Partial Characterization of a Renin-Releasing Factor from Plasma and Hypothalamus

Louis D. Van de Kar, Janice H. Urban, Mark S. Brownfield, and William H. Simmons

SUMMARY Previous studies have indicated that administration of the serotonin releaser p-chloroamphetamine HCl produces a dose-dependent increase in renin secretion through a blood-borne renin-releasing factor. The present studies were designed to partially characterize this renin-releasing factor using an in vitro kidney slice method for the bioassay of renin-releasing activity. Plasma from p-chloroamphetamine-treated, nephrectomized rats was used to obtain the renin-releasing factor, which was fractionated by ultrafiltration into fractions of molecular weight ranges of 1000 to 5000, 5000 to 10,000, and 10,000 to 20,000. The molecular weight range of the renin-releasing factor was determined to be between 5000 and 10,000. Since previous studies have shown that lesions in the hypothalamus prevent the effect of p-chloroamphetamine on renin secretion, we tested whether a hypothalamic extract can release renin from kidney slices. Addition of extracts of boiled rat hypothalamic tissue to the kidney slices caused an increase in renin release. Addition of cerebellar extracts produced a smaller increase in renin release, whereas addition of pituitary extracts had no effect. Fractionation by ultrafiltration of bovine hypothalamic extract revealed that the fraction with a molecular weight range of 5000 to 10,000 possessed the highest renin-releasing ability. The 1000 to 5000 (molecular weight) fraction possessed a sizeable renin-releasing activity, but the 10,000 to 20,000 fraction had no renin-releasing activity. Both bovine hypothalamus fractions (molecular weights between 1000-5000 and 5000-10,000) and plasma fraction lost their renin-releasing activity after digestion with pronase, suggesting that the renin-releasing factor or factors are peptides. These results suggest that a renin-releasing factor may originate in the hypothalamus.

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KEY WORDS • renin-releasing factor • serotonin neurons • kidney slices • hypothalamic extract • p-chloroamphetamine • rat

The serotonin releaser p-chloroamphetamine (PCA) has been used extensively to study the effect of brain serotonin neurons on corticosterone, prolactin, and renin secretion. The effect of PCA on renin secretion is prevented by pretreatment with the serotonin-depleting drug p-chlorophenylalanine and by chemical lesions of serotonergic neurons in the mesencephalic dorsal raphe nucleus. Mechanical destruction of the medio-basal hypothalamus also prevented the effect of PCA on renin secretion. Destruction of the sympathetic nervous system combined with removal of the adrenal medulla did not inhibit the effect of PCA on renin secretion, suggesting a nonneural mediator for the effect of PCA. In previous studies, addition of a plasma fraction that was obtained from PCA-treated rats (PCA-plasma fraction) to kidney slices produced a dose-dependent increase in renin release. Boiling of this plasma fraction for 20 minutes did not affect its renin-releasing activity. The present studies were designed to further characterize the renin-releasing factor using an improved and very reproducible in vitro method for the measurement of renin release from vibratome-cut, coronal kidney slices. The possibility that the renin-releasing factor...
is a peptide was investigated by incubation of the factor with a mixture of proteases (pronase), to test whether this treatment would destroy its renin-releasing activity in vitro.

Previous studies have indicated that structures in the hypothalamus mediate the serotonergic stimulation of renin secretion. Therefore, in the present study, hypothalamic extracts were tested for the presence of a renin-releasing factor.

Materials and Methods

Animals

Male Sprague-Dawley rats (King Animal Laboratories, Oregon, WI, USA) were housed, two per cage, in a light-controlled (12-hour light/dark cycle) and temperature-controlled (22-25 °C) room. Water and rat chow (Wayne Laboratory Blox; Allied Mills, Chicago, IL, USA) were available ad libitum.

