Enhancement of Intracellular Sodium by Vasopressin in Spontaneously Hypertensive Rats

Koji Okada, San-e Ishikawa, Toshikazu Saito

The arginine vasopressin-induced increase in intracellular sodium concentration was augmented in cultured rat vascular smooth muscle cells derived from 12-week-old spontaneously hypertensive rats (SHR) compared with those from 12-week-old normotensive Wistar-Kyoto (WKY) rats. This difference was enhanced by treatment with a Na⁺,K⁺-ATPase inhibitor, ouabain. The calcium-free state did not affect the basal intracellular sodium concentration but completely blocked the arginine vasopressin-induced increase in intracellular sodium concentration in both cell groups. The arginine vasopressin-mobilized cytosolic free calcium was enhanced in SHR compared with WKY rats. This enhancement was diminished but not completely inhibited in the calcium-free state. Also, arginine vasopressin-produced intracellular alkalization was augmented in SHR. Pretreatment of both cell groups with a calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, completely blocked arginine vasopressin-induced intracellular alkalization and increased intracellular sodium concentration. Scatchard analysis showed that the V₁ receptor number of either quiescent or proliferative cells of SHR was five to seven times greater than that of WKY rats, without any change in receptor affinity. These findings therefore indicate that the arginine vasopressin-induced increase in intracellular sodium concentration is augmented in vascular smooth muscle cells of SHR mediated through the enhancement of the mobilization of cytosolic free calcium and the activity of sodium-hydrogen exchange, which depends on an increase in V₁ receptor number. (Hypertension. 1993;22:300-305.)

In increases in cellular Ca²⁺ flux, intracellular sodium concentration ([Na⁺]), and vasoconstrictor hormone-induced mobilization of cytosolic free Ca²⁺ ([Ca²⁺]) have been shown in spontaneously hypertensive rats (SHR) compared with normotensive Wistar-Kyoto (WKY) rats.¹⁻² The cellular Na⁺ uptake and the activity of Na⁺,K⁺-ATPase are suggested to be augmented in vascular smooth muscle cells (VSMCs) of SHR compared with those of WKY rats.²⁻⁶ The angiotensin II-induced stimulation of Na⁺-H⁺ exchange, which is a major pathway for Na⁺ influx in VSMCs,² is greater in VSMCs of SHR than in those of WKY rats.⁸ Our previous study showed that arginine vasopressin (AVP)-induced increase in [Na⁺], in rat VSMCs is dependent on an increase in cellular Na⁺ uptake regulated by both the AVP-induced mobilization of [Ca²⁺], and the activity of Na⁺-H⁺ exchange.⁹ Taken together, the vasoconstrictor hormone-induced increase in [Na⁺], is suggested to be enhanced in SHR VSMCs when compared with WKY VSMCs, but little is known about the difference in the vasoconstrictor hormone-induced increase in [Na⁺], between the two groups of VSMCs.

The present study therefore was undertaken to determine whether there is any difference in the AVP-induced increase in [Na⁺], between VSMCs of SHR and WKY rats by the direct measurement of [Na⁺], with a fluorescence indicator dye, sodium-binding benzofuran isophthalate (SBFI). We also examined an AVP V₁ receptor analysis and the measurement of [Ca²⁺], mobilization, as well as changes in intracellular pH (pHᵢ), in response to AVP in VSMCs of both rat strains to explore the cellular mechanism.

