Regulation of brain renin-angiotensin system by benzamil-blockable sodium channels

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1Department of Clinical and Laboratory Medicine, Kyoto Prefectural University of Medicine, Kyoto 602-0841; 2First Department of Internal Medicine, Shiga University of Medical Sciences, Shiga 520-2152; and 3Department of Clinical Sciences and Laboratory Medicine, Kansai Medical University, Osaka 570-0074, Japan

Nishimura, Masato, Ken Ohtsuka, Naoharu Iwai, Hakuo Takahashi, and Manabu Yoshimura. Regulation of brain renin-angiotensin system by benzamil-blockable sodium channels. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1416–R1424, 1999.—Changes in the renin-angiotensin system (RAS) mRNAs in the brain and the kidney of rats after administration of DOCA and/or sodium chloride were assessed by use of a competitive PCR method. Benzamil, a blocker of amiloride-sensitive sodium channels, was infused intracerebroventriculally or intravenously for 7 days in DOCA-salt or renal hypertensive rats, and the effects of benzamil on the brain RAS mRNAs were determined. Renin and ANG I-converting enzyme (ACE) mRNAs were not downregulated in the brain of rats administered DOCA and/or salt; however, these mRNAs were decreased in the kidney. Intracerebroventricular infusion of benzamil decreased renin, ACE, and ANG II type 1 receptor mRNAs in the brain of DOCA-salt hypertensive rats but not in the brain of renal hypertensive rats. The gene expression of the brain RAS, particularly renin and ACE, is regulated differently between the brain and the kidney in DOCA-salt hypertensive rats, and benzamil-blockable brain sodium channels may participate in the regulation of the brain RAS mRNAs.

The lesion of the anteroventral third ventricle that contains osmosensitive sites reportedly reduces hypertension in salt-sensitive hypertensive models such as DOCA-salt (39) and Dahl salt-sensitive hypertensive rats (12). These findings suggest that the mechanism that is involved in the perception of changes in brain sodium concentrations is important in the pressor mechanism of salt-sensitive hypertension. We have recently reported that brain sodium channels that are blocked by benzamil hydrochloride, one of the amiloride analogs and a specific inhibitor of amiloride-sensitive sodium channels, may be involved in the central pressor mechanisms of salt-sensitive hypertensive models such as DOCA-salt-treated and stroke-prone spontaneously hypertensive rats, possibly by participating in the regulation of the sympathetic nervous activity or AVP release (31). Amiloride-sensitive sodium channels reportedly play an important role not only in transmembrane transport of sodium but also in sodium taste transduction as a sodium receptor in the lingual epithelial cells (1, 14). Therefore, these benzamil-blockable brain sodium channels are expected to play a role as one of the brain sodium receptors that perceive the subtle changes in sodium concentration of cerebrospinal fluid or brain tissue after chronic sodium load in the salt-sensitive hypertensive models.

ANG II in the kidney affects urinary sodium excretion by regulating sodium reabsorption in the renal tubules, and brain ANG II is presumed to participate in the central cardiovascular regulation, including sympathetic nervous activity or AVP release (7, 8, 35, 38) via brain ANG II type 1 (AT1) receptor in rats (10). Therefore, the tissue renin-angiotensin system (RAS) in both the brain and the kidney is likely to play a role in salt-induced hypertension. Our earlier study showed that high sodium intake stimulated by renin gene expression in the hypothalamus and brain renin mRNA was not downregulated in DOCA-salt hypertensive rats, whereas it was markedly downregulated in the kidney (30). In addition, ANG II receptor was reported to be increased in the brain of DOCA-salt hypertensive rats (13). These findings suggest that gene expression of the RAS is regulated differently in the brain and the kidney and suggest that the brain RAS is activated more than the renal RAS in DOCA-salt hypertensive rats. It has not been clarified, however, how other components of the RAS, such as ANG I-converting enzyme (ACE) and angiotensinogen (AGT), are regulated in the brain and the kidney of DOCA-salt hypertensive rats.
In this study, we first compared the changes in expression of the RAS mRNAs induced by administration of either sodium chloride or DOCA between the brain and the kidney to investigate the differential regulation of the RAS mRNAs in these organs in DOCA-salt hypertensive rats. Second, we studied the effects of intracerebroventricular administration of benzamil on the brain RAS mRNAs in DOCA-salt hypertensive rats and renal hypertensive rats to clarify the involvement of benzamil-blockable brain sodium channels in the regulatory mechanism of the brain RAS mRNAs in salt-sensitive hypertensive models.