In Vitro Measurement of Renin-Releasing Ability

In vitro measurement of renin-releasing ability has been described in detail elsewhere. Coronal kidney slices, 400 µm thick, were cut on an Oxford vibratome (Technical Products, St. Louis, MO, USA) and the first slice was discarded. Coronal sectioning ensured that the kidney slices were homogeneous with respect to renin content, since each slice contained both inner and outer cortical tissue. The medullary tissue was removed from each slice by dissection. The slices were cut in half and randomly placed in 10-ml siliconized vials, two halves per vial. Mean slice weight was 18.3 ± 2.55 (SD) mg (n = 24). Then, 2 ml of Krebs-Ringer solution (pH 7.4) was added to each vial, and the vials were placed in a water bath at 37°C. The Krebs-Ringer solution has composition of 118 mM NaCl, 1.22 mM KH2PO4, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 10 mM glucose, and 25 mM NaHCO3. Each vial received its own supply of oxygen (95% O2, 5% CO2) through a needle attached to a plastic tubing that was inserted into each vial through a snap-cap. After a 30-minute equilibration period, the solution was decanted and fresh solution (2.0 ml) was added for the 30-minute preincubation period. After 30 minutes, 0.2-ml samples were withdrawn for the determination of renin concentration. The remaining medium was collected, the pH was measured, and the medium was assayed for the presence of lactate dehydrogenase with a commercial kit (Beckman Liquid-Stat, Palo Alto, CA, USA). In one experiment, the vials were incubated for 3 hours and 0.2-ml samples were taken at 0.5, 1, 2, and 3 hours. An equal volume (0.2 ml) of Krebs-Ringer solution was then added to the vials to compensate for the volume loss. After the incubation period, the vials were refilled with 2.0 ml of fresh Krebs-Ringer solution for the postincubation period. Final 0.2-ml samples were taken at the end of the postincubation period. The determination of renin release in the preincubation and postincubation periods served as a quality control to verify that the slices functioned normally. These data are not shown in the tables because the incubation period (1 hour) was twice as long as the preincubation and postincubation periods (30 minutes), and the data are therefore not comparable.

All the samples were stored at −10°C until determination of renin concentration. Renin concentration was measured by radioimmunoassay of angiotensin I (ANG I) generated from a saturating concentration of renin substrate (angiotensinogen) that was added to the samples. Renin substrate is 0.1 ml of plasma from rats that were nephrectomized and injected with dexamethasone (0.2 mg per rat s.c.) 24 hours before capitation. The ANG I antiserum was used at a dilution of 1:100,000 with 35% binding and 2% intra-assay variability. The detection limit of the radioimunoassay for ANG I is 10 pg per tube, and all the samples of one experiment were analyzed in one assay. Renin release is expressed as nanograms of ANG I per milligram of kidney tissue per hour.

Histological and Immunocytochemical Examination of the Kidney Slices

Vibratome sections of rat kidney cortex were processed for histological and immunocytochemical study either before or following their use in the in vitro renin release assay in order to evaluate their tolerance to the assay procedure. Sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, dehydrated in ethanol and propylene oxide, and embedded in Araldite-Epon plastic. Then, 2-µm sections were obtained with an AO Ultracut microtome (American Optical, Buffalo, NY, USA) and mounted on poly-L-lysine-coated slides, dried, and stained with toluidine blue for routine histology or immunocytochemically stained for renin using a peroxidase-antiperoxidase (PAP) method similar to that published elsewhere. Briefly, for immunocytochemical analysis, sections were treated with sodium metoxide, washed, and sequentially incubated with 1:1000 rabbit anti-hog renin (No. 626; obtained from Dr. Inagami, Vanderbilt University, Nashville, TN, USA), 1:50 goat anti-rabbit IgG, and 1:100 rabbit PAP. Bound PAP was visualized with diaminobenzidine tetrahydrochloride, 30 mg/dl, with 0.01% hydrogen peroxide in 0.1 M Tris, pH 7.6. Immune reagents were diluted in 0.02 M phosphate buffered saline containing 0.1% gelatin, 0.01%
thimerosal, 0.002% neomycin, and 2% normal goat serum. This buffer was also used as a wash solution between incubations with immune reagents.

Preparation of Plasma

In the first experiment, saline or PCA (Regis, Morton Grove, IL, USA; 10 mg/kg i.p.) was administered to conscious rats and the rats were killed by decapitation 60 minutes later. The blood was collected in centrifuge tubes containing heparin (Upjohn, Kalamazoo, MI, USA) 20 U per tube, and centrifuged. The plasma from each rat was divided into three aliquots. One aliquot of 1.0 ml was saved for the determination of plasma renin activity (PRA). The second aliquot (0.2 ml) was saved for the determination of plasma renin concentration. The third aliquot (2.0 ml) was diluted with 1.0 ml of distilled water, placed in a boiling water bath (to denature renin) for 20 minutes, and centrifuged at 13,000 g for 10 minutes. The supernatant was lyophilized to dryness. The dry peptide residues were resuspended in 0.25 ml of saline, and 0.2 ml of the resuspended plasma was added to vials containing 2.0 ml of Krebs-Ringer and kidney slices to test for renin-releasing activity as already described.

