Isolation of Renin-Rich Rat Kidney Cells

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SUMMARY Enzymatic dispersion and density gradient (Percoll) sedimentation were used to isolate a population of renin-containing, granule-laden cells (density 1.067 g/ml) from rat kidney cortex. Using immunohistochemistry (light microscopy) and electron microscopy, we defined the presence and ability of these cells to store renin protein(s). A 1000 base pair rat renin complementary DNA was used to show that these cells express the renin gene. The reverse hemolytic plaque assay defined the functional properties of the renin-containing cell. The data are consistent with the postulated inverse relationship between calcium concentration and release of renin. Thus, we have isolated a population of functional rat kidney cells that synthesize, store, and release renin. (Hypertension 10: 488–496, 1987)

KEY WORDS • renin • juxtaglomerular cell suspensions • renin antibody • renin messenger RNA • hemolytic plaque assay

RENIN is stored in the granules of and presumably synthesized by juxtaglomerular (JG) cells.1,2 The JG cells are modified smooth muscle cells located primarily in the renal afferent arteriole adjacent to the glomerulus.3 Control of renin has been studied in whole animals, in isolated perfused kidneys, in renal cortical slices, in isolated glomeruli, and in cortical cell suspensions.4–11 Most in vitro studies using perfused kidney or slices have been impaired by an inability to isolate the JG cell from the influences of other cell types or structures, such as the macula densa and sympathetic nerves. In addition, in most in vitro preparations, JG cells constitute only a small percentage of the total cell population, and although the composition of the solution bathing the preparation is known, the concentration of endogenous (autacoids) and exogenous substances reaching the JG cell cannot be controlled. Investigations using JG cell suspensions12–16 have yielded the most direct evidence of JG cell release of renin in response to stimuli, but these have not always distinguished between renin synthesis, processing, and release. The ability to isolate functional JG cells for study in suspension or after culture is critical to future investigation of renin control. In the present study, we present evidence for the isolation of a population of rat kidney cells enriched for those cells that synthesize, store, and secrete renin.

Materials and Methods

Isolation of Cells

Male Wistar-Kyoto rats (Charles River Laboratories, Wilmington, MA, USA) weighing 150 to 250 g were decapitated, the abdominal aorta was cannulated, and the kidneys were perfused with enzymes in Krebs buffer of the following composition (mM): 111 NaCl, 5.0 KCl, 1.0 NaH₂PO₄, 11.2 glucose, 25 NaHCO₃, 0.54 MgCl₂, 2.5 CaCl₂ (unless stated otherwise), 0.1% bovine serum albumin (BSA; A-7638; Sigma Chemical, St. Louis, MO, USA), 0.1% collagenase (Type I; Sigma), and DNAase (Type IV; Sigma), 0.017 mg/ml. This enzyme mixture allowed better viability for short-term studies (2–3 hours) with acutely isolated cells. However, the degree of cell death was too high for more chronic studies (10–12 hours), as was determined using the plaque assay (described in a subsequent section), which required the addition of trypsin...

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isolated renin and elastase. These additions did not alter other parameters, such as renin activity, ability to interact with the antirat renin antibody, or cellular morphology (ultrastructure). The buffer was bubbled with 95% O₂, 5% CO₂, to maintain a pH of 7.2 to 7.4.

The kidneys were removed, and the demedullated cortices were minced. Unless otherwise stated, all tissue was kept on ice at approximately 0 to 4°C. The cortices were then incubated in the enzyme solution just described at 37°C for 90 minutes. Enzymatic activity was inhibited by dilution (1:10) with 20% fetal calf serum (HyClone Labs, Logan UT, USA). The cells were filtered through 105-μm then 53-μm nylon mesh and then spun at 250 g for 2 minutes and resuspended in enzyme-free Krebs buffer. Next, 8 ml of standard isosmotic Percoll17 (colloidal silica) was placed in a 30 ml-ultracentrifuge tube and 12 ml of cell suspension was carefully added to form a 40% Percoll solution (vol/vol). The tube was then mixed by gently inverting several times. Usually, cells obtained from the cortices of two kidneys were dispersed, and the cell suspension divided among four tubes (approximately 100 mg of protein/tube). The tubes were spun at 29,000 x g for 20 minutes in a fixed-angle rotor (Model 60 Ti; Beckman Instruments, Fullerton, CA, USA). After ultracentrifugation, the cells were separated into several easily visible layers.18 A tube containing standard density beads (reconstituted according to directions from Pharmacia Fine Chemicals AB, Uppsala, Sweden) was placed in the same rotor and spun simultaneously. The sedimentation density of individual cell layers was determined by comparison with the location of the colored density marker beads (see Figure 1). The individual layers, separated by density, were then pooled, washed free of Percoll by three rinses (1:10 dilution) and then spun at 250 x g for 10 minutes. Cell viability was determined by the ability to exclude trypan blue. One drop of 0.4% trypan blue in 0.9% saline was added to one drop of cell suspension (approximately 10⁶ cells/ml). After 5 minutes, the cells were examined under light microscopy and the cells that excluded trypan blue were then counted with a hemacytometer and expressed as a percentage of the total number of cells. With the use of this marker of cell death, viability was greater than 95%.