**METHODS**

We purchased 5-wk-old male Wistar rats (n = 73, 150-160 g) to make DOCA-salt hypertensive rats and 8 wk-old male Sprague-Dawley rats (n = 24, 165-195 g) to make renal hypertensive rats from Oriental Bio-Service Laboratory (Kyoto, Japan). Sprague-Dawley rats were used for making aortic-ligated renal hypertension because only Sprague-Dawley rats have been used for this method in the original study (36) and in other studies, including ours (5, 29). Rats were housed in plastic cages at a constant temperature (22°C) with a 12:12-h dark-light cycle. During the experiment, animals had free access to water and consumed a diet of rat chow (Oriental Bio-Service Laboratory). The experimental procedure was approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine.

Preparation of DOCA-salt hypertension. DOCA-salt hypertension was induced by treating unilaterally nephrectomized 5-wk-old male Wistar rats with 1% NaCl drinking water and with DOCA at a concentration of 150 mg/kg, administered via a subcutaneous Silastic (Toray Silicone, Tokyo, Japan) implant. To study the effects of administration of DOCA and/or salt on gene expressions of the RAS in the brain and kidney, rats were divided into four groups according to administration of either DOCA or Sham of DOCA and either 1% NaCl solution or distilled water. Rats were maintained for 6 wk (n = 6 for each group) and then killed by decapitation; 2 ml of blood were collected from each animal for measurement of plasma renin activity and creatinine concentration. Plasma renin activity was determined using an RIA to measure the level of ANG I generated (23). Plasma creatinine concentration was measured with an automatic analyzer (Ektachem 700 analyzer, Eastman Kodak, Rochester, NY). Systolic arterial pressure and pulse rate were measured by the tail-cuff method (MK-1000, Muromachi Kikai, Tokyo, Japan). Urine samples were collected during a 24-h period to measure the diurnal urinary excretion of free norepinephrine and AVP; urinary concentrations were measured by HPLC with electrochemical detection or RIA, as described previously (33). For benzamil hydrochloride or hydralazine administration experiments, rats administered with DOCA and 1% NaCl drinking water were used 6 wk after the treatment, at the age of 11 wk, when sustained hypertension developed. The methods are described in detail elsewhere (31).

Preparation of renal hypertension. Renal hypertension was induced by ligating the abdominal aorta between the right and left renal arteries of 8-wk-old male Sprague-Dawley rats. Four weeks after the aortic ligation, at the age of 12 wk, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and an arterial catheter (PE-50) filled with heparinized saline (50 U/ml) was inserted into the ascending aorta through the right carotid artery. The other end of the catheter was pulled through a cut in the skin on the back of the neck at the level of the cervical vertebrae. Arterial pressure was recorded for 10 min once a day by connecting the catheter tip to a small volume displacement pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan). Heart rate was automatically calculated by triggering the femoral arterial pulse pressure with a tachometer (AT-601G, Nihon Kohden). We used direct measurements of arterial pressure and heart rate in these renovascular hypertensive rats because it was difficult to measure them correctly by the tail-cuff method because the abdominal aorta was ligated. The methods are described in detail elsewhere (31).

Continuous benzamil infusion. Under anesthesia with pentobarbital sodium (50 mg/kg ip), rats were placed on a stereotaxic frame. The skin overlaying the midline of the skull was incised, and a small hole was drilled through the appropriate portion of the skull. An L-shaped infusion cannula (26 gauge, stainless steel tubing) was inserted stereotaxically into the right lateral cerebral ventricle (stereotaxic coordinates: +5.6 mm anteroposterior, +1.6 mm lateral, +2.0 mm dorsoventral, with the upper incisor bar set at 5 mm above the interaural line) and fixed to the skull with cyanoacrylate adhesive (Alon Alpha; Toa Gosei Chemical Industries, Tokyo, Japan). An osmotic minipump (Alzet model 2001; Alza, Palo Alto, CA), filled with benzamil hydrochloride or vehicle, was connected to the infusion cannula and implanted subcutaneously into the back of the body. Benzamil hydrochloride [DOCA-salt: 1 nmol·kg⁻¹·day⁻¹ (n = 6) and 10 nmol·kg⁻¹·day⁻¹ (n = 7); aortic ligation: 1 nmol·kg⁻¹·day⁻¹ (n = 6) and 10 nmol·kg⁻¹·day⁻¹ (n = 6)] or vehicle (1 μl/h for DOCA-salt (n = 6) and aortic ligation (n = 6)) was infused intracerebroventricularly for 7 days in DOCA-salt hypertensive rats and aortic-ligated renal hypertensive rats. To rule out the effect of possible leakage of the centrally administered drug into the peripheral blood, the cannula tip of an osmotic minipump containing benzamil hydrochloride (10 nmol·kg⁻¹·day⁻¹) was introduced into the right jugular vein and another osmotic minipump containing vehicle was also implanted for intracerebroventricular infusion (n = 6 rats for both DOCA-salt and aortic ligation experiments). After 24 h, urine samples were collected to measure the diurnal urinary excretion of free norepinephrine and AVP. At the end of the experiments, rats were anesthetized with ether inhalation and killed by decapitation, and 2 ml of blood were collected for the measurement of the plasma concentration of creatinine in DOCA-salt and aortic-ligated renal hypertensive rats and plasma renin activity in renal hypertensive rats.