In each of the following experiments, 40 rats were nephrectomized under halothane anesthesia 20 hours before administration of PCA (n = 24) or saline (n = 16). PCA (12 mg/kg i.p.) and saline were administered, in a volume of 2.0 ml/kg, 1 hour before decapitation. The blood was collected in centrifuge tubes containing heparin and centrifuged in a refrigerated centrifuge. The plasma was pooled into either PCA-plasma (plasma from rats that were injected with PCA) or saline-plasma (plasma from rats treated with saline) pools (of approximately 150 ml each), respectively, placed in a boiling water bath for 20 minutes, and centrifuged at 13,000 g for 20 minutes. Ultrafiltration of the supernatant of the plasma pools was performed at 4°C in a low pressure stirred cell (Nucleopore, Pleasanton, CA, USA), with a low absorption membrane with a molecular weight cutoff of 20,000. The filtered fraction containing solutes with molecular weights below 20,000 was refiltered through a membrane with molecular weight cutoff of 10,000. The latter fraction was then refiltered through a membrane with a molecular weight cutoff of 5000. The remaining fraction containing solutes with molecular weights below 5000 was refiltered through a membrane with a molecular weight cutoff of 1000. The material that was retained on the respective membranes was resuspended in 3 to 4 ml of saline. Thus, three separate fractions were obtained, one that contained solutes in the molecular weight range of 1000 to 5000, one that contained solutes in a molecular weight of 5000 to 10,000, and one that contained solutes with molecular weights of 10,000 to 20,000. All three fractions were stored at −70°C. The hypothalamic ventral surface was exposed, and the hypothalamus was excised with fine curved scissors. The rostral border was the decussation of the optic tracts and the decussation of the anterior commissure. The caudal border was the mamillary bodies. The pituitary was removed from the sella turcica and the cerebellum was removed from the brain. A volume of boiling water (10 ml/g) was added to hypothalamic (0.97 g), cerebellar (0.85 g), or pituitary (0.74 g) tissue, and the tissue was homogenized with a glass tissue grinder, placed in a boiling water bath for 10 minutes, and centrifuged at 38,000 g for 30 minutes. A 4.5-ml aliquot of the supernatant was lyophilized to dryness and resuspended in 0.2 ml of saline. A volume of 0.02 ml was added to the kidney slices to test for renin-releasing activity. This volume corresponds approximately with 45 mg of the original hypothalamic, cerebellar, or pituitary tissue.

Bovine hypothalami were obtained from Pel Freez (Rogers, AR, USA). One hypothalamus (12.3 g) was homogenized in 123 ml of boiling water with a Polytron tissue disrupter (Brinkmann Instruments, Westbury, NY, USA). The supernatant was divided into two aliquots: one aliquot (4.5 ml) was lyophilized to dryness and the remainder of the supernatant (approximately 120 ml) was fractionated by ultrafiltration, as already described, into fractions with molecular weights of 1000 to 5000, 5000 to 10,000, 10,000 to 20,000. The final volume of each fraction was 3 to 4 ml. The extract and fractions were added to the kidney slices in a volume of 0.02 ml to test for renin-releasing activity.

Incubation of the p-Chloroamphetamine–Plasma Fraction and Bovine Hypothalamic Fractions with Pronase

Pronase E (a nonspecific protease, type XIV, isolated from Streptomyces griseus, that has an activity of 4 U/mg; Sigma, St. Louis, MO, USA) was added to the PCA-plasma fraction that had renin-releasing activity (the fraction containing solutes of molecular weights between 5000 and 10,000) and to the equivalent fraction from saline-treated rats. Pronase was dissolved in a 0.1 M borate buffer, pH 7.5, containing 5 mM CaCl₂, at a concentration of 20 mg/200 μl and was added to a volume of 1.6 ml of the PCA-plasma and saline-plasma fractions (molecular weight, 5000–10,000) or to 200 μl of bovine hypothalamic fractions (molecular weight, 1000–5000 and 5000–10,000). The borate buffer vehicle used to dissolve pronase was added, at the same volume (0.2 ml), to an equal aliquot (1.6 ml) of the PCA-plasma and saline-plasma fractions (molecular weight, 5000–10,000) or the bovine hypothalamus fractions, and all the aliquots were incubated at 37°C for 4 hours. After incubation, all aliquots were placed in a boiling water bath for 20 minutes (to denature the pronase) and centrifuged at 13,000 g for 30 minutes. The supernatant was added to the kidney slices in a volume of 0.2 ml per vial (for plasma fractions) or 0.02 ml per vial (for hypothalamic fractions) for the 1-hour incubation period to test for renin-releasing activity.
Statistics

Statistical analysis of the data was performed by analysis of variance (ANOVA), followed by Duncan's new multiple range test for individual comparisons of the means.17

Results

Routine histological analysis of the kidney slices, both before (Figure 1) and after (Figure 2) incubation, revealed that they were very similar. Both showed the presence of healthy-appearing glomeruli and juxtaglomerular apparatus. There was evidence of pronounced swelling in proximal tubular cells in the postincubation as opposed to the preincubation sections; however, swelling was slight in the glomeruli and associated apparatus.

Immunocytochemical localization of renin was not discernibly different between the two groups. Figure 3 shows the kidney slice after incubation for 2.5 hours. Staining was confined to a length of five to seven cells in the afferent arteriole, proximal to its point of entry into the vascular pole of the glomerulus. The immunocytochemical results suggest that juxtaglomerular cell content of immunoreactive renin is not depleted during the incubation of the kidney slices.

