Stimulation of brain Na\(^+\) channels by FMRFamide in Dahl SS and SR rats

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Wang, Hao, Roselyn White, and Frans H. H. Leenen. Stimulation of brain Na\(^+\) channels by FMRFamide in Dahl SS and SR rats. Am J Physiol Circ Physiol 285: H2013–H2018, 2003.—Stimulation of brain Na\(^+\) channels by Phe-Met-Arg-Phe-NH\(_2\) (FMRFamide) increases sympathetic nerve activity and blood pressure (BP) in Wistar rats. Blockade of brain ouabain-like compounds (OLC) by specific antibody Fab fragments prevents these responses to intracerebroventricular FMRFamide. In the present study, we evaluated the effects of high-salt intake on brain FMRFamide levels and the responses of BP and brain OLC to intracerebroventricular infusion of FMRFamide in Dahl salt-sensitive (SS) and salt-resistant (SR) rats. FMRFamide and OLC content was measured with the use of RIA and ELISA, respectively. A high-salt diet (1,370 μmol Na\(^+\)/g) for 2 wk significantly increased BP in Dahl SS but not in SR rats. On a regular salt diet, Dahl SS and SR rats showed similar FMRFamide levels in the whole hypothalamus, pons and medulla, and spinal cord. A high-salt diet for 2 wk did not affect FMRFamide levels in these tissues in both Dahl SS and SR rats. In Dahl SS but not in SR rats, chronic intracerebroventricular infusion of FMRFamide (200 nmol·kg\(^{-1}\)·day\(^{-1}\)) for 2 wk significantly increased BP (mean arterial pressure: 116 ± 5 vs. 100 ± 2 mmHg; \(P < 0.01\)). Chronic intracerebroventricular infusion of FMRFamide significantly increased hypothalamic and pituitary OLC in Dahl SS but not SR rats. These results indicate that Dahl SS rats exhibit enhanced central responses to FMRFamide. In Dahl SS but not in SR rats on a high-salt diet, enhanced Na\(^+\) entry through FMRFamide-activated brain Na\(^+\) channels may increase brain OLC release, thereby leading to hypertension.

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METHODS

Male 4-wk-old Dahl SS and SR rats (Harlan Sprague Dawley, Indianapolis, IN) were housed two per cage in a climatized room on a 12:12-h light-dark cycle at constant room temperature and humidity, and given a diet of standard laboratory chow and tap water ad libitum for 1 wk before entering the study. All procedures were carried out according to the guidelines of the University of Ottawa Animal Committee for the Use and Care of Laboratory Animals.

Experiment 1. FMRFamide Levels in Dahl SS and SR Rats on Regular or High-Salt Diet

Dahl SS and SR rats were placed on either a regular or high-salt diet (rat chow containing either 120 or 1,370 pmol Na+/g, Harlan Sprague Dawley; Madison, WI). Diets were provided for 2 wk, and after measurement of BP (see Arterial BP and heart rate measurements) rats were euthanized by decapitation. The brains and spinal cords were removed and FMRFamide was extracted according to the method of Magjane et al. (21) with some modifications. Briefly, the tissues were put into chilled tubes containing 10 vol of 1 N acetic acid with 0.02 N hydrochloric acid, boiled for 10 min, and then homogenized. The homogenate was centrifuged, the pellet was washed with the same acid mixture, and the two pooled supernatants for each sample were applied to a preconditioned Waters Sep-Pak C18 cartridge. The peptides were eluted with 3 ml 60% acetonitrile in 0.1% trifluoroacetic acid. Phosphate buffer (250 µl, 0.1 M, pH 7.4) and 100 µl 10% glycerol were added to all tubes, which were then dried under vacuum at ambient temperature. Glycerol (100 µl 10%) and acetonitrile (3 ml 60% in 0.1% trifluoroacetic acid) were added to 250 µl aliquots of each standard (diluted in phosphate buffer) which were then dried with the samples. For RIA, all tubes were reconstituted with 250-µl RIA buffer, and transferred to 1.5-ml microtubes for centrifugation at 13,000 revolutions/min for 10 min. A duplicate of the supernatant (100 µl) for each standard and sample was taken for RIA. FMRFamide-specific antibody (100 µl; catalog no. RAS 8755, Peninsula Laboratories) was added to all tubes (except the total counts), followed by 100 µl 125I-labeled YMFamide (Washington State University Peptide Radiodetection Center) containing ~15,000 counts/min and the tubes were then incubated overnight at 4°C. Dextran-coated charcoal (700 µl; 0.6%) was added to all tubes, followed by centrifugation at 3,000 revolutions/min for 30 min at 4°C. The supernatant containing the bound fraction was counted for 3 min with the use of a gamma counter (AutoGamma Cobra II, Canberra-Packard), and the amount of peptide was determined from the standard curve. Extraction efficiency was measured by spiking tissue homogenates with known amounts of labeled peptide. The average recovery was found to be 80%, and the sensitivity of the RIA measurement was 0.25–0.5 pg/tube (2 fmol/sample).

