It has been reported that the increased function of the voltage-dependent calcium channels (VDCC) in the artery is involved in the increase of peripheral resistance in hypertension, and that the sarcoplasmic reticulum (SR) in the artery plays an important role in preventing the development of hypertension via a buffering effect. However, no reports have described the role of VDCC and SR in resistance arterioles in the development or maintenance of hypertension. We investigated the function of VDCC and of SR in the cremaster arterioles of spontaneous hypertensive rats (SHR) and age-matched Wistar Kyoto rats (WKY). The changes in diameter and the intracellular calcium ion concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in the microdissected arterioles, using fluorescent dyes, were measured with videomicroscopy. The KCl concentration-response curves were analyzed in 4- to 5- and 7- to 8-week-old SHR and WKY. The changes in the vascular diameter and [Ca\textsuperscript{2+}]\textsubscript{i} in response to ryanodine, an α-1 adrenoceptor, and angiotensin-II stimulation were compared between the 7- to 8-week-old SHR and WKY. We found an increase in the Ca\textsuperscript{2+} influx by VDCC in the early hypertensive stage, but not in prehypertensive SHR. However, after the onset of hypertension, there were no significant differences from WKY in the SR function mediated by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release or inositol 1,4,5-trisphosphate-induced Ca\textsuperscript{2+} release. In conclusion, an increased influx of Ca\textsuperscript{2+} in the cell membrane, without a buffering effect of SR, was associated with progression of hypertension in the cremaster arterioles of SHR. Am J Hypertens 1999;12:1236–1242 © 1999 American Journal of Hypertension, Ltd.

KEY WORDS: Voltage-dependent calcium channels, sarcoplasmic reticulum, intracellular calcium ion, spontaneous hypertensive rats.
Measurement of the Intracellular Ca^{2+} Concentrations ([Ca^{2+}]i) Measurement of the intracellular Ca^{2+} concentrations have been described previously. After securing a stable intrinsic tone, suffusion was stopped and the cannulated vessels were incubated with Krebs buffer containing 10^{-5} mol/L Fura 2-AM, 0.05% dimethyl sulfoxide, and 0.05% cremophol EL at room temperature. The loading time was 60 min, followed by a 30-min wash period. The vessels were illuminated with a 100-W Xenon lamp, and the light was passed through a quartz collector, a heat filter, and an appropriate excitation filter. A light of an appropriate excitation wavelength was passed to a dichroic mirror. The mirror reflected the excitation light through the objective of the microscope. Fluorescence emission from the sample was passed through a band pass filter (500 to 530 nm) and onto a silicon intensifier tube camera (2400-77, Hamamatsu Photonics). Video images of the vessel were digitized with the image processor and stored on an optical disk for analysis of the fluorescence intensities (Argus 50/Ca image analyzing system; Hamamatsu Photonics). The 340 nm/380 nm ratio was measured in the region of interest that was set at the center of the isolated arteries. The 340 nm/380 nm ratio was used to monitor the relative [Ca^{2+}]i.

Assessment of Voltage-Dependent Calcium Channels The KCl concentration-response curve was plotted and the 340 nm/380 nm ratio was measured at the presence of α-blocker (10^{-7} mol/L prazosin and 10^{-6} mol/L rauwolfa) (n = 6, respectively). Because a significant difference was observed between SHR and WKY at 7 to 8 weeks in a preliminary study, we also compared the KCl response at 4 to 5 weeks (n = 6, respectively) in the prehypertension period. The results of assessment of the vascular responsiveness in SHR and WKY were expressed as the contraction rates, ie, as percentages of the maximal contraction in response to NE 3 × 10^{-5} mol/L, and were calculated by using the following formula: response = [(Dc – Dx)/(Dc – Dm)] × 100, where Dc is the control diameter, Dx is the diameter produced by drug concentration x, and Dm is the diameter obtained during maximal contraction.

There is the possibility that the differences between the rat strains in prehypertensive versus hypertensive states could relate to perfusion pressure. Therefore, we performed KCl concentration response curves in 7- to 8-week-old WKY at 50 mm Hg perfusion pressure (n = 5, respectively), and compared KCl concentration response curves between 7- to 8-week-old SHR and WKY using the same perfusion pressure (50 mm Hg).