Preparation of Renin Substrate

Rat renin substrate was prepared by exsanguination of animals nephrectomized 48 hours earlier. Blood was collected in tubes containing approximately 20 mM EDTA (disodium salt; Sigma) and clarified by low-speed centrifugation (400 g for 5 minutes). Rat renin substrate was isolated from the rat plasma using the method of Khayat et al.14 by precipitation between 1.5 and 2.3 M ammonium sulfate (addition of 4.0 M ammonium sulfate slowly over 1 hour at 5°C). The precipitated protein was dissolved in a minimal amount of 10 mM EDTA and then dialyzed against 10 mM EDTA, pH 7 (3 hours), to inactivate angiotensinases. Finally, the protein was dialyzed against distilled water overnight. After the addition of 1% sodium azide, the solubilized, partially purified renin substrate (pH 6.5) was stored at 0 to 4°C until used. The rat substrate was not contaminated with converting enzyme, as addition of up to 10 mM captopril did not alter the rate of angiotensin I (ANG I) generation. Absence of angiotensinase contamination was determined by incubation of the rat substrate with labeled and unlabeled ANG I followed by thin-layer chromatographic analysis, as described by Ackerly et al.19

Determination of Renin Activity

Cells were sonicated (Kontes, Vineland, NJ, USA) or frozen and thawed three times and centrifuged at 11,800 x g for 10 minutes. Then, 10 μl of the supernatant was incubated with 100 μl of renin substrate (3.5 mg of protein) at pH 6.5 for 30 minutes at 37°C in the presence of 5 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma).14,15 The amount of ANG I generated was determined by radioimmunoassay (New England Nuclear, Boston, MA, USA) and expressed as nanograms of ANG I generated per milligram protein per hour. With this amount of substrate, the reaction was linear with time until 1 ng of ANG I had been generated. Aliquots obtained from the medium used for cell incubation were assayed using the same procedure. Protein concentration was determined by the microbiuret method.

Inhibition of Renin Activity by Antibody

An aliquot (25 μl) of rat renin (cell lysate) was incubated with a 1:30 dilution of rabbit antirat renin antibody20 for 18 to 22 hours at 0 to 4°C. A control tube containing rat renin (25 μl) with the addition of a volume of Krebs buffer equal to that containing antirenin antibody was incubated for a similar time at 0 to 4°C. At the end of the cold incubation period, 100 μl of rat substrate (containing 5 mM EDTA and 2 mM PMSF) was added and the tubes were incubated at 37°C as described for ANG I generation.