Experiment 2. Stability Testing for FMRFamide

To test the stability of FMRFamide in different conditions, the peptide was dissolved in several different vehicles: artificial cerebrospinal fluid (aCSF) pH 7.4, aCSF with bovine serum albumin (BSA; 0.1, 0.5, and 1.0%) pH 7.4, aCSF with ascorbic acid (0.3 and 0.6 mM) pH 7.4, aCSF with pH 5.5 (adjusted with HCl, without BSA or ascorbic acid). These solutions were kept at room temperature or at 37°C for 1 wk, and aliquots were taken at 1- to 2-day intervals for RIA. FMRFamide dissolved in aCSF with pH 7.4 and stored in −20°C was used as control, which was found to be stable for at least 1 yr. Figure 1 shows relevant data. In the vehicle of aCSF with pH 7.4, no more FMRFamide was detectable after 24 h at room temperature or at 37°C. BSA increased the stability slightly, but the peptide was decreased to almost undetectable level within 48 h at room temperature or at 37°C. Ascorbic acid did not increase its stability at all. However, in aCSF with pH 5.5 (without BSA or ascorbic acid), FMRFamide remained at nearly the same level at room temperature or at 37°C for 7 days compared with levels at −20°C.

Experiment 3. Effects of Intracerebroventricular Infusion of FMRFamide on BP, Heart Rate, and OLC in Dahl SS and SR rats

Intracerebroventricular cannulation and infusion of drugs. Under halothane anesthesia and with the use of a stereotaxic frame (Harvard Apparatus), a 23-gauge right-angled stainless-steel cannula was implanted into the left lateral cerebral ventricle and fixed to the skull of the rats with acrylic cement. The cannula was placed 0.4 mm posterior and 1.4 mm lateral to the bregma. The lower end (shorter arm) of the cannula was at a depth of 3.5 mm from the dura, and the upper end (longer arm) was connected to an osmotic minipump (model 2002, Alza; Palo Alto, CA) for intracerebroventricular infusion at 0.5 µl/h for 14 days. Considering the secretion rate of CSF in rats (120–320 µl/h) (3), this low rate of intracerebroventricular infusion is unlikely to affect CSF pressure. For both Dahl SS and SR rats, the pumps were filled with vehicle (aCSF with pH 5.5, n = 8 in Dahl SS and n = 7 in SR rats) or FMRFamide (200 nmol·kg⁻¹·day⁻¹, pH 5.5, n = 8 in Dahl SS and n = 7 in SR rats) and implanted subcutaneously on the back of the rats. The animals were then returned to their original cages and given regular food and water.

Arterial BP and heart rate measurements. Thirteen days after the intracerebroventricular cannulation, carotid arterial cannulation was performed under halothane anesthesia. A polyethylene-50 catheter filled with heparinized saline (100 IU/ml) was inserted into the left carotid artery. The next morning, the carotid arterial catheters were connected to pressure transducers. The rats were allowed to rest for ~30 min, and then BP and heart rate were recorded for 30 min with an on-line computer equipped with AcqKnowledge for Windows (Biopac Systems). After the BP and heart rate measurements, blood was withdrawn from the arterial line in conscious rats, placed into an ice-chilled tube containing EDTA, and centrifuged at 3,000 g for 10 min. Plasma was
separated, and electrolytes were determined with the use of an ion-selective electrode (model 917, Hitachi).

ELISA for OLC. Endogenous OLCs were extracted from the brain regions and plasma and the levels quantified by ELISA (31, 32). Briefly, tissue/plasma samples or ouabain standards and rabbit anti-ouabain antiserum were added to each well in an ovalbumin-ouabain-coated enzyme immunoassay plate. Plates were then incubated at 37°C for 2 h and then rinsed. Anti-ouabain antibodies remaining bound to the ovalbumin-ouabain were reacted with goat anti-rabbit IgG-peroxidase conjugate (Sigma) for 1 h at 37°C. After being washed, the peroxidase enzyme remaining in each well was determined by the addition of 3,3'-5,5'-tetramethylbenzidine base substrate solution. The absorbance in each well was measured at 450 nm with the use of a microplate recorder. The concentration of OLCs in each sample was calculated from the reduction in absorbance according to the ouabain standard curve.

