Enhanced $[\text{Ca}^{2+}]_i$ in renal arterial smooth muscle cells of pregnant rats with reduced uterine perfusion pressure

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Murphy, Jason G., Jason N. Herrington, Joey P. Granger, and Raouf A. Khalil. Enhanced $[\text{Ca}^{2+}]_i$ in renal arterial smooth muscle cells of pregnant rats with reduced uterine perfusion pressure. Am J Physiol Heart Circ Physiol 284: H393–H403, 2003. First published September 12, 2002; 10.1152/ajpheart.00247.2002.—Reduction of uterine perfusion pressure (RUPP) during late pregnancy has been suggested to trigger increases in renal vascular resistance and lead to hypertension of pregnancy. We investigated whether the increased renal vascular resistance associated with RUPP in late pregnancy reflects increases in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) and contraction of renal arterial smooth muscle. Single smooth muscle cells were isolated from renal interlobular arteries of normal pregnant Sprague-Dawley rats and a rat model of RUPP during late pregnancy. The cells were loaded with fura 2 and both cell length and $[\text{Ca}^{2+}]_i$ were measured. In cells of normal pregnant rats incubated in Hanks’ solution (1 mM $\text{Ca}^{2+}$), ANG II (10$^{-7}$ M) caused an initial increase in $[\text{Ca}^{2+}]_i$ to 414 ± 13 nM, a maintained increase to 149 ± 8 nM, and 21 ± 1% cell contraction. In RUPP rats, the initial ANG II-induced $[\text{Ca}^{2+}]_i$ (431 ± 18 nM) was not different from pregnant rats, but both the maintained $[\text{Ca}^{2+}]_i$ (225 ± 9 nM) and cell contraction (48 ± 2%) were increased. Membrane depolarization by 51 mM KCl and the $\text{Ca}^{2+}$-channel agonist BAY K 8644 (10$^{-6}$ M), which stimulate $\text{Ca}^{2+}$ entry from the extracellular space, caused maintained increases in $[\text{Ca}^{2+}]_i$ and cell contraction that were greater in RUPP rats than control pregnant rats. In $\text{Ca}^{2+}$-free (2 mM EGTA) Hanks’ solution, the ANG II- and caffeine (10 mM)-induced $[\text{Ca}^{2+}]_i$, transient and cell contraction were not different between normal pregnant and RUPP rats, suggesting no difference in $\text{Ca}^{2+}$ release from the intracellular stores. The enhanced maintained ANG II-, KCl- and BAY K 8644-induced $[\text{Ca}^{2+}]_i$, and cell contraction in RUPP rats compared with normal pregnant rats suggest enhanced $\text{Ca}^{2+}$ entry mechanisms of smooth muscle contraction in resistance renal arteries and may explain the increased renal vascular resistance associated with hypertension of pregnancy.

vascular resistance; hypertension; calcium; vascular smooth muscle; contraction

NORMAL PREGNANCY is often associated with increases in plasma volume and renal blood flow and decreases in systemic vascular resistance, renal vascular resistance, and arterial pressure (8, 11, 16, 19, 58). The changes in the systemic and renal hemodynamics during normal pregnancy have been attributed in part to increases in the expression of nitric oxide synthases and increased nitric oxide production by many cell types, including vascular and renal cells (1, 4, 7, 13, 40, 46, 56).

In 5–7% of pregnancies, women develop a condition called preeclampsia, which is characterized by increased intravascular coagulation, proteinuria, increased systemic and renal vascular resistance, and hypertension (21, 22, 35, 36, 41, 51). Although hypertension of pregnancy is a major cause of maternal and fetal morbidity and mortality, the exact mechanism of this disorder has not yet been clearly identified. Because of the difficulty of performing mechanistic studies in pregnant women, several animal models of hypertension of pregnancy have been developed (3, 15, 17, 20, 28, 38, 50). Studies (20, 38) in these animal models have led to the hypothesis that reduction in the uteroplacental blood flow and the ensuing placental ischemia and hypoxia during late pregnancy represent possible initiating events that eventually lead to increased systemic and renal vascular resistance and hypertension of pregnancy. In support of this hypothesis, we and others (3, 17, 22, 38) have found that reduction of uterine perfusion pressure (RUPP) in late pregnant rats and rabbits is associated with significant increases in renal vascular resistance and arterial pressure; however, the vascular and cellular mechanisms involved are unclear.