Methods

Cell Culture

Rat VSMCs derived from SHR and WKY rats were isolated using the modified method of Chamley et al.⁹⁻¹² Briefly, rat thoracic aortas were dissected from eight SHR and WKY rats (12 weeks old) (Charles River Japan Inc, Kanagawa, Japan) and then incubated in Eagle’s minimum essential medium (MEM) (ICN/Flow Laboratories Inc, Costa Mesa, Calif) containing 2 mg/mL collagenase (Worthington Biochemical Corp, Freehold, NJ) for 1 hour at 37°C. After the removal of adventitia and small fragments of the outer membrane, the aortas were minced and incubated in Eagle’s MEM containing 2 mg/mL collagenase for 2 hours at 37°C. The freshly isolated cells were resuspended in Eagle’s MEM containing 1 μmol/L L-glutamine, 100 U/mL penicillin, 137 μmol/L streptomycin, and 10% fetal bovine serum (FBS), pH 7.4. The cells were kept in a humidified incubator at 37°C with 95% air and 5% CO₂. All experiments were done in subcultured cells from the third to 10th passages. The subculture was performed by trypsin (0.25%) treatment. Cells grown on thin glass...
slides (13 mm in diameter; Matsunami Kogyo Co, Osaka, Japan) were used for the measurements of [Na\(^+\)], [Ca\(^{2+}\)], and pH. Cells were subjected to the studies on days 7 through 10 of the subculture. The VSMCs of both SHR and WKY rats appeared morphologically similar under phase-contrast microscopy. The medium was changed to fresh Eagle’s MEM containing 10% FBS 1 day before the studies, except for the receptor study. The receptor study was performed using the cells of SHR and WKY rats preincubated with Eagle’s MEM in the presence or absence of 10% FBS for 24 hours to examine the difference between proliferative and quiescent cells.

Measurement of [Na\(^+\)],

The experimental procedure was similar to that used in our previous studies.\(^9\)-\(^13\)-\(^15\) Rat VSMCs were rinsed twice with physiological saline solution (PSS) (mmol/L: NaCl, 140; KCl, 4.6; MgCl\(_2\), 1; CaCl\(_2\), 2; glucose, 10; and HEPES, 10, pH 7.4) and loaded with 10 \(\mu\)mol/L SBFI acetoxymethyl esters (SBFI-AM; Molecular Probes, Inc, Eugene, Ore) for 2 hours at 37°C. SBFI-AM was dissolved in PSS containing 0.02% pluronic F-127, a nonionic surfactant. After loading, the cells on glass slides were rinsed with PSS and then placed in a 1×1-cm quartz cuvette with a special holder in a fluorescence spectrophotometer (CAF-100, Japan Spectroscopic Co, Tokyo, Japan). The complete hydrolysis of SBFI-AM was judged by changes in the excitation and emission spectra. The fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in the ratio mode. AVP (Sigma Chemical Co, St Louis, Mo) was added after a stable fluorescence signal (R) was achieved. From the ratio of fluorescence at 340 and 380 nm, [Na\(^+\)] was determined by the relation between the ratio and the authentic [Na\(^+\)].\(^9\)

Measurement of [Ca\(^{2+}\)],

This experimental procedure was similar to that described in our previous studies.\(^9\)-\(^13\)-\(^16\) Cells were loaded with PSS containing 5 \(\mu\)mol/L fura 2-AM (Dojin Biochemicals, Kumamoto, Japan) for 60 minutes at 37°C. After aspiration of the fura 2-AM solution, the glass slides were rinsed and then placed in a quartz cuvette at 37°C in a fluorescence spectrophotometer. The dual-wavelength excitation method for the measurement of fura 2 fluorescence was also used. Fluorescence was monitored at 500 nm, with excitation wavelengths of 340 and 380 nm in the ratio mode. AVP was added after incubation, cells were rinsed four times with 2 mL ice-cold binding buffer containing 10 mmol/L sodium dodecyl sulfate (SDS)-alkaline solution (1% SDS and 0.1 mol/L NaOH). Samples (0.1 mL) were stored at 4°C until the time of protein assay, and protein was measured by the method of Lowry et al.\(^9\) The radioactivity of the SDS-alkaline solution was counted with a liquid scintillation counter (Aloka LSC-671, Tokyo, Japan).