Simultaneous measurement of PRA, plasma renin concentration, and the plasma concentration of renin-releasing factor was performed in rats that received injections of either saline (2 ml/kg i.p.) or PCA (10 mg/kg i.p.). PCA produced a significant increase in PRA, plasma renin concentration, and renin-releasing factor 60 minutes after the injection (Table 1).

Table 2 shows that all the saline-plasma fractions and the PCA-plasma fractions had renin-releasing activity. The PCA-plasma fraction that contained substances with a molecular weight range of 5000 to 10,000 caused a threefold increase in renin release when compared with all other fractions ($F_{6,8} = 9.84$, $p < 0.01$). Since the PCA-plasma also contained a high

![Figure 1](https://example.com-figure1.png)

**Figure 1.** Photomicrograph of a 2-μm thick kidney slice section that was fixed before incubation in the renin release assay. Toluidine blue-stained section shows normal histological appearance of the glomerulus (G), macula densa (MD), and surrounding proximal and distal tubules. Note the general absence of cellular swelling and that numerous cell nuclei (arrows) appear pale and have diffuse euchromatin staining and a discernible nucleolus. (Original magnification ×500.)

![Figure 2](https://example.com-figure2.png)

**Figure 2.** Photomicrograph of a 2-μm thick kidney slice section after incubation (2.5 hours) in the in vitro assay procedure. Note that the same general histological characteristics are present as in Figure 1. There is some slight swelling of tubular cells, but their nuclei remain lightly stained with the exception of one or two per field that show early pyknotic changes. The glomerulus (G) and juxtaglomerular apparatus show no changes. (Original magnification ×500.)

![Figure 3](https://example.com-figure3.png)

**Figure 3.** Photomicrograph showing immunohistochemical localization of renin in juxtaglomerular cells (JG) of the afferent arteriole in a section taken from a kidney slice after a 2.5-hour incubation. The glomerulus (G) and adjacent structures exhibit only background staining. (Original magnification ×750.)
concentration of serotonin, we added serotonin, as well as PCA and fenfluramine, to a separate group of kidney slices. None were effective in stimulating renin release. Lactate dehydrogenase in the medium was also determined and was 0.034 ± 0.006 μmol/ml/min for the saline-plasma (molecular weight, 5000-10,000) group and 0.032 ± 0.006 μmol/ml/min for the PCA-plasma (molecular weight, 5000-10,000) group.

The duration of the effect of renin-releasing factor was investigated by incubating the plasma fractions with kidney slices for 3 hours. Addition of the PCA-plasma fraction containing substances with molecular weights between 5000 and 10,000 produced a significant increase in renin release from the kidney slices (F13, 50 = 18.54; p < 0.01). The amount of renin in the incubation medium reached a maximum after 2 to 3 hours of incubation (Figure 4). Addition of saline or the plasma fraction (molecular weight, 5000-10,000) that was obtained from saline-treated rats produced a small, statistically insignificant (F13, 50 = 18.54; Duncan’s test: minimal difference at p < 0.05 was 7.8) increase in renin release from the kidney slices (see Figure 4).

A control experiment was performed to verify that the effect of the PCA-plasma fraction (molecular weight, 5000-10,000) was not due to activation of inactive renin that could have been released by the kidney slices. For this purpose, the kidney slices were removed from the incubation medium (2.0 ml of Krebs-Ringer) after 1 hour of incubation, and the incubation medium was then incubated with either saline (0.2 ml) or plasma fractions (molecular weight, 5000-10,000) from saline-treated (saline-plasma; 0.2 ml) or PCA-treated (PCA-plasma; 0.2 ml) rats. As can be seen in Table 3, there was no activation of inactive renin by the plasma fractions since there was no change in the concentration of renin (measured as the ability to generate ANG I in the presence of a saturating concentration of renin substrate).

### TABLE 1. Effect of Saline or p-Chloroamphetamine (10 mg/kg i.p.) on PRA, Plasma Renin Concentration, and Plasma Renin-Releasing Factor Concentration in Conscious Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PRA (ng ANG I/mg kidney/hr)</th>
<th>PRC (ng ANG I/ml/hr)</th>
<th>RRF (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.4 ± 0.9</td>
<td>4.9 ± 0.9</td>
<td>10.4 ± 1.4</td>
</tr>
<tr>
<td>PCA</td>
<td>19.1 ± 1.8*</td>
<td>23.4 ± 4.5*</td>
<td>18.4 ± 2.6*</td>
</tr>
</tbody>
</table>

Data are means ± SEM. PRA = plasma renin concentration; RRF = renin-releasing factor; PCA = p-chloroamphetamine.

* p < 0.05 (by Student’s t test, two-tailed for unpaired observations), compared with values for saline.