It is widely accepted that vascular smooth muscle contraction is triggered by increases in intracellular free $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) due to $\text{Ca}^{2+}$ release from the intracellular stores and $[\text{Ca}^{2+}]_i$ entry from the extracellular space (29, 32, 34). In addition, several $\text{Ca}^{2+}$-dependent protein kinases such as myosin light chain kinase and some protein kinase $\text{C}$ isoforms have been suggested to contribute to smooth muscle contraction (26, 27, 57). Previous studies (43) have shown that vascular smooth muscle contraction and $[\text{Ca}^{2+}]_i$ are

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 Protocol for RUPP. On day 14 of the pregnancy, pregnant rats destined to be in the RUPP group were anesthetized with isoflurane, the abdominal cavity was opened by a midline incision, the lower abdominal aorta was exposed, and a silver clip (0.23 mm ID) was placed around the aorta above the iliac bifurcation. This procedure has been shown to reduce uterine perfusion pressure in the gravid rat by ~40% (20). Because compensation of blood flow to the placenta occurs in pregnant rats through an adaptive increase in ovarian blood flow (47), a silver clip (0.1 mm ID) was also placed on the main uterine branches of both the right and left ovarian arteries. Control pregnant rats were sham operated. Arterial catheters were placed in the carotid artery for measurement of mean arterial pressure in conscious rats with the use of a pressure transducer (Cobe model CDX III, Sema; Birmingham, AL). The arterial pressure was measured on day 19 of pregnancy.

With the use of this protocol, the arterial pressure was 96 ± 2 mmHg in normal pregnant rats and was significantly increased (P = 0.001) to 126 ± 8 mmHg in RUPP rats. RUPP rats, in which the clipping procedure resulted in maternal death or total reabsorption of the fetuses, were excluded from the study. Some of the RUPP rats were allowed to deliver and were studied 3 days postpartum.

 Tissue preparation. On the day of the experiment (day 19 of pregnancy in normal pregnant and RUPP rats or the equivalent in virgin rats or 3-day postpartum rats), the rats were anesthetized by inhalation of isoflurane. The kidneys were rapidly removed and placed in oxygenated Krebs solution. The main branches of the right and left renal arteries were carefully dissected under microscopic visualization down to the interlobular renal arteries (≈150 μm diameter).

 Single cell isolation. Single renal arterial smooth muscle cells were freshly isolated with a gentle procedure, specifically avoiding aspiration through a pipette or centrifugation, as previously described (30, 42, 43). Interlobular renal arterial strips (50 mg) were placed in a siliconized flask containing a tissue digestion mixture of collagenase type II (236 U/mg protein activity, Worthington; Freehold, NJ), elastase grade II (3.25 U/mg protein activity; Boehringer-Mannheim; Indianapolis, IN), and trypsin inhibitor type II soybean (10, 000 U/ml, Sigma; St. Louis, MO) in 7.5 ml of Ca2+- and Mg2+-free Hanks’ solution supplemented with 30% bovine serum albumin (Sigma). Inclusion of albumin in the enzyme digestion medium preserves the response to agonists such as phenylephrine and ANG II in the isolated cells, suggesting that albumin may protect against excessive damage to the plasmalemmal receptors (28). The tissue was incubated three consecutive times in the tissue digestion mixture to yield three batches of cells. For batch 1, the tissue was incubated with 5 mg collagenase, 4 mg elastase, and 147 μl trypsin inhibitor for 60 min. For batches 2 and 3, the tissue was incubated with 2.5 mg collagenase, 4 mg elastase, and 122 μl trypsin inhibitor for an additional 30 min. The tissue preparation was placed in a shaking water bath at 34°C in an atmosphere of 95% O2-5% CO2. At the end of each incubation period, the preparation was rinsed with 12.5 ml of Hanks’ solution with albumin. The first batch containing digested endothelial cells, damaged smooth muscle, and other unwanted material was discarded. Cells from both batch 2 and 3 were used and were poured over glass coverslips placed in wells and cooled to 2°C. With the use of gravitational force, the cells were allowed to settle and adhere to the glass coverslips. Ca2+ was gradually added back to the preparation to avoid the “calcium paradox” (45).

 Cell contraction. Coverslips with the attached cells were placed on the stage of an inverted Nikon Diaphot-300 micro-

reduced in female compared with male rats. Also, studies (42) in female rats have shown that vascular smooth muscle contraction and [Ca2+]i are reduced in pregnant rats compared with virgin rats. However, whether the increased vascular resistance and arterial pressure in RUPP rats compared with normal pregnant rats involves alterations in vascular smooth muscle contraction and [Ca2+]i remains unclear.

Although a previous study (17) has suggested an enhancement of the vascular reactivity in RUPP rats, that study was performed on vascular strips of large conduit arteries such as the thoracic aorta, and therefore the findings of that study may not apply to the physiologically more relevant small resistance vessels. Of these resistance vessels, the changes in the small resistance renal vessels are of particular importance because they contribute not only to the total vascular resistance but also to the long-term renal control mechanisms of arterial pressure (1, 22, 39, 54). Also, because pregnancy may be associated with multiple changes in various types of vascular cells, studying the cellular mechanisms of pregnancy-associated changes in vascular resistance in a multicellular vascular preparation could be difficult and thus makes it important to measure [Ca2+]i in single vascular smooth muscle cells.

In the present study, we used a pregnant rat model with RUPP to test the central hypothesis that reduction in uterine perfusion pressure during late pregnancy is associated with increases in contraction and [Ca2+]i in resistance renal arterial smooth muscle cells. Experiments were designed to investigate the following: 1) whether the contraction of renal arterial smooth muscle cells is enhanced in RUPP rats compared with normal pregnant rats; 2) whether the enhanced contraction of renal arterial smooth muscle cells of RUPP rats reflect changes in [Ca2+]i; and 3) whether the changes in [Ca2+]i in renal arterial smooth muscle cells of RUPP rats are due to changes in Ca2+-release from the intracellular stores and/or Ca2+ entry from the extracellular space.