Hydralazine administration. Either hydralazine (15 mg·kg⁻¹·day⁻¹, n = 6) or vehicle (n = 6) was administered by gavage for 7 days to DOCA-salt hypertensive rats. The hydralazine dose was found in a preliminary study to exert a similar hypotensive effect as intracerebroventricular infusion of benzamil (10 nmol·kg⁻¹·day⁻¹). For that study, 12 DOCA-salt hypertensive rats were divided into three groups of 4 rats each; the three groups received different hydralazine doses of 5, 15, or 25 mg·kg⁻¹·day⁻¹, respectively, for 2 wk. Systolic arterial pressure and pulse rate were measured by the tail-cuff method (MK-1000, Muromachi Kikai). After this procedure, a dose of 15 mg·kg⁻¹·day⁻¹ hydralazine was chosen because it had an antihypertensive effect similar to that of intracerebroventricular infusion of 10 nmol·kg⁻¹·day⁻¹ benzamil.

Isolation and analysis of RNA. Immediately after the decapitation, the brain (hypothalamus and lower brain stem) and the kidney were removed, frozen in dry ice, and stored at −80°C until extraction. Sample RNA was isolated by the
CsCl-guanidine thiocyanate method, as described previously (29). RNA was denatured with glyoxal and dimethylsulfoxide and electrophoresed on 1.1% agarose gel in 10 mM NaH₂PO₄- Na₂HPO₄ (pH 7.0). After deoxyxilumination by alkali, the gels were stained with ethidium bromide to confirm the quality of the sample RNA.

Quantitative analysis of the expression levels of the AT₁ (AT₁a + AT₁b) receptor mRNA was performed by using a competitive PCR method. The plasmid pSPORT-1, which contained a full-length cDNA fragment for the AT₁b receptor (16), was digested by restriction enzyme Msc I and then self-ligated. The resultant plasmid contained a cDNA fragment that lacked a region encompassing between nt 187 and nt 475. The deletion-mutated cRNA for the AT₁b receptor mRNA was synthesized from this plasmid by using SP6 RNA polymerase after the plasmid was linearized by restriction enzyme Hind III. Template plasmid DNA was completely digested by DNAse I, and degraded DNA and free nucleotides were removed by repeated washing in Centricon 30. The deletion-mutated cRNA for the AT₁b receptor mRNA was amplified in a total reaction mixture of 25 µl containing 1× PCR buffer [50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM MgCl₂, and 0.01% (wt/vol) gelatin], 0.2 mM dNTP, 50–100 nM PCR primer, 0.1 mM [γ-³²P]dCTP (3,000 Ci/mmol), 0.5 units Taq DNA polymerase (Stratagene, La Jolla, CA), and 25 pmol sense and antisense primers. The amounts of competitor cRNA used in this study are shown in Table 1. The resulting cDNA mixtures were purified by phenol-chloroform extraction and two rounds of ethanol precipitation with ammonium acetate and dissolved in 20 µl water. Five microliters of the cDNA mixture were digested by DNase I, and degraded DNA and free nucleotides were removed by repeated washing in Centricon 30. The digested cDNA was amplified in a total reaction mixture of 25 µl containing 1× PCR buffer [50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM MgCl₂, and 0.01% (wt/vol) gelatin], 0.2 mM dNTP, 50–100 nM PCR primer, 0.1 mM [γ-³²P]dCTP (3,000 Ci/mmol), 0.5 units Taq DNA polymerase (Stratagene, La Jolla, CA), and 25 pmol sense and antisense primers. Primer sequences and the sizes of the PCR products in each gene are shown in Table 1. Because these two primers correspond to the regions where no sequence divergence is noted between AT₁a and AT₁b receptors, the PCR product indicates AT₁ (AT₁a + AT₁b) receptor mRNA. The reaction profile included an initial denaturating step at 95°C for 1 min and 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. A trace amount of [α-³²P]-dCTP was included in the PCR reaction. Fragments of 607 and 419 bp could be synthesized by PCR from the native rat AT₁ receptor mRNA and the deletion-mutated cRNA, respectively (Table 1).