### TABLE 2. Effect of Saline or Plasma Fractions from Saline-Treated and p-Chloroamphetamine-Treated Nephrectomized Rats on Renin Release from Kidney Slices

<table>
<thead>
<tr>
<th>Variable</th>
<th>Renin release (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA-plasma fraction</td>
<td></td>
</tr>
<tr>
<td>Mol wt, 1000-5000 (n = 10)</td>
<td>12.0 ± 1.8</td>
</tr>
<tr>
<td>Mol wt, 5000-10,000 (n = 17)</td>
<td>39.5 ± 5.6*</td>
</tr>
<tr>
<td>Mol wt, 10,000-20,000 (n = 6)</td>
<td>12.3 ± 1.6</td>
</tr>
<tr>
<td>Saline-plasma fraction</td>
<td></td>
</tr>
<tr>
<td>Mol wt, 1000-5000 (n = 5)</td>
<td>11.3 ± 1.5</td>
</tr>
<tr>
<td>Mol wt, 5000-10,000 (n = 10)</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td>Mol wt, 10,000-20,000 (n = 5)</td>
<td>10.9 ± 1.9</td>
</tr>
<tr>
<td>Saline (n = 13)</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>10⁻⁶ M serotonin (n = 4)</td>
<td>8.8 ± 1.2</td>
</tr>
<tr>
<td>10⁻⁶ M PCA (n = 4)</td>
<td>8.6 ± 1.1</td>
</tr>
<tr>
<td>10⁻⁶ M fenfluramine (n = 4)</td>
<td>9.5 ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SEM. PCA = p-chloroamphetamine.

* p < 0.01, compared with values for all other groups (by ANOVA and Duncan’s new multiple range test).

### FIGURE 4. Effect on renin release of a 3-hour incubation of kidney slices with saline or a plasma fraction that was obtained from saline-treated or p-chloroamphetamine (PCA)-treated rats. Data represent mean ± SEM of six determinations. Star indicates significant difference compared with the corresponding saline or saline-plasma group (p < 0.01, by ANOVA and Duncan’s new multiple range test). AL = angiotensin I; MW = molecular weight.

### TABLE 3. Test for Nonselective Activation of Renin in the Incubation Medium (Krebs-Ringer) by the Fractions of Plasma from Saline-Treated or p-Chloroamphetamine-Treated Rats

<table>
<thead>
<tr>
<th>Fraction added</th>
<th>Renin activity (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA-plasma fraction (mol wt, 5000-10,000)</td>
<td>231.2 ± 19.0</td>
</tr>
<tr>
<td>Saline-plasma fraction (mol wt, 5000-10,000)</td>
<td>276.0 ± 8.8</td>
</tr>
<tr>
<td>Saline</td>
<td>238.6 ± 22.8</td>
</tr>
</tbody>
</table>

Values are means ± SEM of six determinations. PCA = p-chloroamphetamine.

The Krebs-Ringer samples (2 ml) were preincubated with kidney slices for 1 hour to release renin. The kidneys were removed, and 0.2 ml of either saline, saline-plasma, or PCA-plasma was added to the medium. The samples were incubated for 1 additional hour, and 0.2-ml aliquots were taken for the determination of renin activity.
To determine whether the renin-releasing factor or factors are peptides, the PCA-plasma and saline-plasma fractions (molecular weight, 5000–10,000) were incubated with pronase. We tested the activity of pronase and pronase mixed with the plasma fraction using a casein plate to verify its proteolytic activity. Incubation of the PCA-plasma fraction (molecular weight, 5000–10,000) with pronase completely eliminated its renin-releasing activity (\(F_{3,28} = 29.84, \ p < 0.01\)), while the low renin-releasing activity of the saline-plasma fraction was not diminished (Table 4). These data suggest that the renin-releasing factor is a peptide.

Extracts of rat hypothalamic, cerebellar, and pituitary tissues were added to the kidney slices to test whether these extracts contain renin-releasing substances. The tissue extracts that were added to the slices corresponded to 45 mg of tissue. As can be seen from Table 5, the hypothalamic extract produced a significant increase in renin release (\(F_{4,36} = 11.47, \ p < 0.01\)), suggesting that the hypothalamus contains a renin-releasing factor. The cerebellar extract also significantly increased renin release from kidney slices.

This effect was not statistically significant when compared with the saline group, but it was significant in comparison with the pituitary extract. The pituitary extract did not increase but instead produced a slight but statistically insignificant decrease in renin release from kidney slices (see Table 5).