The resting cell length and basal [Ca2+]i and the changes in cell contraction and [Ca2+]i in response to activators with different mechanisms of action were measured and compared in single smooth muscle cells freshly isolated from renal interlobular arteries of normal pregnant and RUPP rats and loaded with the Ca2+-indicator fura 2. Contraction and [Ca2+]i were also measured in cells isolated from virgin rats as a reference and in cells of 3-day postpartum rats to demonstrate whether the changes in cell contraction and [Ca2+]i are reversible with delivery.

METHODS

Animals. Virgin and time-pregnant (day 12) Sprague-Dawley rats (12 wk of age) were purchased from Harlan Sprague Dawley, housed individually in the animal facility, and maintained on ad libitum standard rat chow and tap water in a 12:12-h light-dark cycle. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee at the University of Mississippi Medical Center and the American Physiological Society.
scope and viewed using a Nikon ×100 objective (total magnification ×1,000). The cells were bathed in 0.5 ml of Hanks’ solution that remained stationary during the data recording. Only viable, healthy, and spindle-shaped cells ≥40 μm in length were selected. Viable, healthy cells adhered to the glass coverslips and appeared bright, with a halo along the periphery, and without a visible nucleus when viewed with phase-contrast optics. The viability of the smooth muscle cells was confirmed by their exclusion of trypan blue (0.2%, Sigma) and their consistent and significant contraction in response to ANG II and KCl. Cell images were acquired with a PXL charge-coupled device camera and displayed on a personal computer (PC) with image analysis software (PMIS, Photometrics; Tucson, AZ). The number of pixels corresponding to the cell length in the cell image was transformed into micrometers using a calibration bar. Three different smooth muscle activators were used. ANG II was used to stimulate both Ca2+ release from the intracellular stores and Ca2+ entry from the extracellular space (5, 10). Caffeine was used to activate the Ca2+-induced Ca2+ release mechanism in Ca2+-free solution (34). Membrane depolarization by high-KCl solution caused the Ca2+ chelator BAY K 8644 to be used to activate Ca2+ entry from the extracellular space (31, 32). The changes in cell length in response to ANG II (10−7 M), caffeine (10 mM), KCl (51 mM), and BAY K 8644 (10−6 M) were consistently measured after 10 min of stimulation, and the magnitude of cell contraction was expressed as [(Lf − L)/Lf] × 100, where Lf is the initial cell length and L is the final cell length. All cell contraction measurements were made at 37°C.

Measurement of [Ca2+]i. [Ca2+]i was measured in fura 2-loaded single renal arterial smooth muscle cells using the ratio method, as previously described (29, 42, 43, 54, 62). The cells were incubated in the fura 2 loading solution for 30 min at 34°C. The loading solution was made of normal Hanks’ solution supplemented with 1 μM of the cell permeant fura 2-AM (Molecular Probes; Eugene, OR) and 0.01% Pluronic F-127 (Sigma). The fura 2-AM was diluted from a 1 mM stock solution in DMSO so that the final concentration of DMSO in the loading solution was 0.1%. The fura 2-loaded cells were washed twice and further incubated in normal Hanks solution washed for at least 30 min to allow complete deesterification of the dye. Nonspecific intracellular esterases hydrolyze the fura 2-AM esters and liberate the Ca2+-sensitive indicator fura 2 (25). Because of the photosensitivity of the fura 2 molecule, precautionary measures were taken throughout the procedure to avoid extensive photobleaching, and the excitation light was blocked by a shutter when no fluorescence measurements were recorded.

The fura 2-loaded cells were viewed through a Nikon CF Fluar ×100 oil immersion objective (numerical aperture 1.3) on an Nikon Diaphot-300 microscope. The Ca2+ indicator was excited alternately at 340 and 380 nm with a filter wheel that alternates between the two filters at a frequency of 0.5 Hz, i.e., a 2-s exposure period at each excitation filter. The emitted light was collected at 510 nm to a photomultiplier tube R928 (Ludl Electronic Products; Hawthorne, NY) through a pinhole aperture 1 μm in diameter positioned 1 μm from the plasma membrane and 1 μm from the nucleus. The fluorescent signal was digitized with a module (Mac 2000, Luddl) and analyzed on a PC with the use of Image-analysis software. The signal-to-noise ratio was improved by setting the Mac 2000 module to acquire and average eight consecutive fluorescent intensity readings from the photomultiplier tube during the 2-s exposure period at each excitation filter. The background signal was measured at the end of each experiment by treating the cells with digitonin (10 μM) to release the intracellular fura 2 and with MnCl2 (1 mM) to quench fura 2 fluorescence, followed by a normal Hanks’ solution wash. The background signal was subtracted from the recorded fluorescence signal. Spectral shifts that result from binding of the Ca2+ ion allow the fura 2 indicator to be used ratiometrically and thus make the measurement of [Ca2+]i less sensitive to changes in cell thickness or the membrane thickness loading the cell. Caffeine (10 mM) was used to induce Ca2+ release from the intracellular stores and Ca2+ entry from the extracellular space (5, 10). Caffeine was used to activate the Ca2+ release mechanism in Ca2+-free solution (34). Membrane depolarization by high-KCl solution caused the Ca2+ chelator BAY K 8644 to be used to activate Ca2+ entry from the extracellular space (31, 32). The changes in cell length in response to ANG II (10−7 M), caffeine (10 mM), KCl (51 mM), and BAY K 8644 (10−6 M) were consistently measured after 10 min of stimulation, and the magnitude of cell contraction was expressed as [(Lf − L)/Lf] × 100, where Lf is the initial cell length and L is the final cell length. All cell contraction measurements were made at 37°C.