The gene expression levels of renin, ACE, and AGT mRNAs were determined by competitive RT-PCR methods as previously reported (18, 20, 30). The mutated cRNA for ACE has a 4-bp insertion at the Avr II site, the PCR product from the mutated cRNA lacks this Avr II site. The PCR product from native ACE mRNA should liberate 195- and 122-bp fragments by Avr II (TaKaRa Syuzo, Tokyo, Japan) digestion. The PCR amplification profiles included an initial denaturing step at 95°C for 1 min and 40 cycles at 95°C for 30 s, 52°C (renin) or 58°C (AGT, ACE) for 30 s, and 72°C for 1 min.

The PCR products were electrophoresed on a 2% agarose gel for visual inspection and a 5% polyacrylamide gel for precise quantitative analyses. The gel was dried at 80°C for 2 h with a gel dryer (model 543, Bio-Rad Laboratories, Richmond, CA) and exposed to XAR-5 X-ray film (Eastman Kodak) with two intensifying screens (Cronex, E. I. du Pont de Nemours, Wilmington, DE). Radioactive standards (ARC-146 ¹⁴C standard; American Radiolabeled Chemicals, St. Louis, MO) were included to determine relative optical densities. Several exposures were obtained to achieve optimal autoradiography signals that could be visualized within the range of optical gray densities provided by ¹⁴C standards. Autoradiographic signals were photographed by a DC 40 camera (Eastman Kodak), and fragment intensity was analyzed by a computer imaging system (BioMax 1D, Eastman Kodak). The expression levels of the mRNAs were calculated as follows:

expression level (molecules/µg) = amount of mutated cRNA (molecules) × (Cₙ/Cₜ) × (Cₜ/Cₚ), where Iₙ and Iₚ represent the intensity of the PCR product from native and mutated RNAs, respectively, and Cₚ and Cₜ represent the content of dCTP in the PCR product from native and mutated RNA, respectively. The details of this calculation were previously described (19, 20, 30).

Agents. Benzamid 3,5-diamino-[amino-(benzylamino)methylene]-6-chloropyrazin carbamoxylic acid hydrochloride; Research Biochemicals International, Natick, MA; dissolved in 10% propylene glycol and 0.9% saline, and the pH was adjusted to 7.5. The vehicle solution of benzamid was 0.9% saline with 10% propylene glycol (pH 7.5). Hydralazine (Sigma Chemical, St. Louis, MO) was diluted in 0.9% saline solution, and 0.9% saline was used as the vehicle of hydralazine.

Statistical analysis. Data are expressed as means ± SE. Differences between experimental and control groups were evaluated by one-way ANOVA and followed by application of Duncan’s new multiple-range test. P < 0.05 was accepted as statistically significant.

Table 1. PCR methods to detect renin-ANG system mRNAs

<table>
<thead>
<tr>
<th>Target</th>
<th>Competitor Amount, molecules/µg</th>
<th>PCR Product Size, bp</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin</td>
<td>4.1 × 10⁵ (1.0 × 10⁶)</td>
<td>372 (native)</td>
<td>5’-CTGGGAGGACCATGACACCTACTATACAG-3’</td>
</tr>
<tr>
<td>ACE</td>
<td>5.5 × 10⁴ (1.8 × 10⁵)</td>
<td>263 (competitor)</td>
<td>5’-GAGGCACGATGACGTAGCATGTCGTCC-3’</td>
</tr>
<tr>
<td>AT₁R</td>
<td>4.0 × 10⁴ (1.6 × 10⁵)</td>
<td>317 (native)</td>
<td>5’-CCTGACATACGAGGTTGGCCAG-3’</td>
</tr>
<tr>
<td>AGT</td>
<td>2.5 × 10⁴ (4.0 × 10⁵)</td>
<td>321 (competitor)</td>
<td>5’-GCCAGCTTCCGCCAGGAAAAGGACGC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>810 (native)</td>
<td>5’-GCCGCGGTTAATACTACCCCCCTTATCCTCC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>733 (competitor)</td>
<td>5’-GTCACCCAGGACTTCCGAGGCGCCATGTCG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>607 (native)</td>
<td>5’-GGAAAGCCATTGGTTGGTG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>593 (competitor)</td>
<td>5’-GCCAATCCGCTTATATCC-3’</td>
</tr>
</tbody>
</table>

ACE, ANG I-converting enzyme; AT₁R, angiotensinogen; AT₁,R, ANG II type-1 receptor. Amounts of competitor were used for determination of brain mRNA concentrations. Values in parentheses show amount of competitor used for determination of kidney mRNA concentrations.