Sarcoplasmic Reticulum Function

Ca^{2+}-Induced Ca Release (CICR) To eliminate the influence of influx of the extracellular Ca^{2+}, the extra-
cellular perfusion fluid was changed to Ca$^{2+}$-free Krebs buffer (34°C, 40 min), the 7- to 8-week-old SHR and WKY vascular response (n = 5, respectively) to 10$^{-5}$ mol/L ryanodine was compared, and the changes in the 340 nm/380 nm ratio were assessed. In the Ca$^{2+}$-free Krebs buffer, CaCl$_2$ was replaced with 1 mmol/L EGTA.$^{11}$ The results of assessment of the vascular responsiveness in SHR and WKY were expressed as in the earlier-mentioned KCl response.

**IP$_3$-Induced Ca$^{2+}$ Release: α-1 Adrenoceptor (AR)**

Whether α-1 AR are altered by the high blood pressure was investigated before assessing IP$_3$ stimulation in response to α-1 AR stimulation. The concentration-response curve (CRC) of norepinephrine (NE) in the presence of rauwolscine (10$^{-6}$ mol/L) was then compared in 7- to 8-week-old SHR and WKY (n = 7, respectively). The bath solution contained propranolol (10$^{-6}$ mol/L) to block the β-adrenoreceptors. To eliminate the influence of influx of the extracellular Ca$^{2+}$, the extravascular perfusion solution was changed to Ca$^{2+}$-free Krebs buffer (34°C, 40 min) in the presence of EGTA. IP$_3$ stimulation was performed by α-1 AR stimulation, and the vascular responses in SHR and WKY were compared (n = 6, respectively). The effect of α-1 AR was expressed as the percentage displayed in relation to the blood vessel diameter before exposure to NE (10$^{-7}$mol/L) in the presence of rauwolscine (10$^{-6}$ mol/L). (When Ca$^{2+}$ was 0, constriction was no longer observed after that caused by the first α-1 AR stimulation, even if α-1 AR stimulation was continued, and therefore, we only assessed NE [10$^{-7}$mol/L]).

**Measurement of [Ca$^{2+}$]$_i$** After Fura 2 loading and washout, a change was made to Ca$^{2+}$-free Krebs buffer (34°C, 40 min) in the presence of EGTA to eliminate the influx of the extracellular Ca$^{2+}$, and the effect of α-1 AR was assessed by observing the changes in the 340 nm/380 nm in response to NE (10$^{-7}$mol/L) in the presence of rauwolscine (10$^{-6}$ mol/L) (n = 6, respectively).

**Angiotensin-II** The vascular perfusion fluid was changed to Ca$^{2+}$-free Krebs buffer (34°C, 40 min) in the presence of EGTA, IP$_3$ stimulation was performed with angiotensin-II (10$^{-9}$ to 10$^{-7}$ mol/L), and the response of the 7- to 8-week-old SHR and WKY vessels were compared (n = 5, respectively). IP$_3$-induced Ca$^{2+}$ channels were stimulated with angiotensin-II (10$^{-9}$ to 10$^{-7}$mol/L), and the changes in the 340 nm/380 nm were assessed (n = 5, respectively).

**Statistical Analysis** Bonferroni’s procedures were used for multiple comparisons among various concentration-response curves, and the unpaired two-tail t test was used to compare the changes in the 340 nm/380 nm ratio of Fura 2 fluorescence. The results are expressed as the mean ± standard error of the mean (SEM), with $P < .05$ representing significance.

**RESULTS**

**Voltage-Dependent Calcium Channels** There was no significant difference in the KCl concentration-response curve between 4- to 5-week-old SHR, before the onset of hypertension, and age-matched WKY (Figure 1A). At 7 to 8 weeks, in the early period of hypertension, however, the KCl response was greater in SHR than in WKY (Figure 1B). The KCl response at the same perfusion pressure was greater in SHR than in WKY (Figure 1C).

The change in the 340 nm/380 nm ratio ([Ca$^{2+}$]$_i$) during the KCl response at 7 to 8 weeks showed a significant increase in SHR (227% ± 13%) compared with WKY (174% ± 10%) ($P < .0001$) (Figure 2).

**Sarcoplasmic Reticulum Function** This was measured only in 7- to 8-week-old SHR during the early period of hypertension and in age-matched WKY.

**CICR in Response to Ryanodine** The vascular responsiveness of SHR and WKY to 10$^{-5}$ mol/L ryanodine was not significantly different (Figure 3, left panel). Because vasodilatation was observed from 6 min onward when ryanodine contraction was induced under Ca$^{2+}$-free conditions, contraction was assessed up to 5 min in this study.

The change in the 340 nm/380 nm ratio ([Ca$^{2+}$]$_i$) was 141% ± 6% in SHR and 138% ± 4% in WKY, and the difference was not significant (Figure 3, right panel).