Cellular \(^{45}\text{Ca}^{2+}\) Release

The cellular \(^{45}\text{Ca}^{2+}\) release study was performed in confluent monolayer cells grown on 35×10-mm culture dishes according to our previous report.\(^11\) Cells were washed twice with 2 mL PSS and then incubated with 2 mL PSS containing 2 \(\mu\)Ci/mL \(^{45}\text{Ca}^{2+}\) (specific activity, 25.9 mCi/mg; New England Nuclear) for 60 minutes at 37°C. After incubation, the cells were rapidly rinsed 10 times during 1 minute with 2 mL PSS. The solution was placed into a counting vial and replaced with 2 mL PSS at 1-minute intervals for 6 minutes. PSS containing 10 mmol/L or 1 \(\mu\)mol/L AVP or vehicle was added at 6 minutes, and the samples were collected at 1-minute intervals for an additional 6 minutes. Cells were extracted with 2 mL SDS-alkaline solution, and then the radioactivity was counted with a liquid scintillation counter (Aloka LSC-671). The results are expressed as
FIG 1. Bar graph shows difference in peak levels of arginine vasopressin (AVP)–increased intracellular sodium concentration in vascular smooth muscle cells of spontaneously hypertensive rats (filled bars) and normotensive Wistar-Kyoto rats (open bars). Values are mean±SEM, n=4. *P<.05, **P<.01.

percent release from the resting intracellular 40Ca2+ counts at the measured times.

**Statistical Analysis**

Results are expressed as mean±SEM. The unpaired Student’s t test and an analysis of multiple variance using Scheffe’s method were used for statistical comparison. A value of P<.05 was considered significant.

### Results

**Difference in Arginine Vasopressin–Induced Increase in [Na+]i Between VSMCs of SHR and WKY Rats**

Our previous study has shown that AVP at a concentration of 1 nmol/L or higher increases [Na+]i in a dose-dependent manner in cultured rat VSMCs.9 [Na+]i gradually increased and reached maximum levels approximately 5 minutes after the addition of AVP, maintaining similar levels during at least the 10-minute observation period.9 In the present study, submaximal (10 nmol/L) and maximal (1 μmol/L) doses of AVP were used. The difference in the AVP-induced increase in [Na+]i, between VSMCs of SHR and WKY rats is shown in Fig 1. There was no difference in the basal [Na+]i level between the two groups of cells, but the AVP-induced increase in [Na+]i was greater in VSMCs of SHR than in those of WKY rats. This difference was enhanced by the treatment of both cell groups with 0.1 mmol/L ouabain for 10 minutes without any change in the basal [Na+]i levels (Table 1). Otherwise, a few minutes of exposure of both cell groups to Ca2+-free solution containing 0.1 mmol/L EGTA did not affect the basal [Na+]i but completely inhibited the AVP-induced (1 μmol/L) increase in [Na+]i (Table 1). Pretreatment of both cell groups with 50 μmol/L W7 for 10 minutes also totally blocked the AVP-induced increase in [Na+]i, without any change in the basal [Na+]i levels (Table 1). Similar results were obtained in the third to 10th passages of subcultured VSMCs derived from both SHR and WKY rats (data not shown).

**Effect of Arginine Vasopressin on Ca2+ Kinetics Between VSMCs of SHR and WKY Rats**

Our previous study has shown that AVP at a concentration of 1 nmol/L or higher also induces the mobilization of [Ca2+]i. The submaximal (10 nmol/L) and maximal (1 μmol/L) doses of AVP were also used in the present study. There was no difference in basal [Ca2+]i levels between both cell groups, but the AVP-induced mobilization of [Ca2+]i was greater in VSMCs of SHR than in those of WKY rats (Fig 2). Ca2+-free solution containing 0.1 mmol/L EGTA decreased the basal [Ca2+]i levels and the AVP-mobilized (1 μmol/L) [Ca2+]i in both cell groups (Fig 2). The enhancement of [Ca2+]i mobilization by AVP was still obtained in VSMCs derived from SHR under a Ca2+-free state. The effect of AVP on cellular 40Ca2+ release from VSMCs of SHR and WKY rats is shown in Table 2. The basal cellular 40Ca2+ release and AVP-increased cellular 40Ca2+ release were significantly enhanced in VSMCs of SHR compared with those of WKY rats.