*Sensitive to Avr II digestion. †Resistant to Avr II digestion.
RESULTS

Changes in arterial pressure, pulse rate, plasma renin activity, and urinary excretion of vasopressin and norepinephrine by the administration of DOCA and/or sodium chloride. The systolic arterial pressure was elevated significantly in rats treated with DOCA-water and DOCA-salt (Table 2). The pulse rate increased in the groups administered with salt, and the urinary excretions of AVP and norepinephrine were elevated in rats treated with DOCA-salt. The plasma renin activities were suppressed when DOCA or salt was administered, particularly in the DOCA-salt group compared with the control group.

Responses of gene expressions of the RAS in the brain and the kidney to DOCA and/or sodium chloride administration. Renin mRNA levels in the hypothalamus and the lower brain stem were not affected by the administration of DOCA and/or salt, whereas renin mRNA levels in the kidney were suppressed by the administration of DOCA and/or salt, compared with control rats administered with sham-DOCA and distilled water (Fig. 1A); these changes in renin mRNA levels in the kidney were consistent with those in plasma renin activity (Table 2). In the same manner as renin mRNA, ACE mRNA levels in the brain were not downregulated when DOCA and/or salt was administered and were upregulated in the lower brain stem in DOCA-salt-treated rats compared with control rats, although administration of DOCA and/or salt decreased ACE mRNA levels in the kidney (Table 2). In the same manner as renin mRNA, ACE mRNA levels in the brain were not downregulated when DOCA and/or salt was administered and were upregulated in the lower brain stem in DOCA-salt-treated rats compared with control rats, although administration of DOCA and/or salt decreased ACE mRNA levels in the kidney (Table 2).

Changes in arterial pressure, pulse rate, and urinary excretion of AVP and norepinephrine by intracerebroventricular infusion of benzamil. Continuous intracerebroventricular infusion of benzamil (1 or 10 nmol·kg·day⁻¹) for 7 days attenuated hypertension in DOCA-salt hypertensive rats; this was accompanied by reduction of urinary excretion of AVP and norepinephrine (Table 3). Intravenous infusion of benzamil (10

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Systolic Arterial Pressure, mmHg</th>
<th>Pulse Rate, beats/min</th>
<th>Plasma Renin Activity, ng ANG I·mL⁻¹·h⁻¹</th>
<th>Urinary AVP, ng·day⁻¹·g body wt⁻¹</th>
<th>Urinary Free Norepinephrine, pg·day⁻¹·g body wt⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (−), Na (−)</td>
<td>386 ± 10</td>
<td>155 ± 6</td>
<td>376 ± 6</td>
<td>2.2 ± 0.5</td>
<td>18 ± 2.3</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>D (+), Na (−)</td>
<td>364 ± 8</td>
<td>190 ± 8†</td>
<td>353 ± 19</td>
<td>0.8 ± 0.2†</td>
<td>29 ± 5.9</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>D (−), Na (+)</td>
<td>357 ± 12</td>
<td>164 ± 3</td>
<td>427 ± 16†</td>
<td>0.6 ± 0.3†</td>
<td>44 ± 9.4</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>D (+), Na (+)</td>
<td>295 ± 16†</td>
<td>200 ± 9†</td>
<td>438 ± 14†</td>
<td>0.2 ± 0.1†</td>
<td>234 ± 45.4†</td>
<td>3.4 ± 0.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for each group. D (+), with DOCA; D (−), sham DOCA; Na (+), 1% NaCl drinking water; Na (−), distilled water; AVP, arginine vasopressin. *P < 0.05, †P < 0.01 vs. D (−), Na (−) group.
nmol·kg⁻¹·day⁻¹) for 7 days affected neither arterial pressure nor urinary excretion of AVP and norepinephrine in this type of hypertension (Table 3). In aortic-ligated renal hypertensive rats (basal mean arterial pressure of 194 ± 8 mmHg, basal heart rate of 430 ± 11 beats/min, basal urinary excretion of AVP of 24 ± 4 pg·day⁻¹·g body wt⁻¹, basal urinary excretion of norepinephrine of 1.8 ± 0.6 ng·day⁻¹·g body wt⁻¹), a 7-day intracerebroventricular or intravenous infusion of benzamil (1 or 10 nmol·kg⁻¹·day⁻¹) did not affect mean arterial pressure, heart rate, or urinary excretion of AVP and norepinephrine (data are not shown). The plasma concentration of creatinine was within the normal range in all DOCA-salt hypertensive rats (0.3 ± 0.08 mg/dl) and renal hypertensive rats (0.4 ± 0.09 mg/dl). There was no significant difference in plasma renin activity in renal hypertensive rats (10 ± 3, 11 ± 3, 9 ± 2, and 10 ± 3 ng·ml⁻¹·h⁻¹ for vehicle, 1 nmol·kg⁻¹·day⁻¹ benzamil, 10 nmol·kg⁻¹·day⁻¹ benzamil, and 10 nmol·kg⁻¹·day⁻¹ intravenous benzamil, respectively).