**Results**

**Resting cell length and ANG II-induced contraction in renal arterial smooth muscle cells.** In resting cells of virgin rats, the average cell length was 57.5 ± 1.2 μm (Fig. 1A). The resting cell length was significantly longer in normal pregnant compared with virgin rats (P = 0.002), but significantly shorter in RUPP rats compared with pregnant rats (P < 0.001) or virgin rats.
and enhanced in RUPP rats compared with virgin rats. However, because the cells showed considerable variability in their initial length and magnitude of contraction, the cell contraction was normalized to the initial cell length. ANG II caused 32 ± 2.9% contraction in cells of virgin rats (Fig. 1B). The ANG II-induced cell contraction was significantly reduced in pregnant rats compared with virgin rats (P = 0.002), but significantly enhanced in RUPP rats compared with normal pregnant (P < 0.001) or virgin rats (P < 0.001) (Fig. 1B). The ANG II-induced cell contraction was not significantly different (P = 0.656) between 3-day postpartum rats and virgin rats (Fig. 1B).

Basal and ANG II-induced changes in [Ca\(^2+\)]\(_i\), in presence of external Ca\(^{2+}\). In cells of virgin rats, the basal [Ca\(^{2+}\)]\(_i\) was 86 ± 6 nM. ANG II caused a transient initial peak in [Ca\(^{2+}\)]\(_i\), followed by a steady-state increase that was maintained for at least 10 min (Fig. 2). The initial and maintained ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were abolished in cells treated with the ANG II AT\(_1\) receptor antagonist losartan (10\(^{-6}\) M) (Fig. 2A). Also, the ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were significantly inhibited in cells treated with the phospholipase C inhibitor neomycin (0.5 mM) (Fig. 2B) or U-73122 (10\(^{-5}\) M) (data not shown).

The sources of the ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were investigated. The maintained ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were abolished in Ca\(^{2+}\) Fig. 2. Effect of ANG II (10\(^{-7}\) M) on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in renal arterial smooth muscle cells of virgin rats. Single smooth muscle cells were incubated in normal Hanks' solution (1 mM Ca\(^{2+}\)). Data bars represent the means ± SE of measurements in 36–72 cells of 6–12 rats of each group. L\(_i\), initial cell length; L\(_f\), final cell length. *P < 0.05, significantly different from respective measurements in virgin rats; †P < 0.05, significantly different from respective measurements in normal pregnant rats.

(P < 0.001) (Fig. 1A). The resting cell length in 3-day postpartum rats was not significantly different (P = 0.65) from that in virgin rats (Fig. 1A).

ANG II (10\(^{-7}\) M) caused contraction in cells of all groups of rats. The absolute ANG II-induced contraction (in μm) appeared to be reduced in pregnant rats and enhanced in RUPP rats compared with virgin rats. However, because the cells showed considerable variability in their initial length and magnitude of contraction, the cell contraction was normalized to the initial cell length. ANG II caused 32 ± 2.9% contraction in cells of virgin rats (Fig. 1B). The ANG II-induced cell contraction was significantly reduced in pregnant rats compared with virgin rats (P = 0.002), but significantly enhanced in RUPP rats compared with normal pregnant (P < 0.001) or virgin rats (P < 0.001) (Fig. 1B). The ANG II-induced cell contraction was not significantly different (P = 0.656) between 3-day postpartum rats and virgin rats (Fig. 1B).

Basal and ANG II-induced changes in [Ca\(^{2+}\)]\(_i\), in presence of external Ca\(^{2+}\). In cells of virgin rats, the basal [Ca\(^{2+}\)]\(_i\) was 86 ± 6 nM. ANG II caused a transient initial peak in [Ca\(^{2+}\)]\(_i\), followed by a steady-state increase that was maintained for at least 10 min (Fig. 2). The initial and maintained ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were abolished in cells treated with the ANG II AT\(_1\) receptor antagonist losartan (10\(^{-6}\) M) (Fig. 2A). Also, the ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were significantly inhibited in cells treated with the phospholipase C inhibitor neomycin (0.5 mM) (Fig. 2B) or U-73122 (10\(^{-5}\) M) (data not shown).