Table 6 shows that bovine hypothalamic fractions contain renin-releasing substances. The highest effect was seen with the fraction with a molecular weight range of 5000 to 10,000 (\(F_{3,326} = 6.8098, \ p < 0.01\)). The second highest effect was seen with the fraction with substances with molecular weights of 1000 to 5000 (\(p < 0.05\)), whereas the 10,000 to 20,000 (molecular weight) fraction was devoid of significant renin-releasing ability. Isoproterenol (10\(^{-6}\) M) was also tested to indicate the maximal range of renin release in this bioassay (see Table 6). Digestion of the bovine hypothalamic fractions (molecular weight, 1000–5000 and 5000–10,000) with pronase for 4 hours completely eliminated the renin-releasing activity of both fractions (Table 7).

Rat hypothalamic, cerebellar, and pituitary extracts and bovine hypothalamic extract and fractions (molecular weight, 1000–5000, 5000–10,000), and saline-plasma and PCA-plasma fractions (molecular weight, 5000–10,000) were added to Krebs-Ringer solution in

### Table 4. Ability of Pronase-Digested p-Chloroamphetamine-Plasma or Saline-Plasma Fractions to Release Renin from Kidney Slices

<table>
<thead>
<tr>
<th>Fraction added*</th>
<th>Renin release (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated PCA-plasma fraction (n = 9)</td>
<td>15.7 ± 1.2†</td>
</tr>
<tr>
<td>Pronase-treated PCA-plasma fraction (n = 10)</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>Vehicle-treated saline-plasma fraction (n = 6)</td>
<td>10.6 ± 1.3</td>
</tr>
<tr>
<td>Pronase-treated saline-plasma fraction (n = 6)</td>
<td>11.7 ± 0.9</td>
</tr>
<tr>
<td>Saline (n = 9)</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>10(^{-6}) M isoproterenol (n = 6)</td>
<td>19.6 ± 1.9†</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Number of determinations is shown in parentheses. PCA = p-chloroamphetamine.

*Molecular weight range of PCA-plasma and saline-plasma fractions was 5000 to 10,000.

†\(p < 0.01\) (by ANOVA and Duncan's new multiple range test), compared with values in saline or corresponding pronase-treated group.

### Table 5. Effect of Rat Hypothalamic, Cerebellar, and Pituitary Extracts on Renin Release from Kidney Slices

<table>
<thead>
<tr>
<th>Substance added</th>
<th>Renin release (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n = 13)</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>Hypothalamic extract (n = 16)</td>
<td>19.1 ± 2.1*</td>
</tr>
<tr>
<td>Cerebellar extract (n = 10)</td>
<td>15.3 ± 2.1†</td>
</tr>
<tr>
<td>Pituitary extract (n = 8)</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>10(^{-6}) M isoproterenol (n = 5)</td>
<td>19.7 ± 2.6*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number of determinations is presented in parentheses.

*\(p < 0.01\), †\(p < 0.05\) (by ANOVA and Duncan's new multiple range test), compared with values for pituitary extract.

*\(p < 0.05\) (by ANOVA and Duncan's new multiple range test), compared with values for saline.

### Table 6. Effect of Bovine Hypothalamic Fractions on Renin Release from Kidney Slices

<table>
<thead>
<tr>
<th>Bovine hypothalamic fraction</th>
<th>Renin release (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>14.8 ± 1.1</td>
</tr>
<tr>
<td>Mol wt, 1000–20,000</td>
<td>12.6 ± 3.0</td>
</tr>
<tr>
<td>Mol wt, 5000–10,000</td>
<td>22.7 ± 4.0*</td>
</tr>
<tr>
<td>Mol wt, 1000–5000</td>
<td>18.6 ± 3.5†</td>
</tr>
<tr>
<td>Saline</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td>10(^{-5}) M isoproterenol</td>
<td>31.4 ± 3.2*</td>
</tr>
</tbody>
</table>

Values are means ± SEM of six determinations.

*\(p < 0.01\), †\(p < 0.05\) (by ANOVA and Duncan's new multiple range test), compared with values for saline.

### Table 7. Ability of Pronase-Digested Fractions from Bovine Hypothalamus to Release Renin from Kidney Slices

<table>
<thead>
<tr>
<th>Fraction added*</th>
<th>Renin release (ng ANG I/mg kidney/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated fraction 1 (mol wt, 1000–5000)</td>
<td>16.0 ± 2.4*</td>
</tr>
<tr>
<td>Pronase-digested fraction 1 (mol wt, 1000–5000)</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>Vehicle-treated fraction 2 (mol wt, 5000–10,000)</td>
<td>15.4 ± 0.6*</td>
</tr>
<tr>
<td>Pronase-digested fraction 2 (mol wt, 5000–10,000)</td>
<td>8.2 ± 0.7</td>
</tr>
<tr>
<td>Saline</td>
<td>8.5 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SEM of six determinations.