Responses of gene expressions of the brain RAS to intracerebroventricular infusion of benzamil. In DOCA-salt hypertensive rats, continuous intracerebroventricular infusion of benzamil (1 or 10 nmol·kg⁻¹·day⁻¹) for 7 days lowered the expression levels of renin, ACE, and AT₁ receptor mRNAs in the hypothalamus and the lower brain stem almost to the levels of nonhypertensive control rats (sham-DOCA and distilled water) but elevated the expression levels of AGT mRNA (Figs. 4 and 5). Intravenous infusion of benzamil (10 nmol·kg⁻¹·day⁻¹) for 7 days did not affect the expression levels of the brain RAS mRNAs in DOCA-salt hypertensive rats (Figs. 4 and 5). On the other hand, gene expressions of renin, ACE, AGT, and AT₁ receptor were not affected by intracerebroventricular or intravenous infusion of benzamil in aortic-ligated renal hypertensive rats (data are not shown).

Effects of attenuation of hypertension by hydralazine on expression levels of the brain RAS mRNAs in DOCA-salt hypertensive rats. Oral administration of hydralazine (15 mg·kg⁻¹·day⁻¹) for 7 days decreased systolic arterial pressure in DOCA-salt hypertensive rats [202 ± 8 and 148 ± 7 mmHg for vehicle (n = 6) and hydrala-

![Fig. 2. Blots showing the effects of administration of DOCA (D) and/or sodium chloride (Na) on the expression of ACE (A) or ANG II type 1 receptor mRNA (AT₁R) (B) in the hypothalamus of rats. mut.ACE and del.AT₁R are competitor RNAs in competitive RT-PCR method.](image)

![Fig. 3. Changes in the expression of AT₁R mRNA (A) or angiotensinogen (AGT) mRNA (B) in the brain (hypothalamus and lower brain stem) and kidney induced by administration of DOCA (D) and/or sodium chloride (Na) in rats. *P < 0.05, **P < 0.01, compared with D (−), Na (−).](image)
Table 3. Responses of systolic arterial pressure, pulse rate, and urinary excretion of vasopressin and norepinephrine to a 7-day intracerebroventricular infusion of benzamil hydrochloride or vehicle and to intravenous infusion of benzamil in DOCA-salt hypertensive rats

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 6)</th>
<th>1 nmol·kg⁻¹·day⁻¹ ICV</th>
<th>10 nmol·kg⁻¹·day⁻¹ ICV</th>
<th>10 nmol·kg⁻¹·day⁻¹ ICV</th>
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<tr>
<td>Systolic AP, mmHg</td>
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<tr>
<td>Control</td>
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<td>195 ± 10</td>
<td>184 ± 8</td>
<td>194 ± 8</td>
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<tr>
<td>3rd Day</td>
<td>199 ± 9</td>
<td>163 ± 8*</td>
<td>152 ± 6†</td>
<td>198 ± 7</td>
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<tr>
<td>5th Day</td>
<td>195 ± 8</td>
<td>165 ± 6*</td>
<td>143 ± 5†</td>
<td>193 ± 9</td>
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</tr>
<tr>
<td>7th Day</td>
<td>203 ± 11</td>
<td>160 ± 8†</td>
<td>141 ± 6†</td>
<td>191 ± 12</td>
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<tr>
<td>Pulse rate, beats/min</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>417 ± 10</td>
<td>407 ± 11</td>
<td>404 ± 5</td>
<td>398 ± 9</td>
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<tr>
<td>3rd Day</td>
<td>400 ± 9</td>
<td>382 ± 13</td>
<td>377 ± 13</td>
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<tr>
<td>5th Day</td>
<td>398 ± 14</td>
<td>362 ± 13</td>
<td>346 ± 14*</td>
<td>391 ± 8</td>
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<tr>
<td>7th Day</td>
<td>401 ± 14</td>
<td>361 ± 16</td>
<td>359 ± 12*</td>
<td>390 ± 11</td>
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<td>Urinary AVP, pg·day⁻¹·g body wt⁻¹</td>
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<tr>
<td>Control</td>
<td>186 ± 14</td>
<td>187 ± 21</td>
<td>186 ± 38</td>
<td>195 ± 19</td>
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<tr>
<td>3rd Day</td>
<td>174 ± 21</td>
<td>129 ± 17</td>
<td>86 ± 14†</td>
<td>130 ± 29</td>
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<tr>
<td>5th Day</td>
<td>161 ± 19</td>
<td>126 ± 24</td>
<td>85 ± 8†</td>
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<tr>
<td>7th Day</td>
<td>155 ± 11</td>
<td>96 ± 18*</td>
<td>71 ± 10†</td>
<td>143 ± 20</td>
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<td>Urinary NE, ng·day⁻¹·g</td>
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<tr>
<td>Control</td>
<td>4.6 ± 1.1</td>
<td>4.9 ± 0.8</td>
<td>4.8 ± 0.6</td>
<td>6.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>3rd Day</td>
<td>6.1 ± 0.7</td>
<td>2.7 ± 0.4†</td>
<td>2.7 ± 0.4†</td>
<td>5.0 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>5th Day</td>
<td>6.5 ± 0.7</td>
<td>3.7 ± 0.7*</td>
<td>3.2 ± 0.3†</td>
<td>5.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>7th Day</td>
<td>5.7 ± 0.9</td>
<td>3.0 ± 0.4*</td>
<td>2.5 ± 0.6†</td>
<td>4.6 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. AP, arterial pressure; AVP, arginine vasopressin; NE, free norepinephrine. *P < 0.05, †P < 0.01, vs. the group of vehicle infusion.