The sources of the ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were investigated. The maintained ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were abolished in Ca\(^{2+}\)}
free (2 mM EGTA) Hanks’ solution (Fig. 3A) and in the presence of the Ca\(^{2+}\) channel blocker NiCl\(_2\) (1 mM) (Fig. 3B) or diltiazem (1 \(\mu\)M) (Fig. 3C), suggesting that the maintained increases in \([\text{Ca}^{2+}]_i\) are due to \Ca^{2+}\ entry from the extracellular space. On the other hand, the transient ANG II-induced increase in \([\text{Ca}^{2+}]_i\) was still present under these conditions (Fig. 3, right), suggesting that it is mainly due to \Ca^{2+}\ release from the intracellular stores.

The basal and ANG II-induced changes in \([\text{Ca}^{2+}]_i\) were compared in the different groups of rats. The basal \([\text{Ca}^{2+}]_i\) was significantly reduced in pregnant rats compared with virgin rats \((P = 0.013)\), but significantly elevated in RUPP rats when compared with normal pregnant \((P < 0.001)\) or virgin rats \((P = 0.013)\) (Fig. 4, A and B). The basal \([\text{Ca}^{2+}]_i\) was not significantly different \((P = 0.765)\) between 3-day postpartum rats and virgin rats (Fig. 4, A and B).

Fig. 3. Effect of removal of extracellular Ca\(^{2+}\) (A) and the Ca\(^{2+}\) entry blockers (B and C) on ANG II (10\(^{-7}\) M)-induced \([\text{Ca}^{2+}]_i\), in renal arterial smooth muscle cells of virgin rats. Cells were incubated in Hanks’ solution (1 mM Ca\(^{2+}\)) and then stimulated with ANG II. The bathing solution was rapidly changed to Ca\(^{2+}\)-free (2 mM EGTA) Hanks’ solution (A, left) or the cells were incubated in Ca\(^{2+}\)-free Hanks’ solution for 1 min before ANG II (A, right) was added. In other cells, the effect of topical application (left) or pretreatment of the cells (right) for 5 min with 1 mM NiCl\(_2\) (B) or 10 \(\mu\)M diltiazem (C) on the ANG II-induced changes in \([\text{Ca}^{2+}]_i\), was measured. Representative traces of experiments in 6 cells from 6 virgin rats are shown.

Fig. 4. Basal and ANG II (10\(^{-7}\) M)-induced transient and maintained \([\text{Ca}^{2+}]_i\), in renal arterial smooth muscle cells of virgin, pregnant, RUPP, and 3-day postpartum rats and incubated in Hanks’ solution (1 mM Ca\(^{2+}\)). The traces are representative of \([\text{Ca}^{2+}]_i\), measurements (A). Dashed lines were drawn at the smallest basal and maintained ANG II-induced increase in \([\text{Ca}^{2+}]_i\), recorded in cells of pregnant rats to facilitate comparison with the other groups of rats. Horizontal bars below the traces represent time of application of ANG II. The data bars represent the means \(\pm\) SE of the basal (B) and ANG II-induced initial peak \([\text{Ca}^{2+}]_i\), (C) and maintained \([\text{Ca}^{2+}]_i\), (D) in 12–24 cells from 6 to 12 rats of each group. *\(P < 0.05\), significantly different from respective measurements in virgin rats; †\(P < 0.05\), significantly different from respective measurements in normal pregnant rats.
($P = 0.004$), but significantly enhanced in RUPP rats when compared with normal pregnant ($P < 0.001$) or virgin rats ($P = 0.001$) (Fig. 4D), suggesting pregnancy-associated differences in Ca$^{2+}$ entry from the extracellular space. On the other hand, the maintained ANG II-induced [Ca$^{2+}$]$_{i}$ was not significantly different ($P = 0.724$) between 3-day postpartum rats and virgin rats (Fig. 4D).

**Cell contraction and [Ca$^{2+}$]$_{i}$ in Ca$^{2+}$-free medium.** To further investigate the role of the intracellular Ca$^{2+}$ release mechanisms, the ANG II response was measured in Ca$^{2+}$-free (2 mM EGTA) Hanks’ solution. In cells of virgin rats incubated in Ca$^{2+}$-free (2 mM EGTA) Hanks’ solution for 1 min, the basal [Ca$^{2+}$]$_{i}$ was reduced to 33 ± 2 nM, which was not significantly different from that in normal pregnant, RUPP and 3-day postpartum rats. Also, in cells of virgin rats, ANG II ($10^{-7}$ M) caused 10 ± 1.3% cell contraction (Fig. 5A) and a transient increase in [Ca$^{2+}$]$_{i}$ to 329 ± 15 (Fig. 5, B and C), which were not significantly different from the respective measurements in the other groups of rats. Similarly, in Ca$^{2+}$-free (2 mM EGTA) Hanks’ solution, caffeine (10 mM) caused 8 ± 1.2% cell contraction (Fig. 6A) and a transient increase in [Ca$^{2+}$]$_{i}$ to 434 ± 14 (Fig. 6, B and C) in cells of virgin rats, which were not significantly different from the respective measurements in 12 cells from 6 rats of each group. Horizontal bars below the traces represent time of application of caffeine.