*\(p < 0.01\) (by ANOVA and Duncan's new multiple range test), compared with values for the corresponding pronase-digested group and saline.
the same concentration as in the previous experiments and incubated with renin substrate for 1 hour to test whether they would generate ANG I in the absence of renin. As can be seen in Table 8, these fractions and extracts generated extremely low levels of ANG I, which had to be measured at the sensitivity limit of the radioimmunoassay. Note that normal rat plasma tested at the same concentration produced a five to 17 times greater generation of ANG I. These data suggest that the effect of the plasma hypothalamic fractions on renin release from kidney slices is not due to nonspecific interaction with the renin release bioassay.

Discussion

The major findings of the present experiments are that the renin-releasing factor is a relatively large peptide (molecular weight, 5000–10,000) and that a substance or substances with renin-releasing ability are present both in rat and bovine hypothalamus.

The histological and immunocytochemical analysis of the kidney slices was performed to test the viability of the slices and showed that the kidney slices remained healthy and contained renin even after 2.5 hours of incubation. Furthermore, the values for the cytosolic marker enzyme, lactate dehydrogenase, in the medium were very low, and there was no difference between the saline-plasma and PCA-plasma group, even though the kidney slices that were incubated with the PCA-plasma fraction (molecular weight, 5000–10,000) released three times more renin (see Table 2) than did those incubated in the saline-plasma fraction.

Studies in humans, and conscious rats and anesthetized dogs have indicated that brain serotoninergic neurons stimulate the secretion of renin. The serotonin agonist quipazine and the serotonin releasers PCA and fenfluramine produce dose-dependent increases in PRA. The effects of PCA and fenfluramine were prevented by pretreatment with the serotonin synthesis inhibitor p-chlorophenylalanine and by serotonin uptake blockers. Chemical lesions of the serotoninergic neurons in the mesencephalic dorsal raphe nucleus by intraraphe injections of the neurotoxin 5,7-dihydroxytryptamine prevented the effect of PCA on renin secretion, suggesting that brain serotoninergic neurons mediate the effect of PCA. Electrophysiological lesions of the dorsal raphe nucleus also prevented the effect of stress on PRA. The dorsal raphe nucleus innervates nuclei in the hypothalamus, including the arcuate and ventromedial hypothalamic nuclei.

The exact location of the serotoninergic nerve terminals that regulate renin secretion is not known; however, mechanical ablation of the mediobasal hypothalamus or posterolateral deafferentation of the hypothalamus prevents the PCA-induced increase in PRA.

We have previously presented evidence that the serotoninergic stimulation of renin secretion is not mediated by either the sympathetic nervous system or adrenal catecholamines. The sympathetic blocker bretylium tosylate did not inhibit the effect of PCA on renin secretion, and chemical sympathectomy combined with adrenal medullectomy did not inhibit the effect of PCA on PRA. Furthermore, spinal transection between the first and second thoracic vertebrae (proximal to the renal nerves) did not prevent the effect of PCA on PRA, suggesting that the sympathetic nervous system does not mediate the serotoninergic stimulation of renin secretion.

De Vito et al. presented evidence for a blood-borne renin-releasing factor in dogs; the factor appeared in the blood of the dogs in response to hypovolemia. However, Polomski et al. could not replicate these results. Thus, the hypothesis was tested that stimulation of brain serotoninergic receptors triggers the release of an endocrine factor that releases renin directly from the kidney. Recently, we have presented data showing that administration of PCA to nephrectomized rats causes the appearance of a blood-borne renin-releasing factor that stimulates renin secretion in a dose-dependent manner. The renin-releasing factor was found to be heat-stable. It is possible, though not very likely, that the renin-releasing factor stimulates renin secretion by activating β-adrenergic receptors in the kidney.

The increase in renin release produced by the plasma and brain fractions most likely is not due to a nonspecific activation of inactive renin or by a substance such as cathepsin D, which would cleave ANG I from angiotensinogen, since the incubation of hypothalamic, PCA-plasma, or saline-plasma fractions with renin substrate (angiotensinogen) resulted in barely measurable amounts of ANG I (close to the blank values in the radioimmunoassay; see Table 8). In addition, these fractions did not seem to activate the inactive renin that
may have been released from the kidney slices (see Table 3). Since both the plasma and hypothalamic extracts were boiled during the extraction procedures, it is not likely that proteolytic enzymes would have survived. Finally, the fraction with the highest renin-releasing activity had a molecular weight (5000–10,000) that is lower than that of most proteolytic enzymes. The other fractions also had some renin-releasing activity, probably because fractionation by ultrafiltration provides only relative enrichment rather than complete separation of renin-releasing factor from other substances.