DISCUSSION

We show in the present study that gene expressions of brain renin and ACE are not downregulated in rats administered with DOCA and/or salt; in particular, the ACE gene is upregulated by DOCA-salt treatment in the lower brain stem, whereas these mRNAs are markedly decreased in the kidney when DOCA and/or salt is administered. Gene expression of AT1 receptor is upregulated by DOCA-salt treatment in both the brain and kidney compared with control rats; however, the extent of upregulation of AT1 receptor mRNAs by DOCA-salt treatment seems to be greater in the brain than in the kidney. On the other hand, changes in brain AGT mRNA levels induced by DOCA-salt treatment are almost the same as those in renal AGT mRNA levels. These findings indicate that gene expressions of the RAS, particularly renin and ACE, are differently regulated in the brain and kidney, and the brain RAS is
expected to be activated more than the renal RAS in DOCA-salt hypertensive rats from the standpoint of gene expression.

Continuous intracerebroventricular infusion of benzamil decreased the mRNA levels of brain renin, ACE, and AT1 receptor of DOCA-salt hypertensive rats almost to the levels of normotensive control rats; however, brain AGT mRNA levels were increased. Because benzamil is a potent specific inhibitor of amiloride-sensitive sodium channels, these findings strongly suggest that benzamil-blockable brain sodium channels are involved in the regulation of the RAS mRNAs in the brain of DOCA-salt hypertensive rats. It is not clear from this study, however, how the benzamil blockade of brain sodium channels affects the regulation of the brain RAS mRNAs. The decrease in arterial pressure induced by intracerebroventricular infusion of benzamil is thought to have nothing to do with changes in the brain RAS mRNAs in DOCA-salt hypertensive rats because oral administration of hydralazine did not affect the brain RAS mRNA levels despite the attenuation of hypertension in DOCA-salt-treated rats. On the other hand, high sodium concentration is reported to increase the number of ANG II receptors in cultured neuronal cells of rats (6). This finding indicates that high sodium may stimulate the synthesis of ANG II receptors in neuronal cells via their sodium receptors. Benzamil-blockable brain sodium channels may act as one of the sodium receptors in the brain cells that produce the components of the RAS in DOCA-salt hypertensive rats. Further study is needed to clarify this point.

The effects of continuous intracerebroventricular administration of benzamil on the brain RAS mRNAs were completely different in DOCA-salt-treated and renal hypertensive rats. The precise mechanism responsible for this difference is not clear from this study; however, as a possible mechanism to explain this difference, the activity or density of benzamil-blockable amiloride-sensitive brain sodium channels may be different between these two hypertensive rats. The activity or density of amiloride-sensitive sodium channels is reportedly increased by humoral factors such as aldosterone or AVP (2, 9). Aldosterone activates “cryptic” sodium channels to functional ones by membrane methylation and increases sodium-potassium selectivity (42). AVP can insert activated sodium channels with high sodium selectivity into the cell membranes by increasing cAMP (26). DOCA that has a mineralocorticoid action is similar to aldosterone in its pharmacological effects, and AVP is increased in DOCA-salt hypertensive rats but not in renal hypertensive rats. Thus, in DOCA-salt hypertensive rats, the activity, density, or selectivity for sodium of amiloride-sensitive sodium channels is expected to be increased in the brain and other organs, compared with renal hypertensive rats, although we do not have direct evidence to prove this hypothesis.

