mM K^+-induced contraction in strips of femoral arteries from 13-week-old SHR. (a) Typical recordings. Strips were treated with 10^{-5} M ryanodine (Rya 5) plus 20 mM caffeine (Caf) and were then washed out with a Krebs solution, as shown in Figure 1. After a 45 min washout, Nif was added in a cumulative fashion. At the end of experiments, 10^{-4} M papaverine (Pap) was added to identify the position of the maximum relaxation. In other experiments, strips were contracted with 65.9 mM K^+ and Nif was added after the contraction had reached a plateau. Concentrations of Nif are expressed as the negative log of the molar concentration. (b) Concentration-response curves for the relaxant effects of Nif on the elevated resting tone after ryanodine-treatment (○) and the 65.9 mM K^+-induced contraction (●). Relaxations induced by each concentration of Nif are expressed as % of the maximum relaxation induced by Pap. Data points are means of 7 preparations, and s.e.mean are shown by vertical bars.

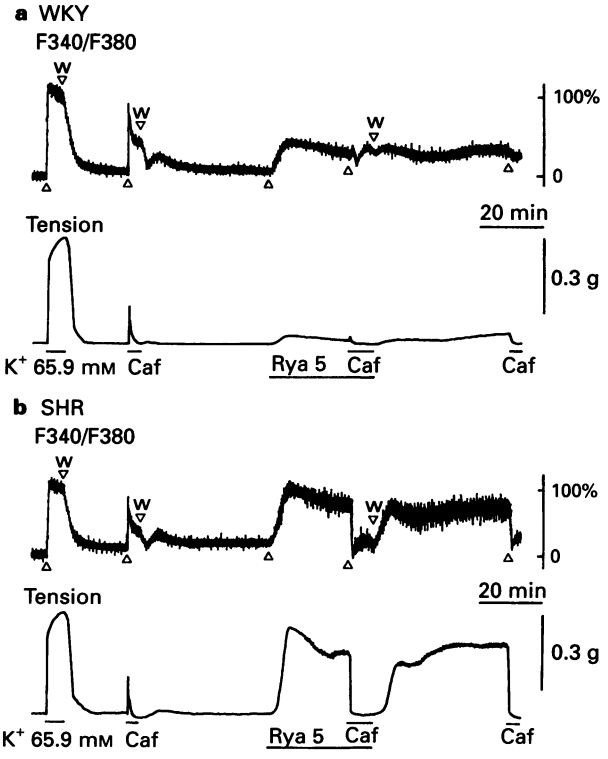

Figure 4 Typical recordings of the changes in $[Ca^{2+}]_i$ (indicated by F340/F380) and tension induced by caffeine and ryanodine in strips of femoral arteries from 13-week-old WKY (a) and SHR (b). After loading with fura-PE3, the strips were exposed to 65.9 mM K^+, 20 mM caffeine (Caf) and 10^{-5} M ryanodine (Rya 5) in a similar manner as in Figure 1. Triangles in each upper panel (F340/F380) denote the addition of each agent and the washout (w) with a Krebs solution.

$[Ca^{2+}]_i$, $n=4$) and SHR (37.5±7.8%, $n=4$). To determine the effects of verapamil on the ryanodine-induced elevation of $[Ca^{2+}]_i$, these values are compared with the values determined in the absence of verapamil (Figure 4). The verapamil-sensitive $[Ca^{2+}]_i$ increase was calculated as 6.3% (WKY) and 51.2% (SHR), respectively.

Resting Ca^{2+} influx and net cellular Ca^{2+} uptake

The resting Ca^{2+} influx in the arteries measured by a 5 min incubation with ^{45}Ca was significantly increased in SHR when compared with WKY (Table 2A). When the arteries were incubated with ^{45}Ca in the presence of 10^{-5} M ryanodine, this influx was significantly decreased in WKY but was unchanged in SHR. Therefore, the resting Ca^{2+} influx in the presence of ryanodine was still significantly increased in SHR. On the other hand, this influx was significantly increased by 160 mM K^+ solution in both WKY and SHR arteries (Table 2A).