The present results confirm our previous results and provide evidence that this renin-releasing factor is a peptide, since it was destroyed by pronase and survived boiling. Pronase is a mixture of proteases that will destroy a wide variety of proteins when incubated at 37°C at pH 7.5. The renin-releasing activity of the 5000 to 10,000 (molecular weight) PCA-plasma fraction, which was incubated in the absence of pronase, was not as high as that found in our previous experiments, probably because this fraction was incubated for the same length of time (4 hours) with the pronase vehicle, which contained 5 mM CaCl2. Since high calcium is known to inhibit renin release, it could partly inhibit the effect of the renin-releasing factor. In all the experiments, the equivalent saline-plasma fraction produced a slight increase in renin release. However, the low renin-releasing activity of the saline-plasma fraction was not affected by pronase, suggesting that the substance or substances in the saline-plasma fraction that affect renin release from kidney slices are not peptides and therefore differ from the renin-releasing factor present in the PCA-plasma fraction.

Since addition of serotonin to the kidney slices did not stimulate renin secretion, we conclude that serotonin is not the renin-releasing factor. This conclusion is also substantiated by the finding that the molecular weight range of the factor was between 5000 and 10,000, whereas the molecular weights of serotonin, PCA, and fenfluramine are 176, 249, and 206, respectively.

The present data demonstrate that the brain contains substances that can release renin (Table 3). Since both the plasma and hypothalamic extracts were boiled during the extraction procedures, it is not likely that proteolytic enzymes would have survived. The fraction with the highest renin-releasing activity had a molecular weight (5000–10,000) that is lower than that of most proteolytic enzymes. The other fractions also had some renin-releasing activity, probably because fractionation by ultrafiltration provides only relative enrichment rather than complete separation of renin-releasing factor from other substances.

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The present data demonstrate that the brain contains a substance or substances with renin-releasing ability. The hypothalamus had the highest renin-releasing activity, but the cerebellum contained a considerable concentration of renin-releasing substances. Conversely, the pituitary extract (which was not fractionated) was devoid of renin-releasing activity and caused a small, insignificant decrease in renin release from kidney slices. Since the pituitary neural lobe contains a high concentration of vasopressin, a substance that is known to inhibit renin secretion, it is possible that the presence of renin-releasing substances in the pituitary could be masked. The bovine hypothalamus also contains substances that can release renin from kidney slices. The hypothalamic fraction with the highest renin-releasing activity (molecular weight, 5000–10,000) may contain the same or similar protease-digestable substances as the plasma fraction (molecular weight, 5000–10,000) obtained from PCA-treated rats. An important difference between rat plasma and the bovine hypothalamic extract is the finding that the lower molecular weight fraction (i.e., 1000–5000) of rat plasma had low renin-releasing activity that was not significantly different from the equivalent fraction from saline-treated rats. In contrast, the bovine hypothalamic fraction (molecular weight, 1000–5000) had a considerable concentration of renin-releasing substances. It is possible that a large renin-releasing peptide may be metabolized to a smaller peptide with renin-releasing activity, though this smaller peptide may not reach the circulation. On the other hand, it could be a completely different substance. Alternatively, a small peptide with renin-releasing activity may be bound to a small plasma protein and thus would be fractionated in the molecular weight range of 5000 to 10,000. The hypothalamic fractions had seemingly lower renin-releasing activity than the plasma fractions; however, this difference is due to a lower amount of starting material. The plasma fractions were obtained from 40 rats, whereas the hypothalamic fractions were obtained from 10 rats or from one cow.

Additional evidence in support of the hypothesis that the renin-releasing substance or substances in the rat and bovine hypothalamus may be similar to the plasma-borne renin-releasing factor is the fact that lesions of the mediobasal hypothalamus prevent the effect of PCA on renin secretion. Recently, Gotoh et al reported that electrolytic lesions in the hypothalamic paraventricular nucleus inhibited the effects of PCA and of immobilization stress on renin secretion, whereas electrolytic lesions in the dorsomedial hypothalamic nucleus had no effect. Since neurons in the paraventricular nucleus send axons to both the neural lobe of the pituitary gland and the median eminence, a renin-releasing substance could be secreted from the hypothalamic paraventricular nucleus into the circulation.

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References
5. Van de Kar LD, Beetha CL. Pharmacological evidence that serotoninergic stimulation of prolactin secretion is mediated via
the dorsal raphe nucleus. Neuroendocrinology 1982;35:225–230
7. Alper RH, Ganong WF. Pharmacological evidence that the sympathetic nervous system mediates the increase in secretion of renin produced by p-chloroamphetamine. Neuropharmacology 1984;23:1237–1240
18. Fray JCS, Lush DJ, Share DS, Valentine AND. Possible role of calmodulin in renin secretion from isolated rat kidneys and renal cells; studies with trifluoperazine. J Physiol (Lond) 1983;343:447–454
25. Van de Kar LD, Lorenz SA. Differential serotonergic innervation of individual hypothalamic nuclei and other forebrain regions by the dorsal and median raphe nuclei. Brain Res 1979;162:45–54
Partial characterization of a renin-releasing factor from plasma and hypothalamus.
L D Van de Kar, J H Urban, M S Brownfield and W H Simmons

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