We did not measure the brain ANG II concentration in this study. In a previous study that used immunocytohistochemical methods, brain ANG II levels were increased in DOCA-salt hypertensive rats compared with control rats (41). In peripheral organs or tissues, ANG II upregulates AGT mRNA (22, 27) but downregulates renin, ACE, and AT1 receptor mRNAs (22, 25). Brain ANG II is presumed to decrease renin gene expression in the brain (24). It is uncertain, however, how brain ANG II affects the gene expression of other components of the brain RAS. Administration of ACE inhibitors in spontaneously hypertensive rats reportedly decreases brain ANG II receptors (3, 28), most of which are AT1 receptors (10), and losartan, a nonpeptide AT1 receptor antagonist, reduces gene expression of brain AT1a receptor (17), which is a major subtype of AT1 receptor in the brain (36); i.e., brain ANG II may upregulate the expression of AT1 receptor mRNA in the brain, unlike in peripheral organs or tissues (17, 25). Thus it is difficult to explain changes in brain renin or AT1 receptor mRNA levels in DOCA-salt hypertensive rats by only the increase or decrease in brain ANG II concentration. Brain ANG II might be involved
in the regulation of the brain RAS mRNAs, but it is not likely to play a major role.

We have shown in a recent study that intracerebroventricular infusion of CV-1974, a nonpeptide AT$_1$ receptor antagonist, attenuates hypertension in DOCA-salt-treated rats, which is accompanied by reduction in urinary excretion of AVP but not norepinephrine (32). Therefore, the decrease in urinary excretion of AVP seen in intracerebroventricular infusion of benzamil may be derived from the downregulation of AT$_1$ receptor as well as of renin and ACE in the brain. However, further study is needed to determine whether the decrease in urinary norepinephrine seen in intracerebroventricular infusion of benzamil is derived from the downregulation of the brain RAS or from other mechanisms that need benzamil-blockable brain sodium channels.

Previous studies indicate that gene expression of AGT does not seem to be influenced by the blood pressure levels (29), ANG II (11, 22), or dexamethasone treatment (21) in both the brain and kidney. Renal AGT mRNA levels are reportedly decreased by a high-salt diet, compared with a low-salt diet (15); in our present study, AGT mRNA levels are also decreased by administration of salt or DOCA-salt in the brain and by that of DOCA and/or salt in the kidney. This downregulation of brain AGT mRNA in DOCA-salt hypertensive rats is abolished by intracerebroventricular administration of benzamil, and the brain AGT mRNA levels appear to be restored to the levels of control rats. These findings suggest that DOCA-salt treatment downregulates AGT mRNA in the brain and the kidney, and benzamil-blockable sodium channels are involved in this downregulation as a sodium receptor. The effects of benzamil on AGT mRNA levels in the kidney require further investigation.

Long-term administration of mineralocorticoid and/or salt suppresses the activities of circulating or tissue RAS in peripheral organs such as the kidney. In these circumstances with low plasma renin activities, a part of the mechanisms to regulate arterial pressure may switch to the brain RAS from the RAS in the peripheral tissues, and benzamil-blockable brain sodium channels are likely to play a role in the maintenance or the increase in the activity of the brain RAS, possibly as one of the brain sodium receptors. Benzamil-blockable brain sodium channels are presumed to be one of the key factors to connect salt intake with the brain RAS in DOCA-salt hypertensive rats.

Perspectives

The main cause of salt-sensitive hypertension has been ascribed only to the ability of sodium excretion from the kidney. Excessive salt intake and lowered sodium excretion elicit sodium retention in body fluid, and continued sodium retention stimulates the centrally mediated pressor mechanisms, such as the increase in sympathetic activity or AVP release in salt-sensitive hypertensive models. However, no precise mechanism has been determined to explain the pathway from sodium retention to the activation of these centrally mediated pressor mechanisms. The results obtained from this study suggest that the brain RAS is activated more than the renal RAS in DOCA-salt hypertensive rats, and benzamil-blockable brain sodium channels are involved in the pressor mechanism of this type of salt-sensitive hypertension by participating in regulation of the brain RAS mRNAs. Benzamil-blockable brain sodium channels are presumed to be an important factor in solving the mechanism of salt-sensitive hypertension.

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