**Effect of KCl and BAY K 8644.** Membrane depolarization by high KCl and the Ca$^{2+}$ channel agonist BAY K 8644 are known to stimulate Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels (29). In cells of virgin rats, KCl (51 mM) and BAY K 8644 ($10^{-6}$ M) caused significant cell contraction and increase in [Ca$^{2+}$]$_{i}$. Although the BAY K 8644 responses were slower in onset than KCl, the KCl- and BAY K 8644-induced contraction reached a plateau in ~5 min, and the increase in [Ca$^{2+}$]$_{i}$ was maintained for at least 10 min (Fig. 7). The KCl- and BAY K 8644-induced contraction and [Ca$^{2+}$]$_{i}$ were abolished by the nonselective Ca$^{2+}$ entry blocker NiCl$_2$ (1 mM) or the Ca$^{2+}$ channel blocker diltiazem ($10^{-6}$ M) (Fig. 7), suggesting that they are due to Ca$^{2+}$ entry from the extracellular space.

The average KCl-induced cell contraction and [Ca$^{2+}$]$_{i}$ after 5 min of stimulation were compared in the different groups of rats. KCl caused 31 ± 1.8% contrac-
Fig. 7. Effect of membrane depolarization by 51 mM KCl and the Ca\(^{2+}\) channel agonist BAY K 8644 on [Ca\(^{2+}\)], in renal arterial smooth muscle cells of virgin rats. Cells were stimulated with 51 mM KCl (A) or 10 \(^{-6}\) M BAY K 8644 (B), and the changes in [Ca\(^{2+}\)], were recorded. The effect of topical application (left) or pretreatment of the cells (right) for 5 min with 1 mM NiCl\(_2\) or 10 \(^{-6}\) M diltiazem on the KCl- and BAY K 8644-induced changes in [Ca\(^{2+}\)], was measured. Representative traces of experiments in 6 cells from 6 virgin rats are shown.

Fig. 8. Effect of 51 mM KCl on cell contraction (A) and [Ca\(^{2+}\)], in renal arterial smooth muscle cells of virgin, normal pregnant, RUPP, and 3-day postpartum rats. The traces are representative of cell contraction and [Ca\(^{2+}\)], measurements and the data bars represent the means ± SE of cell contraction and [Ca\(^{2+}\)], measurements in 12–24 cells from 6 to 12 rats of each group. Dashed lines were drawn at the smallest basal [Ca\(^{2+}\)]i; and 3) the increased [Ca\(^{2+}\)], in cells of RUPP rats compared with normal pregnant rats involves Ca\(^{2+}\) entry from the extracellular space but not Ca\(^{2+}\) release from the intracellular stores.

It has been suggested that the renin-angiotensin system is activated during normal pregnancy to maintain blood pressure. Although the plasma levels of renin and ANG II do not appear to be elevated in hypertensive pregnant women or in pregnant dogs or rats with reduced uteroplacental perfusion pressure, the vasopressor responses to ANG II appear to be increased in preeclamptic women and in rat models of “hypertension during pregnancy” produced by inhibition of NO synthesis (22). Nevertheless, little is known regarding the effects of ANG II on the mechanisms of

DISCUSSION

The main findings of the present study are the following: 1) renal arterial smooth muscle cell contraction is enhanced in RUPP rats compared with normal pregnant rats; 2) the enhanced renal arterial smooth muscle cell contraction in RUPP rats is associated with increases in [Ca\(^{2+}\)]; and 3) the increased [Ca\(^{2+}\)], in cells of RUPP rats compared with normal pregnant rats involves Ca\(^{2+}\) entry from the extracellular space but not Ca\(^{2+}\) release from the intracellular stores.
vascular smooth muscle contraction during normal pregnancy and during reduction of uterine perfusion pressure in late pregnancy.

The present study showed that the ANG II-induced contraction of renal arterial smooth muscle was reduced in pregnant rats when compared with virgin rats. The results are consistent with previous reports that the pressor response and vascular reactivity to vasoconstrictor stimuli are reduced during late pregnancy (31, 42, 44). Although the observed decrease in vascular smooth muscle reactivity to ANG II during late pregnancy can be explained by a decrease in the ANG II affinity to ANG II receptors, it could also be due to inhibition of signaling mechanisms downstream from ANG II receptor activation.

The interaction of an agonist such as ANG II with its receptor is known to activate phospholipase C and to increase the hydrolysis of phosphatidylinositol 4,5-bisphosphate into d-myoinositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and diacylglycerol (5, 9). Ins(1,4,5)P₃ stimulates Ca²⁺ release from the intracellular stores (59) and diacylglycerol stimulates protein kinase C (27, 48). In addition, ANG II may enhance Ca²⁺ entry through the plasma membrane Ca²⁺ channels (39, 55).