The anti-ouabain antibody was raised from rabbits immunized with the commercially available cardenolide ouabain conjugated with BSA. This antibody has a high-antibody titer (1:1.6 × 10⁶), full cross reactivity with ouabain, 8% cross reactivity with digoxin, and minimal cross reactivity with numerous common endogenous steroids and peptides (32). In addition, the OLC is eluted with 25% acetonitrile in water from Sep-Pak C₁₈ cartridges and common adrenocortical steroids such as corticosterone or aldosterone are too hydrophobic to be eluted at this acetonitrile concentration.

Statistical Analysis

Values are expressed as means ± SE. Differences between groups were evaluated by two-way ANOVA, followed by Newman-Keuls post hoc multiple comparison. The level of significance was set at a value of $P < 0.05$.

RESULTS

**FMRFamide Levels in Dahl SS and SR Rats on Regular or High-Sodium Diet**

High salt intake for 2 wk significantly increased BP in Dahl SS but not in Dahl SR rats (Table 1). On regular salt diet, FMRFamide levels were similar in the whole hypothalamus, pons and medulla, and spinal cord of Dahl SS and SR rats. FMRFamide levels in the hypothalamus were higher than in other tissues. A high-salt diet for 2 wk did not affect FMRFamide levels in these tissues in either Dahl SS or SR rats (Table 1).

**Responses to Intracerebroventricular Infusion of FMRFamide in Dahl SS and SR Rats**

All rats developed normally over the duration of study. No differences in body weight gain (Table 2) and food and water intake (data not shown) were observed among rats receiving different treatments.

Figure 2 shows the responses of BP to intracerebroventricular infusion of FMRFamide for 2 wk in Dahl SS and SR rats. In Dahl SS rats, chronic intracerebroventricular infusion of FMRFamide in aCSF with pH 5.5 for 2 wk significantly increased MAP by 16 mmHg (116 ± 5 vs. 100 ± 2 mmHg; $P < 0.01$). However, the same intracerebroventricular infusion of FMRFamide did not change BP in Dahl SR rats. Intracerebroventricular infusion of FMRFamide did not change heart rate in either Dahl SS or SR rats (Table 2).

Figure 3 shows the responses of brain and peripheral OLC levels to intracerebroventricular infusion of

<table>
<thead>
<tr>
<th>Dahl SS</th>
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</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>MAP, mmHg</td>
<td>HR, beats/min</td>
<td></td>
</tr>
<tr>
<td>Regular (Reg)</td>
<td>8</td>
<td>107 ± 3</td>
<td>467 ± 12</td>
</tr>
<tr>
<td>High</td>
<td>8</td>
<td>122 ± 5*</td>
<td>483 ± 17</td>
</tr>
<tr>
<td>Dahl SR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular (Reg)</td>
<td>7</td>
<td>100 ± 2</td>
<td>414 ± 12</td>
</tr>
<tr>
<td>High</td>
<td>7</td>
<td>96 ± 4</td>
<td>447 ± 13</td>
</tr>
</tbody>
</table>

Values are means ± SE. HR, heart rate; MAP, mean arterial pressure; Dahl SS, Dahl salt-sensitive rats; Dahl SR, Dahl salt-resistant rats; Reg, regular salt diet (120 μmol Na⁺/g food); High, high-salt diet (1,370 μmol Na⁺/g food). *$P < 0.05$ vs. regular diet in the same strain.