The net cellular Ca^{2+} uptake in the arteries measured by a 30 min incubation with ^{45}Ca was not significantly different between WKY and SHR (Table 2B). When the arteries were incubated with ^{45}Ca in the presence of 10^{-5} M ryanodine, this uptake was significantly decreased in both strains. The Ca^{2+} uptake in the presence of ryanodine was found to be significantly increased in SHR when compared with WKY. On the other hand, this uptake was significantly increased by 160 mM K^+ solution in both WKY and SHR arteries (Table 2B).

After treatment of the arteries with 10^{-5} M ryanodine plus 20 mM caffeine and washout for 40 min, the resting tone and $[Ca^{2+}]_i$ were elevated in both WKY and SHR, as shown in Figures 1 and 3. Under these conditions, the resting Ca^{2+} influx was significantly decreased in WKY and SHR arteries when compared with the resting Ca^{2+} influx determined in each artery after treatment with only 20 mM caffeine and the washout for 40 min (Table 2C). The Ca^{2+} influx under these conditions was significantly increased in SHR when compared with WKY.

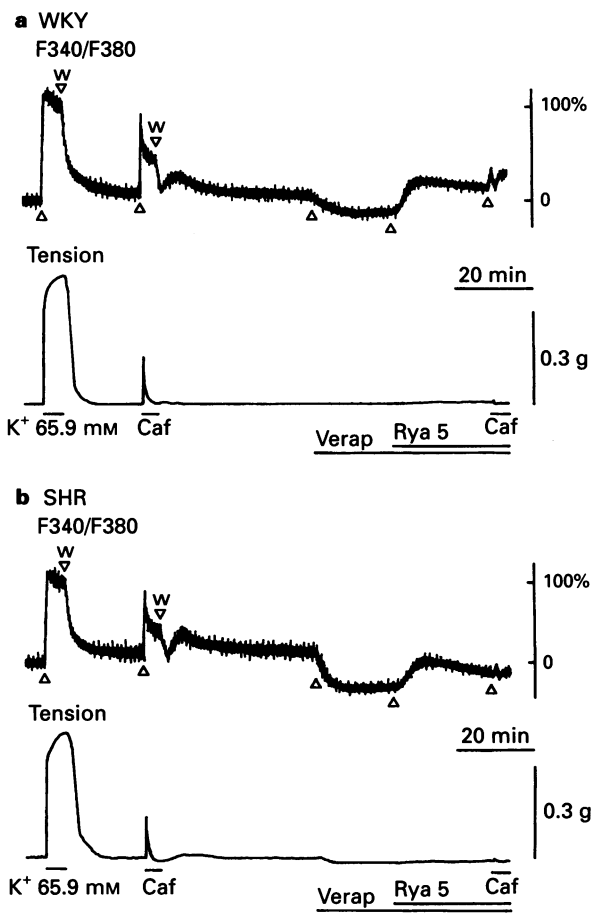

Figure 5 Typical recordings of the effects of verapamil on ryanodine-induced changes in $[Ca^{2+}]_i$ (indicated by F340/F380) and tension in strips of femoral arteries from 13-week-old WKY (a) and SHR (b). After loading with fura-PE3, the strips were exposed to 65.9 mM K^+ and 20 mM caffeine (Caf) in a similar manner as in Figure 4. Following washout for 40 min, 5×10^{-6} M verapamil (Verap) was added for 20 min, and then 10^{-5} M ryanodine (Rya 5) and Caf were added as indicated. Triangles in each upper panel (F340/F380) denote the addition of each agent and the washout (w) with a Krebs solution.

When the arteries were incubated with ^{45}Ca for 5 min in the presence of 10^{-7} M nifedipine, the resting Ca^{2+} influx was unchanged in WKY, but was significantly decreased in SHR (Table 2D). The resting Ca^{2+} influx in the presence of nifedipine was still significantly increased in SHR.