The observation that the ANG II-induced [Ca²⁺]ᵢ was abolished by the ANG II antagonist losartan suggests that the ANG II responses are mediated via ANG II receptors. Also, the significant inhibition of ANG II-induced [Ca²⁺]ᵢ by the phospholipase C inhibitors neomycin and U-73122 provides evidence that the ANG II responses involve activation of PLC and thereby the hydrolysis of plasma membrane phospholipids. Additionally, the observation that ANG II still causes an initial [Ca²⁺]ᵢ transient in Ca²⁺-free solution and in cells treated with the Ca²⁺ channel blockers NiCl₂ and diltiazem provides evidence that the initial peak [Ca²⁺]ᵢ is mainly due to Ca²⁺ release from the intracellular stores. On the other hand, the inhibition of the maintained ANG II-induced increase in [Ca²⁺]ᵢ in Ca²⁺-free solution or in cells treated with NiCl₂ or diltiazem provides evidence that the maintained increase in [Ca²⁺]ᵢ involves Ca²⁺ entry from the extracellular space.

The reduced smooth muscle cell contraction in pregnant rats does not appear to be due to changes in Ca²⁺ uptake to or Ca²⁺ release from intracellular stores because the ANG II- and caffeine-induced cell contraction and [Ca²⁺]ᵢ in Ca²⁺-free solution, which are often used as a measure of releasable intracellular Ca²⁺ stores, are not significantly different between pregnant and virgin rats. We should note that the initial ANG II response in normal Hanks’ solution appears to involve both Ca²⁺ release from the intracellular stores and Ca²⁺ influx from the extracellular space. This is supported by the observation that the ANG II-induced [Ca²⁺]ᵢ transient was smaller in Ca²⁺-free solution and in the presence of NiCl₂. We observed that the initial ANG II response in normal Hanks’ solution was slightly reduced in pregnant rats and slightly increased in RUPP rats compared with virgin rats; however, the changes did not reach significant levels (Fig. 4C). This may be related to the possibility that the initial ANG II response in normal Hanks’ solution is mainly due to Ca²⁺ release from intracellular stores. This is supported by the present observation that the ANG II-induced [Ca²⁺]ᵢ transient in Ca²⁺-free solution was >75% of the ANG II initial response in normal Hanks’ solution.

On the other hand, the reduced smooth muscle cell contraction in pregnant rats appears to involve Ca²⁺ entry from the extracellular space because the maintained ANG II-induced cell contraction and [Ca²⁺]ᵢ were reduced in pregnant rats compared with virgin rats. The present results with ANG II are consistent with previous reports (42) that the maintained phenylephrine-induced cell contraction and [Ca²⁺]ᵢ are reduced in cells of pregnant rats and provide further evidence that Ca²⁺ entry from the extracellular space is reduced during pregnancy.
To investigate the possible Ca\(^{2+}\) entry pathways involved, we compared the ANG II response with that induced by KCl and BAY K 8644. Membrane depolarization by high KCl and the Ca\(^{2+}\) channel agonist BAY K 8644 are known to stimulate Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (31, 32). The observations that the KCl- and BAY K 8644-induced cell contraction and [Ca\(^{2+}\)]\(_i\) are inhibited by the Ca\(^{2+}\) channel blockers NiCl\(_2\) and diltiazem support the contention that the KCl and BAY K 8644 responses involve Ca\(^{2+}\) entry from the extracellular space. Also, the KCl- and BAY K 8644-induced cell contraction and [Ca\(^{2+}\)]\(_i\) were reduced in normal pregnant rats compared with virgin rats, suggesting that Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels is reduced during pregnancy. The reduced Ca\(^{2+}\) entry from the extracellular space could be related to the possibility that either the Ca\(^{2+}\) permeability or the number of Ca\(^{2+}\) channels is reduced during pregnancy. However, the latter is more readily envisioned because the unitary conductance of an ion channel is not generally subject to physiological regulation or pathological alteration.

An increase in endogenous NO production by various cells including vascular endothelial during late pregnancy has been suggested to cause a decrease in vascular reactivity (40, 56) perhaps through increased formation of cGMP in vascular smooth muscle (12, 13), and cGMP has been shown to reduce smooth muscle [Ca\(^{2+}\)]\(_i\), by decreasing Ca\(^{2+}\) entry from the extracellular space (37, 52, 60). However, the pregnancy-associated changes in the endothelium-dependent NO and cGMP production are less likely involved in the present observations in isolated vascular smooth muscle cells. More likely mechanisms may involve dysregulation of Ca\(^{2+}\) channels in a manner that might alter their voltage-sensitivity, i.e., by changes in protein kinase C activity. Other alternative mechanisms may involve changes in the membrane potential due to upregulation of K\(^+\) channel activity/expression during normal pregnancy.