**FMRFamide Levels, pmol/g tissue**

<table>
<thead>
<tr>
<th></th>
<th>Hypothalamus</th>
<th>Pons + medulla</th>
<th>Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.35 ± 0.03</td>
<td>0.23 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.36 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.38 ± 0.04</td>
<td>0.26 ± 0.02</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.36 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

Table 2. Effects of intracerebroventricular infusion of FMRFamide for 2 wk on body weight gain, heart rate, and plasma electrolytes in Dahl SS and Dahl SR rats

<table>
<thead>
<tr>
<th></th>
<th>Body Weight Gain, g</th>
<th>HR, beats/min</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl SS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>8</td>
<td>67 ± 3</td>
<td>465 ± 20</td>
<td>144 ± 1</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>FMRF</td>
<td>8</td>
<td>59 ± 4</td>
<td>463 ± 22</td>
<td>141 ± 1</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Dahl SR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>7</td>
<td>64 ± 4</td>
<td>424 ± 22</td>
<td>145 ± 1</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>FMRF</td>
<td>7</td>
<td>61 ± 4</td>
<td>409 ± 21</td>
<td>141 ± 1</td>
<td>4.3 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Veh, intracerebroventricular (icv) infusion of vehicle; FMRF, icv infusion of FMRFamide (200 nmol·kg⁻¹·day⁻¹).
FMRFamide for 2 wk in Dahl SS and SR rats. Chronic intracerebroventricular infusion of FMRFamide in vehicle with pH 5.5 increased hypothalamic and pituitary OLC by 60–70% in Dahl SS rats. There were no changes of adrenal or plasma OLC levels by intracerebroventricular FMRFamide in Dahl SS rats. In Dahl SR rats, chronic intracerebroventricular infusion of FMRFamide did not affect either brain or peripheral OLC levels.

Intracerebroventricular infusion of FMRFamide with vehicle of pH 5.5 for 2 wk did not change plasma Na\(^+\), K\(^+\), or Cl\(^-\) concentrations in Dahl SS or SR rats (Table 2).

**DISCUSSION**

In the present study, no differences in FMRFamide levels were found in the whole hypothalamus, pons and medulla, and spinal cord between Dahl SS and SR rats on a regular salt diet. A high-salt diet for 2 wk did not affect brain FMRFamide levels in Dahl SS or SR rats.

In contrast, responsiveness to central FMRFamide between Dahl SS and SR rats did differ. Chronic intracerebroventricular infusion of FMRFamide caused hypertension associated with significant increases in brain OLC in Dahl SS rats but not in SR rats. These findings suggest that Dahl SS rats are more sensitive to FMRFamide than SR rats and FMRFamide-activated brain sodium channels might contribute to increases in brain OLC and subsequent hypertension caused by a high-salt diet. FMRFamide is an excitatory neuropeptide present in both invertebrate and vertebrate nervous systems (4, 25). In the rat brain, FMRFamide-like immunoreactive material has been found in nerve cells and terminals, particularly in the hypothalamus, brain stem, and pituitary gland (4, 33). Intracerebroventricular administration of FMRFamide increases sympathetic nerve activity and BP in rats (12, 23). In snails, FMRFamide induces a fast excitatory depolarizing response due to the direct activation of an amiloride-sensitive sodium channel (20). This FMRFamide-activated sodium channel is blocked by amiloride and benzamil hydrochloride, an amiloride analog, and specific inhibitor of amiloride-sensitive sodium channels but not by ethylisopropylamiloride, an efficient Na\(^+\)/H\(^+\) exchanger inhibitor. Efforts to find and clone this channel in rats have so far been unsuccessful (24). However, similar sodium channels do appear to exist in the rat brain. First, FMRFamide is present in the rat brain. In the present study, FMRFamide levels were determined with a very sensitive and specific RIA. Consistent with other studies (4, 33), the concentration of FMRFamide was much higher in the hypothalamus than in other tissues. Second, intracerebroventricular infusion of FMRFamide increases sympathetic activity and BP in rats, consistent with the presence of FMRFamide receptors (presumably FMRFamide-activated sodium channels) in the rat brain. These sodium channels are similar to those found in snails and could contribute to the hypertensive effects of FMRFamide in rats.
channels, and/or possibly other kinds of benzamil-blockable sodium channels, such as ENaC (29), appear to play a major role in salt-sensitive hypertension. Gomez-Sanchez (8, 9) first reported that intracerebroventricular infusions of benzamil prevent mineralocorticoid-induced hypertension in Wistar rats and salt-induced hypertension in Dahl SS rats. Recently, we (12) showed that intracerebroventricular infusion of benzamil markedly decreases renal sympathetic nerve activity, BP, and heart rate in a dose-related manner in SHR on a high-salt diet. Further studies are clearly needed to clarify what type of sodium channel is blocked by benzamil in rat brain.