Discussion

As shown in the present study, the addition of ryanodine to the resting tone of femoral arteries caused a contraction and an elevation of $[Ca^{2+}]_i$, which were much larger in SHR than in WKY. The large ryanodine action in the SHR artery may be due to its higher resting Ca^{2+} influx via L-type VDCCs, which would be expected to cause a larger action if the function of the SR is abolished. Since the myogenic tone was maintained in the resting state of the SHR artery, this tone also appears to be due to the resting Ca^{2+} influx which exceeded the Ca^{2+} buffering ability of SR in this artery.

The mechanism of action of ryanodine is considered to be due to this alkaloid binding to CICR channels that are in an open state, and then locking them open (Fleischer *et al.*, 1985; Rousseau *et al.*, 1987; Iino *et al.*, 1988). The initial action of ryanodine could thus be acceleration of Ca^{2+} release. After full development of the ryanodine action, the SR becomes empty and cannot accumulate Ca^{2+} any longer, so that the SR cannot buffer Ca^{2+} which enters cells from extracellular space, nor release Ca^{2+} from there. Ryanodine binds CICR channels practically irreversibly (Fleischer *et al.*, 1985). Therefore, once the CICR channels have been opened by the ryanodine, they remain open for a long period even after the ryanodine has been washed out from the medium. These actions of ryanodine were confirmed in the present study and are shown in Figure 1. When CICR channels in SR were locked open by ryanodine, an elevation of resting tone occurred which was accompanied by a considerable loss of caffeine-releasable Ca^{2+} in both SHR and WKY arteries. Therefore, the Ca^{2+} released from the SR may be considered to contribute to the ryanodine-induced contractions. However, this is unlikely, because the ryanodine-induced contractions were due to Ca^{2+} influx via L-type VDCCs. This conclusion was drawn from the following observations: (1) the ryanodine-induced contractions were abolished by blockers of L-type VDCCs (nifedipine, verapamil and diltiazem) or a Ca^{2+}-free solution; (2) these contractions were abolished by hyperpolarization of the membrane through the opening of K^+ channels by cromakalim; and (3) the caffeine-induced contractions were not affected by nifedipine. Fur-

Table 2 Effects of ryanodine, high K^+ solution and nifedipine on resting Ca^{2+} influx and net cellular Ca^{2+} uptake in femoral arteries from 13-week-old WKY and SHR

^{45}Ca incubation condition[a]	*Ca^{2+} taken up by the tissue* (nmol g^{-1} wet tissue) *WKY*	*SHR*
A K^+ 5.4 mM for 5 min (control)	86.4 ± 2.2 (18)	103.1 ± 2.2# (18)
+ Ryanodine 10^{-5} M	79.6 ± 1.6* (16)	98.2 ± 2.2# (20)
K^+ 160 mM for 5 min	166.2 ± 4.9* (12)	190.3 ± 6.8#* (12)
B K^+ 5.4 mM for 30 min (control)	164.0 ± 4.9 (14)	177.9 ± 5.9 (14)
+ Ryanodine 10^{-5} M	135.8 ± 5.2* (14)	158.4 ± 6.6#* (14)
K^+ 160 mM for 30 min	283.6 ± 6.8* (21)	328.1 ± 6.5#* (21)
C K^+ 5.4 mM for 5 min (control)	87.2 ± 2.2 (10)	104.1 ± 1.2# (10)
After ryanodine treatment	69.4 ± 1.7* (10)	88.5 ± 4.7#* (10)
D K^+ 5.4 mM for 5 min (control)	82.1 ± 2.4 (15)	103.1 ± 3.4# (15)
+ Nifedipine 10^{-7} M	84.3 ± 2.8 (15)	94.1 ± 2.0#* (15)

[a] Arteries were incubated for 5 min (Ca^{2+} influx) or 30 min (net cellular Ca^{2+} uptake) in each solution to which ^{45}Ca had been added. (A) Effects of ryanodine and high K^+ solution on the resting Ca^{2+} influx. (B) Effects of ryanodine and high K^+ solution on the net cellular Ca^{2+} uptake. (C) The resting Ca^{2+} influx after treatment with 10^{-5} M ryanodine plus 20 mM caffeine and washout for 40 min. (D) Effects of nifedipine on the resting Ca^{2+} influx. Nifedipine (10^{-7} M) was added 30 min before the application of and also during the ^{45}Ca incubation period. 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' ($P<0.05$).