**IP$_3$-Induced Ca$^{2+}$ Release** With α-1 AR The alpha-1 AR CRC in response to NE was compared in SHR and WKY, but the difference was not significant (Figure 4). The absence of changes in α-1 AR receptors between SHR and WKY allowed investigation of the SR function. We therefore reduced the extracellular Ca$^{2+}$ to 0 and assessed the vasoconstriction by α-1 AR. The percentage change in the vessel diameter was 9.5% ± 1.3% in SHR and 8.2% ± 1.4% in WKY, with no significant differences between them (Figure 5, left panel). Furthermore, the change in the 340 nm/380 nm ratio ([Ca$^{2+}$]$_i$) was 117% ± 7% in SHR and 110% ± 8% in WKY, and the difference was not significant (Figure 5, right panel).

**With Angiotensin-II** When we reduced the [Ca$^{2+}$]$_i$ to 0, and assessed the vasoconstriction with angiotensin-II, constriction was observed in both SHR and WKY, but the difference between them was not significant (Figure 6, upper panel). The change in the 340 nm/380 nm ratio ([Ca$^{2+}$]$_i$) in response to angiotensin-II 10$^{-9}$ mol/L was 3.0% ± 0.8% in SHR and 3.0% ± 0.6% in WKY, and the difference between them was not significant (Figure 6, bottom panel).
DISCUSSION

The principal finding in this study was not the increase in the Ca$^{2+}$ influx via VDCC in arterioles of SHR before the onset of hypertension, but that the onset of hypertension was associated with an increase in the Ca$^{2+}$ influx via VDCC. However, after the onset of hypertension, there were no significant differences from WKY in the SR function mediated by CICR or IP3-induced Ca$^{2+}$ release.

Voltage-Dependent Calcium Channels. The intravascular pressure was set at the same level for WKY and SHR in some previous studies that used similar in vitro systems. However, the arterial pressures of SHR differ from those of WKY, hence the in vivo pressure of the respective animals should be used to reproduce the physiologic conditions. Therefore,
based on the report of Bohlen et al., who described the direct measurement of in vivo pressure in the first-order artery of the cremaster muscle, we applied the pressure of 40 mm Hg to a resistance vessel in 4- to 5-week-old and 7- to 8-week-old WKY, and in 4- to 5-week-old SHR. We applied the pressure of 50 mm Hg in the 7- to 8-week-old SHR. However, there is the possibility that the differences between the rat strains in prehypertensive versus hypertensive states could relate to perfusion pressure. Therefore, we compared KCl concentration response curves between 7- to 8-week-old SHR and WKY using the same perfusion pressure (50 mm Hg). These data also showed that the KCl response was greater in SHR than in WKY.

A study on the femoral artery strips of SHR showed an increase in the KCl response. Moreover, although this study was performed on Day 1 to 3 azygos vein muscle in SHR, it has been reported that Ca^{2+} currents by whole-cell voltage clamping showed a greater proportion of L-currents than in WKY. In addition, in a study on the femoral artery, an increase in the Ca^{2+} influx was observed in 4-week-old SHR, in the early period of hypertension, whereas in a study on the mesenteric artery of 5-week-old SHR, potassium-induced contractions were similar in both SHR and WKY. The results of the later study were consistent with the data in the prehypertensive period in our own study. However, the results of our study showed an increase in the KCl contraction response after the onset of hypertension (7 to 8 weeks).
potential of the vascular smooth muscle in the resting state tended to be more depolarized in SHR than in WKY, and this depolarization may induce an increase in Ca\(^{2+}\) influx in the vascular smooth muscle of SHR and an associated increase in contraction. The increase in KCl response in SHR after the onset of hypertension in our study may also be attributed to the tendency toward depolarization of the vascular smooth muscle membrane potential in SHR. (However, the resting potential of the arterioles was not measured in this study.) Nevertheless, this is controversial because a report stated that there were no significant differences from WKY in the changes of the basal Ca\(^{2+}\) concentrations and norepinephrine- and K\(^{+}\)-induced Ca\(^{2+}\) concentrations in the mesenteric artery of 14- to 18-week-old SHR. Moreover, as the role of VDCC in the resistance arterioles of SHR during the pre- and early-hypertensive stages of hypertension and in age-matched WKY has not been previously investigated, this is the first report of the changes in VDCC in the resistance arterioles of SHR during those stages.

Because salt-sensitive hypertension in humans is associated with an exaggerated uptake of calcium from the extracellular space, it would be interesting to know whether the presence of a similar exaggerated extracellular calcium uptake is associated with salt sensitivity in our hypertensive model rats. However, our model has been characterized as salt-resistant SHR rats. Therefore, increased Ca influx in our SHR model is not associated with salt sensitivity.