In 1991, Hamlyn et al. (10) isolated from human plasma an endogenous sodium pump inhibitor, which was determined to be indistinguishable from the cardiac glycoside ouabain by mass spectrometric or biochemical means. However, its exact structure is still unresolved (16). The hypothalamus may be one of the sources of OLC (16, 19, 34). OLC in the brain plays a critical role in genetic models of salt-sensitive hypertension, such as Dahl SS rats and SHR (1). Blockade of brain OLC by specific Fab fragments blocks the sympathetic hyperactivity and hypertension in Wistar rats by chronic intracerebroventricular infusion of Na\(^+\)-rich aCSF or in Dahl SS rats and SHR by high salt intake (11, 14). Blockade of brain OLC also blocks sympathoexcitatory and pressor responses to a brief intracerebroventricular infusion of FMRFamide in rats (12), consistent with the concept that FMRFamide causes release of OLC and thereby sympathoexcititation. The present study shows that chronic intracerebroventricular infusion of FMRFamide increases BP associated with clear increases in brain OLC in Dahl SS rats but does not affect either in Dahl SR rats. In previous studies (30, 31), we showed that benzamil inhibits increases in brain OLC in Wistar rats by chronic intracerebroventricular infusion of Na\(^+\)-rich aCSF or in Dahl SS rats by high salt intake, suggesting that open sodium channels in the brain are essential for brain OLC production and release. Benzamil also blocks sympathoexcitatory and pressor responses to both CSF Na\(^+\) and FMRFamide but not to ouabain (12). One may speculate that in the brain FMRFamide activates benzamil-blockable sodium channels and that the resulting acutely or chronically enhanced Na\(^+\) entry leads to increases in brain OLC release and thereby sympathoexcitation and hypertension. Activation of peripheral mechanisms by possible spillover of FMRFamide is very unlikely because the dose for peripheral administration needed to increase BP is much higher than the dose used for intracerebroventricular infusion in this study (28).

Genetic control of the mechanisms linking CSF Na\(^+\) with brain OLC appears to be altered in Dahl SS rats toward hypertension. Two differences between Dahl SS and Dahl SR rats appear to contribute to their different sodium sensitivity. First, the control of CSF Na\(^+\) is disturbed in SS versus SR rats (27), and a high-salt diet causes CSF Na\(^+\) concentration to increase by 5–6 mmol/l in SS but not SR rats (13, 22). We have demonstrated that increases in CSF Na\(^+\) of this magnitude are sufficient to increase brain OLC and thereby to cause hypertension (14, 30). Second, increases in brain OLC as well as sympathoexcitatory and pressor responses for same increases in CSF Na\(^+\) are clearly larger in SS versus SR rats (15). In normotensive Wistar rats, FMRFamide enhances responses to CSF Na\(^+\) or high salt intake (23). The present study shows that FMRFamide increases hypothalamic and pituitary OLC as well as BP only in Dahl SS and not in SR rats. Altogether, these findings may indicate that genetically determined differences in ENaCs and/or FMRFamide-gated Na\(^+\) channels contribute to both.

**Limitations of Present Study**

Although high salt intake did not affect FMRFamide levels in the whole hypothalamus or hindbrain, further studies are needed to assess content and/or release in specific nuclei. The present studies were only done in young Dahl rats. The increased responsiveness to CSF Na\(^+\) in SS versus SR rats diminishes with maturation (15). If indeed enhanced responsiveness to FMRFamide mediates the enhanced responsiveness to CSF Na\(^+\) in SS versus SR rats, one may expect a parallel change in responses to both FMRFamide and CSF Na\(^+\) with maturation. Both of these issues require further follow-up studies.

In summary, no differences in FMRFamide levels were found in the whole hypothalamus, pons and medulla, and spinal cord of Dahl SS and Dahl SR rats on either a regular or high-salt diet. However, chronic intracerebroventricular infusions of FMRFamide increase brain OLC and BP in Dahl SS rats but not in SR rats. These findings support the concept that in Dahl SS rats but not in SR rats on a high-salt diet, genetically determined enhanced Na\(^+\) entry through benzamil-blockable brain sodium channels increases brain OLC release (and content) and thereby sympathetic outflow and BP.

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**DISCLOSURES**

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28. Thiemannmann C, al-Damluji S, Hecker M, and Vane JR. Specificity of FMRFamide and four FaRPs (FMRFamide-related peptides), including the mammalian-derived FaRPs F-8-Famide (NPFF) and A-18-Famide, for opioid mu, delta, kappa 1, kappa 2a, or kappa 2b receptors. Peptides 15: 401–404, 1994.