thermore, the size of the caffeine-induced contraction in the SHR artery was only 14% larger than that in the WKY artery, this amount is clearly insufficient to account for the dramatically larger effect of ryanodine in the SHR. The involvement of Ca^{2+} influx via L-type VDCCs in the ryanodine-induced contractions may suggest the direct action of ryanodine on L-type VDCCs. This conclusion has already been drawn from the functional experiments in the SHR artery (Kojima *et al.*, 1994). However, this is also unlikely, because the studies using a 5 min incubation with ^{45}Ca showed that ryanodine failed to increase the resting ^{45}Ca influx. Moreover, the net cellular ^{45}Ca uptake was decreased during the addition of ryanodine.

Therefore, another explanation is needed to account for the ryanodine-induced contractions. As shown in Table 2, the nifedipine-sensitive ^{45}Ca influx was found only in the resting state of the SHR artery. The resting ^{45}Ca influx was increased in SHR when compared with WKY, 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 the elevated $[Ca^{2+}]_i$ (Spieker *et al.*, 1988; Jelicks & Gupta, 1990; Sada *et al.*, 1990) have been obtained. Although the precise mechanism that is responsible for the increased resting Ca^{2+} influx in the SHR artery is not clear, a possible explanation 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 found in the resting state of several arteries from SHR (Hermsmeyer, 1976; Cheung, 1984; Stekiel *et al.*, 1986). Therefore, the possible mechanism of the ryanodine-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 removal of SR by ryanodine caused a potent contraction in this artery (for details, see below).

Both the myogenic tone and the ryanodine-induced contraction in the SHR artery were sensitive to inhibition by blockers of L-type VDCCs and 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 essence of this hypothesis is that: (1) Ca^{2+} which enters the cell across the plasmalemma is taken up into SR by Ca^{2+}-ATPase before it can reach the myofilaments; (2) the Ca^{2+}-ATPase of the SR contributes to Ca^{2+} extrusion from the cells due to vectorial release of SR Ca^{2+} towards the inner surface of the plasmalemma, from where it is extruded by the Na^+-Ca^{2+} exchanger and the Ca^{2+}-ATPase of the plasmalemma; and (3) a Ca^{2+} gradient in the peripheral cytoplasm is created by these processes. In the resting state of the SHR artery, it is likely that a relatively large amount of Ca^{2+} is taken up into the SR, as estimated by the contractile action of ryanodine. This assumption was also supported by the finding that caffeine caused a larger contraction in SHR than in WKY. Recently, Kanagy *et al.* (1994) have examined the effects of agents that interact with the function of SR in aortic strips from stroke-prone SHR (SHRSP), and concluded that because of increased plasma membrane permeability, additional free Ca^{2+} enters the cell and is sequestered into the SR, resulting in a large caffeine-releasable store. Thus, the SR in SHR arteries serves as an effective barrier to Ca^{2+} entry by utilizing the Ca^{2+} uptake mechanism.

However, it is also possible that some Ca^{2+} can reach the myofilaments even in the resting state of the SHR artery, since the myogenic tone was maintained in this artery, as shown in Figure 5. Because the myogenic tone was abolished by nifedipine or verapamil, a small part of the resting Ca^{2+} influx via L-type VDCCs is involved in the maintenance of the myogenic tone. Studies in which a 5 min incubation with ^{45}Ca was used showed that the amount of Ca^{2+} influx via L-type VDCCs was relatively small when compared with the total resting Ca^{2+} influx in the SHR artery. It has been reported that the magnitude of 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 influx via L-type VDCCs, then the Ca^{2+} uptake by the SR is less able to compete with the Ca^{2+} influx with the result that the delivery of Ca^{2+} to the myofilaments would be more effective in initiating contraction. Therefore, the myogenic tone in the resting state of the SHR artery appears to be due to resting Ca^{2+} influx via L-type VDCCs which exceeded the Ca^{2+} buffering ability of SR in this artery.