**Sarcoplasmic Reticulum** Caffeine and ryanodine have been assessed in terms of CICR, but because caffeine interferes with the Ca\(^{2+}\) influx and there is an increase in cAMP and an intensification of the Ca pump activity, caffeine may not be suitable for investigating the SR function. Therefore, we did not use caffeine in this study.

It has been reported that ryanodine contraction is increased in the femoral artery of 13-week-old SHR compared with WKY, but that increase was attributed to the Ca\(^{2+}\) influx mediated by VDCC and was considered to be an extracellular Ca\(^{2+}\)-dependent contraction. However, in our study, ryanodine produced contractions even in the Ca\(^{2+}\)-free solution, and it seems very likely that Ca\(^{2+}\) release from SR plays an important role in ryanodine contraction. However, although at 7 to 8 weeks KCl contractions were larger in SHR, ryanodine contractions in SHR and WKY were not significantly different. In spite of this, Asano et al reported that ryanodine contractions in the femoral artery of 13-week-old SHR were increased compared with the contractions in WKY. Their results are consistent with those reported by Kojima et al. Moreover, although their study was conducted using aortic strips of SHRSP, Kanagy et al showed that caffeine contractions were increased in SHRSP compared with WKY, and they proposed that SR storage in SHRSP was greater. Toyoda et al demonstrated that the function of SR buffer was increased in SHR, and that it had a preventive effect against the progression of hypertension. However, there was no difference between SHR and WKY in the ryanodine contractions in the cremaster arteriole in our study. The difference from their results may be due to the methods of evaluating vasoconstriction, the size of the blood vessels, the site, or the age. Further studies are necessary to elucidate this point.

Upregulation of the IP3-receptor system has been observed in a study on the aorta. To investigate IP3-receptor-mediated SR function in our study, we assessed the α-1 AR response after reducing the extracellular Ca\(^{2+}\) concentration to 0. However, if the number and affinity of α-1 AR in the vascular smooth muscle of SHR in the early period of hypertension differed from their number and affinity in WKY, it would be difficult to evaluate the actual IP3-receptor-mediated function. However, because the dose-dependent contraction curve of the α-1 AR in the arteriole of SHR in the early period of hypertension was not significantly different from the curve in WKY (Figure 4), we could evaluate the IP3-receptor-mediated SR function. However, this IP3-receptor-mediated SR function after the onset of hypertension was not significantly different from that in WKY.

The contribution of SR to the modulation of [Ca\(^{2+}\)]\(_i\) is dependent on the size of SR, and is greater in the conduit vessels than in the resistance vessels. These data suggest that SR function in relation to the changes in Ca\(^{2+}\) concentration does not have a greater effect than VDCC on the arterioles. There may be differences in the mechanism of Ca\(^{2+}\) modulation in the arterioles as compared with the aorta and other large blood vessels. This may be an important reason for the difference between our data and those of previous studies that analyzed the differences in the function of SR in the SHR and WKY.

**Study Limitation** The blood vessels used in this experiment were the cremaster arterioles. These vessels have the advantage of being easy to dissect from the surrounding connective tissue, making it easy to obtain blood vessels having a suitable vessel diameter for use in this experiment. Because the cremaster arterioles have often been used in previous studies on the peripheral blood vessel responsiveness, we used them in this experiment. The SR function before the onset of hypertension should be investigated in further studies.

Measurements of Ca\(^{2+}\) signals were performed in intact arterioles. Therefore we could not distinguish Ca\(^{2+}\) signals of the vascular smooth muscle cells from...
those of nonvascular smooth muscle cells, endothelial cells, and adventitial cells. However, because endothelial cells do not have voltage-gated \( \text{Ca}^{2+} \) channels, we can neglect the \( \text{Ca}^{2+} \) signal from endothelial cells during KCl constriction. Moreover, because KCl concentration response curves in vitro showed the response of vascular smooth muscle cells, our \( \text{Ca}^{2+} \) signal data also depend mainly on vascular smooth muscle cells, but not endothelial or adventitial cells.

There is the possibility that some of the differences between the two strains could relate to the amount of Fura 2 gaining access to the cytosol or getting into intracellular organelles. This would alter the \( \text{Ca}^{2+} \) signal due to the \( \text{Ca}^{2+} \) buffering effect of Fura 2. Further studies are necessary to elucidate this point.

In conclusion, an increased influx of \( \text{Ca}^{2+} \) in the cell membrane, without a buffering effect of SR, was found to be associated with progression of the high blood pressure in the cremaster arterioles of SHR.

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