**Difference in Arginine Vasopressin–Induced pH, Changes Between VSMCs of SHR and WKY Rats**

Our previous studies have shown that AVP induces a rapid and transient intracellular acidification, followed by sustained intracellular alkalization in rat VSMCs.9,12 The submaximal (10 nmol/L) and maximal (1 μmol/L) doses of AVP were used in the present studies. The difference in the AVP-induced pH changes between VSMCs of SHR and WKY rats is shown in Fig

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**Table 1. Interaction Between Arginine Vasopressin–Increased [Na+]i and Ouabain, Calcium-Free Condition, and W7 in Rat Vascular Smooth Muscle Cells**

<table>
<thead>
<tr>
<th></th>
<th>[Na+]i (mmol/L)</th>
<th>[Na+]i (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>AVP (1 μmol/L)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>12.1±0.3</td>
<td>28.1±1.6</td>
</tr>
<tr>
<td>WKY</td>
<td>11.9±0.4</td>
<td>36.2±2.9</td>
</tr>
<tr>
<td>SHR</td>
<td>12.7±0.7</td>
<td>35.4±1.7*</td>
</tr>
<tr>
<td>Ouabain (0.1 mmol/L)</td>
<td>13.0±1.2</td>
<td>47.5±2.8*</td>
</tr>
<tr>
<td>WKY</td>
<td>12.2±0.4</td>
<td>12.3±0.5*</td>
</tr>
<tr>
<td>SHR</td>
<td>12.8±0.5</td>
<td>12.0±0.6*</td>
</tr>
<tr>
<td>W7 (50 μmol/L)</td>
<td>12.5±0.7</td>
<td>12.4±0.3*</td>
</tr>
<tr>
<td>SHR</td>
<td>12.2±0.5</td>
<td>11.3±0.5*</td>
</tr>
</tbody>
</table>

[Na+]i, intracellular sodium concentration; AVP, arginine vasopressin; WKY, normotensive Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; [Ca2+]i, extracellular calcium concentration; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide. Values are mean±SEM, n=4. Pretreatment with ouabain enhanced augmented AVP-induced increase in [Na+]i in vascular smooth muscle cells of SHR, but the calcium-free state and preincubation with W7 completely blocked AVP-increased [Na+]i in both cell groups.

*P<.01 vs vehicle.
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3. The basal pH of SHR was similar to that of WKY rats (7.26±0.13 vs 7.33±0.13, n=6, NS). An intracellular acidification induced by 1 μmol/L AVP was significantly greater in VSMCs of SHR than in those of WKY rats. There was no difference in AVP-induced (10 nmol/L) intracellular acidification between the two cell groups.

The sustained cellular alkalinization was also markedly enhanced in VSMCs of SHR when compared with those of WKY rats. Pretreatment with 50 μmol/L W7 for 10 minutes completely blocked the AVP-produced (1 μmol/L) intracellular alkalinization but did not affect the basal pH (data not shown).

**Difference in Arginine Vasopressin V1 Receptor Between VSMCs of SHR and WKY Rats**

Scatchard analysis of [3H]AVP receptor binding using quiescent cells of SHR and WKY rats is shown in Fig 4. Cells were preincubated for 24 hours with fresh serum-free Eagle’s MEM before the start of experiments. The receptor number (Bmax) of SHR was 273 fmol/mg protein, which was greater than the 42 fmol/mg protein of WKY rats. There was no significant difference in Kd values between SHR and WKY rats (0.910 vs 0.909 nmol/L, n=3, NS). Similar results were obtained in the proliferative cells of SHR and WKY rats, which were preincubated with fresh Eagle’s MEM supplemented with 10% FBS for 24 hours before the start of experiments (Bmax, 74 vs 400 fmol/mg protein, n=3, P<.01; Kd, 0.435 vs 0.588 nmol/L, n=3, NS).

**Discussion**

In the present study we showed that the AVP-increased [Na+]i is enhanced in VSMCs of SHR compared with those of WKY rats by the fluorescence measurement of [Na+]i.91314 Because our previous study showed that the AVP-induced increase in [Na+]i is associated with an increase in cellular Na+ uptake regulated by the mobilization of [Ca2+], and the activity of Na+H+ exchange,9 the effects of AVP on the mobilization of [Ca2+], and the changes in pH, were examined to reveal the cellular mechanisms that produce the difference in the AVP-increased [Na+]i, between VSMCs of SHR and WKY rats.