In contrast to the reduced vascular resistance and arterial pressure in pregnant rats, significant increases in vascular resistance and arterial pressure have been shown in the RUPP rats (3). The RUPP rats also show proteinuria as indicated by increased urinary protein excretion, impaired renal function as indicated by reduction in glomerular filtration rate and renal plasma flow, and decreased litter size and pup weight, as previously described (3,17). In search of the mechanisms of the increased vascular resistance during reduction of uterine perfusion pressure in late pregnancy, we (17) have previously found that the vascular reactivity is enhanced in aortic strips of RUPP rats compared with normal pregnant rats, and the increased vascular reactivity has been attributed in part to a reduction in endothelium-dependent vascular relaxation in the RUPP rats. However, in that study, the vascular reactivity was still greater in endothelium-denuded aortic strips of RUPP rats compared with normal pregnant rats, suggesting an additional endothelium-independent component of the enhanced vascular reactivity in RUPP rats (17). The present results in resistance renal arterial smooth muscle cells are consistent with the previous study in large aortic strips and further suggest that vascular smooth muscle contraction is enhanced in RUPP rats compared with normal pregnant rats.

Because [Ca\(^{2+}\)]\(_i\) is a major determinant of smooth muscle contraction, we investigated whether the enhanced vascular contraction in RUPP rats reflect changes in smooth muscle [Ca\(^{2+}\)]\(_i\). We found that the ANG II-induced [Ca\(^{2+}\)]\(_i\), was enhanced in RUPP rats compared with normal pregnant or virgin rats. The increased ANG II-induced cell contraction and [Ca\(^{2+}\)]\(_i\), in RUPP rats can be due to increased Ca\(^{2+}\) release from the intracellular stores and/or enhanced Ca\(^{2+}\) entry from the extracellular space. The ANG II- and caffeine-induced [Ca\(^{2+}\)]\(_i\), transient and contraction in Ca\(^{2+}\)-free solution were not significantly different between RUPP rats and pregnant rats, suggesting that the enhanced cell contraction in RUPP rats is not due to changes in Ca\(^{2+}\) uptake to or Ca\(^{2+}\) release from intracellular stores. On the other hand, the observed increase in the maintained ANG II-induced cell contraction and [Ca\(^{2+}\)]\(_i\) in RUPP rats compared with normal pregnant or virgin rats suggests that Ca\(^{2+}\) entry from the extracellular space may be stimulated. The enhanced Ca\(^{2+}\) entry in cells of RUPP rats could be related to an increase in the permeability/number of Ca\(^{2+}\) channels, possible alteration of the voltage sensitivity of the Ca\(^{2+}\) channels by changes in protein kinase C activity, or changes in the membrane potential due to possible downregulation of the K\(^+\) channels in RUPP rats.

The present results suggest that the enhanced cell contraction and [Ca\(^{2+}\)]\(_i\), in RUPP rats may involve stimulation of Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels because the cell contraction and [Ca\(^{2+}\)]\(_i\), in response to KCl and BAY K 8644, which activate the voltage-gated Ca\(^{2+}\) channels, were enhanced in RUPP rats compared with normal pregnant rats. However, the present results cannot exclude the possibility that other types of Ca\(^{2+}\) channels such as the receptor-operated Ca\(^{2+}\) channels (31) may also be involved in the enhanced ANG II-induced cell contraction in RUPP rats. Also, the enhanced ANG II contraction could be due to activation of other Ca\(^{2+}\)-dependent contraction mechanisms in addition to Ca\(^{2+}\) entry. For example, ANG II may activate specific Ca\(^{2+}\)-dependent protein kinase C isoforms through increased formation of diacylglycerol (24, 53).

The present study showed that in 3-day postpartum rats the ANG II-, KCl- and BAY K 8644-induced cell contraction and [Ca\(^{2+}\)]\(_i\), returned to levels not significantly different from those in virgin rats. These data lend support to the contention that the enhanced cell contraction and [Ca\(^{2+}\)]\(_i\), in RUPP rats are reversible on delivery, and thereby further suggest that the changes in [Ca\(^{2+}\)]\(_i\) are related to the reduction in uterine perfusion pressure during late pregnancy.

The question remains as of how a localized reduction in uterine perfusion pressure during late pregnancy...
would cause generalized enhancement of vascular con-
striction and [Ca^{2+}]_i, particularly in renal arterial
smooth muscle cells. It has been hypothesized that
reduction in the uteroplacental blood flow and the
ensuing placent al ischemia-hypoxia during late preg-
nancy may be associated with increased plasma levels
of cytokines, which may then cause generalized vaso-
constriction, increased vascular resistance, and arte-
rial pressure (14, 23, 33, 61, 63). This is supported by
reports (2, 18) indicating that chronic infusion of cyto-
kines such as tumor necrosis factor-α is associated with
increased vascular resistance and arterial pressure in
late pregnant rats. This is also supported by reports (6,
49) showing that tumor necrosis factor-α enhances
Ca^{2+}-dependent signaling mechanisms of smooth mus-
cle contraction.

Thus in renal arterial smooth muscle cells, the
changes in [Ca^{2+}]_i and contraction due to Ca^{2+} entry
from the extracellular space but not Ca^{2+} release from
the intracellular stores are enhanced in late pregnant
rats with reduced uterine perfusion pressure compared
with normal pregnant or virgin rats. The increased
renal arterial smooth muscle cell contraction and
[Ca^{2+}]_i; during reduction of uterine perfusion pressure
in late pregnancy may, in part, explain the increased
renal vascular resistance associated with hypertension
of pregnancy.

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