Because the ryanodine-induced contraction in the SHR artery was abolished by blockers of L-type VDCCs, neither the Ca^{2+} release from the SR nor the resting Ca^{2+} influx through the leak pathway was involved in this contraction. Therefore, it is likely that the distribution of cellular Ca^{2+} which enters the cell via L-type VDCCs is changed during the addition of ryanodine and additional Ca^{2+} reached the myofilaments when compared with the myogenic tone. During the addition of ryanodine, the Ca^{2+} released from the SR appears to cycle through the SR; Ca^{2+} leaked from the open-locked CICR channels around the outer surface of the SR was again taken up into the SR by Ca^{2+}-ATPase. Under these conditions, it is likely that the SR cannot buffer the resting Ca^{2+} influx via L-type VDCCs, thus allowing a greater proportion of the influxed Ca^{2+} to activate the myofilaments. Such a Ca^{2+} cycling by the SR has been described in the resting state of rat mesenteric resistance arteries (Naganobu & Ito, 1994).

The present study also demonstrated the existence of a relatively large compartment of $[Ca^{2+}]_i$ which does not contribute to force generation during the addition of ryanodine. As shown in Figure 5, in the presence of verapamil, ryanodine failed to cause a contraction in the SHR artery, while this alkaloid caused an elevation of $[Ca^{2+}]_i$. These $[Ca^{2+}]_i$ 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 of ryanodine. These compartments were the same for arteries from SHR and WKY, suggesting that during the elimination of Ca^{2+} influx via L-type VDCCs, ryanodine exhibited similar actions on SHR and WKY. When the verapamil-sensitive compartment of $[Ca^{2+}]_i$ during the addition of ryanodine was calculated from the peak values shown in Figures 4 and 5, this compartment was 51.2% (SHR) and 6.3% (WKY), respectively, of the 65.9 mM K^+-induced elevation of $[Ca^{2+}]_i$. These values were comparable with the peak values of the ryanodine-induced contraction shown in Table 1.

In the presence of ryanodine, caffeine caused only a relaxation without a contraction and decreased the $[Ca^{2+}]_i$ in the SHR artery, while it caused a small contraction and a small elevation of $[Ca^{2+}]_i$ in the WKY artery, as shown in Figures 1 and 4. Such a caffeine-induced relaxation has been demonstrated in vascular smooth muscle and the possible mechanism of this action was explained as follows: (1) caffeine increases cyclic AMP content by inhibiting cyclic AMP phosphodiesterase, (2) caffeine inhibits Ca^{2+} influx stimulated by K^+-depolarization and by noradrenaline, and (3) caffeine inhibits myosin light chain kinase and actin-myosin interaction (Sato *et al.*, 1988b; Martin *et al.*, 1989; Ozaki *et al.*, 1990; Hughes *et al.*, 1990; Watanabe *et al.*, 1992).

As shown in Figure 5, the addition of verapamil to the resting tone of the SHR artery decreased the resting $[Ca^{2+}]_i$ which was accompanied by a relaxation from the resting tone, suggesting that the resting $[Ca^{2+}]_i$ was already elevated in SHR. The verapamil-induced decrease in resting $[Ca^{2+}]_i$ was also observed in the WKY artery. This observation may indicate that the L-type VDCCs are activated even in the WKY artery. However, such an activation was not detected in the measurements of resting ^{45}Ca influx. If the activation of L-type VDCCs in the WKY artery is small, such a discrepancy may be

explained. Assuming that L-type VDCCs in the resting state of the WKY artery are slightly activated, the small contraction induced by ryanodine in this artery can also be explained by the 'superficial buffer barrier' hypothesis, as described above.

From the present study, we concluded that ryanodine causes an increase in tension and $[Ca^{2+}]_i$ in the SHR femoral artery by compromising the function of superficial buffer barrier in the face of increased resting Ca^{2+} influx via L-type VDCCs.

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