First, we examined the AVP-induced mobilization of [Ca2+]i in both cell groups. There was no difference in av

**TABLE 2. Difference in Arginine Vasopressin-Produced Cellular [Ca2+]i Release Between Vascular Smooth Muscle Cells From Spontaneously Hypertensive and Wistar-Kyoto Rats**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>Vehicle</td>
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<td></td>
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</tr>
<tr>
<td>WKY</td>
<td>2.2±0.1</td>
<td>2.1±0.1</td>
<td>2.1±0.1</td>
<td>2.0±0.1</td>
<td>2.0±0.1</td>
<td>2.0±0.1</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>SHR</td>
<td>2.6±0.1*</td>
<td>2.6±0.2*</td>
<td>2.5±0.2*</td>
<td>2.4±0.2*</td>
<td>2.6±0.2*</td>
<td>2.5±0.2*</td>
<td>2.6±0.2*</td>
</tr>
<tr>
<td>AVP (10 nmol/L)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>WKY</td>
<td>2.3±0.1</td>
<td>11.8±0.6</td>
<td>12.3±0.5</td>
<td>5.3±0.1</td>
<td>3.4±0.1</td>
<td>2.6±0.1</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>SHR</td>
<td>2.6±0.2</td>
<td>18.1±0.3†</td>
<td>16.9±0.4†</td>
<td>7.4±0.1†</td>
<td>4.5±0.1†</td>
<td>3.7±0.1†</td>
<td>3.7±0.1†</td>
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<tr>
<td>AVP (1 μmol/L)</td>
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<tr>
<td>WKY</td>
<td>2.4±0.1</td>
<td>22.5±0.3</td>
<td>12.6±0.3</td>
<td>5.6±0.1</td>
<td>3.6±0.2</td>
<td>3.0±0.1</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>SHR</td>
<td>2.5±0.1</td>
<td>28.4±0.8†</td>
<td>15.9±0.1†</td>
<td>7.2±0.1†</td>
<td>4.9±0.1†</td>
<td>4.2±0.2†</td>
<td>3.7±0.1†</td>
</tr>
</tbody>
</table>

WKY, normotensive Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; AVP, arginine vasopressin. Values are mean±SEM, n=4. Cellular [Ca2+]i release is shown as percent release of resting intracellular [Ca2+]i contents; time shows time after addition of AVP. Basal and AVP-increased cellular [Ca2+]i releases were enhanced in vascular smooth muscle cells of SHR compared with those of WKY rats. *P<.05, †P<.01 vs WKY.
Intracellular Acidification Intracellular Alkalinization

0.2
0.1
0.0
-0.1
10 nmol/l AVP

10 nmol/l AVP

0.3
0.2
0.1
0.0
Bound AVP (fmol/mg protein)

FIG 3. Bar graphs show difference in arginine vasopressin (AVP)-induced changes in intracellular pH in vascular smooth muscle cells of spontaneously hypertensive rats (filled bars) and normotensive Wistar-Kyoto rats (open bars). Values are mean±SEM, n=4. *P<.05, **P<.01.

FIG 4. Scatchard analysis of arginine vasopressin (AVP) V1 receptor binding in quiescent vascular smooth muscle cells of spontaneously hypertensive rats (●) and normotensive Wistar-Kyoto rats (○). Values are means, n=3.
results from an increase in the mobilization of [Ca^{2+}], and the activity of Na^+-H^+ exchange, which depends on an increase in the B_{max} of AVP V_1 receptors.

In conclusion, we report the first observation that AVP-increased [Na^+]_i was augmented in VSMCs of SHR compared with those of WKY rats mediated through an increase in the mobilization of [Ca^{2+}], and the activity of Na^+-H^+ exchange. Such an enhancement depends on an increase in the B_{max} of AVP V_1 receptors, which enhanced intracellular signal transduction. The present findings provide new insights into the cellular mechanisms of hypertension in the SHR.

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Enhancement of intracellular sodium by vasopressin in spontaneously hypertensive rats.
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