Immunocytochemistry with the Immunoperoxidase Technique

Cell smears were prepared from dispersed cells, separated by density on Percoll by clearing in methanol (-20°C) and plated onto glass slides coated with poly-L-lysine (molecular weight 90,000; 1 mg/ml in deionized water; Sigma). Visualization of renin-containing cells was accomplished using the avidin-biotinperoxidase complex (ABC) method of immunocytochemistry. All ABC reagents were obtained from Vector Laboratories, Burlingame, CA, USA. In brief, slides were washed in 10 mM sodium phosphate-buffered saline (PBS; 0.9%), incubated in 0.3% H₂O₂ (Sigma) in methanol to quench endogenous peroxidase activity, and rinsed well in PBS. Blocking serum (normal goat serum diluted 1:75 in PBS) was applied for 20 minutes, then blotted off. Slides were incubated with rabbit antirat renin antibody20 reconstituted from lyophilized serum and diluted 1:750 in PBS or, for controls, in normal rabbit serum, also diluted 1:750 for 60 minutes. Biotinylated second antibody, diluted...
Tris chloride, 5 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS); pH 7.5; room temperature for 5 hours), to ethanol, and 2 M CsCl₂. The homogenate was applied to 2.75 ml of autoclaved 5.7 M CsCl₀.₁ M Na₂HPO₄, pH 7.2, containing 1 mM EDTA, and sonicated, denatured herring sperm DNA, 100 μg/ml. Following hybridization, the membranes were washed for 10 minutes per cycle in 250 ml of the following solutions: twice in 0.5% BSA, 5% SDS, 40 mM NaH₂PO₄, 1 mM EDTA and four times in 1% SDS, 40 mM NaH₂PO₄, 1 mM EDTA at 65°C. Then, the membranes were briefly rinsed in 40 mM sodium phosphate to remove SDS.

The rat renin complementary DNA (cDNA; 100 ng) was labeled to specific activities of 1 to 2 × 10⁹ cpm/μg DNA by nick-translation. The probe used was a 1000 base pair renin cDNA cloned from a rat kidney library as described by Burnham et al. Signals were detected by autoradiography (Kodak x-ray film; Rochester, NY, USA) in the presence of an image-intensifying screen.

Reverse Hemolytic Plaque Assay
The release of renin was determined by modification of the reverse hemolytic plaque assay of Leong et al. reported for detection of growth hormone released from pituitary cells. Renal cortical cells for the reverse hemolytic plaque assay were dispersed and separated on Percoll gradients using Roswell Park Memorial Institute (RPMI) culture media 1640 (Gibco, Chagrin Falls, OH, USA). The enzyme solution included 100 μg/ml, 100 μl/ml, and streptomycin (Gibco), 100 μg/ml. An equal volume of monodispersed kidney cell suspension (10⁷ cells/ml) was mixed with staphylococcal protein A-conjugated sheep erythrocytes (12% vol/vol) and allowed to attach to poly-L-lysine-coated glass slides (Cunningham chamber). Each chamber held approximately 30 μl of solution. Slides were placed in a large Petri dish lined with moist tissue paper to provide humidity and incubated for 75 minutes in a water-jacketed CO₂ incubator at 37°C with an atmosphere of 95% air, 5% CO₂.

Rabbit antirat renin antibody, diluted 1:60 with RPMI containing 0.1% BSA (Sigma) and either 2.5
mM CaCl₂ or 0.8 mM EGTA was introduced into the Cunningham chamber (completely exchanging the fluid volume) by capillary action and incubated for 2 hours at 37°C. Complement from guinea pig serum was prepared as previously described by Leong et al.²⁶ In brief, guinea pig serum was incubated with protein A-coated sepharose beads to remove antibodies (immunoglobulin) to sheep erythrocytes, which may cause nonspecific background lysis. After centrifugation to remove the sepharose beads, the supernatant was aliquoted and stored at −70°C. Immediately before use, an aliquot was thawed and diluted 1:20 with ice-cold RPMI-BSA medium. Each chamber then received 75 µl of the medium and was returned to the incubator for 15 to 25 minutes. Once plaques (areas of hemolysis) were clearly visible, trypan blue was added to the chambers to determine the viability of the central plaque-forming cells. Chambers were then fixed by infusion with 2% glutaraldehyde (Sigma) in RPMI medium 1640. The coverslips were removed, and the slides were washed 3 times in deionized water and allowed to air dry. Each slide was then stained with 0.05% azure II, 0.02% methylene blue, and 0.05% sodium borate in distilled water for 30 seconds. Plaque number and area were determined by direct microscopic examination (Leitz Orthoplan microscope; Rockleigh, NJ, USA) with the aid of a videocamera (Dage 68, MTI) and a Videoplan computer (Zeiss, Oberkochen, West Germany).

Statistics
Student’s paired or unpaired t test or Duncan’s analysis of variance (followed by Dunnett’s test) was used to determine differences in mean values, and the results are presented as means ± SE. A p value less than 0.05 was considered statistically significant.

Results
When dispersed rat kidney cortical cells were separated by ultracentrifugation according to density on a 40% isosmotic Percoll gradient two, and sometimes three, distinct bands of cells were observed. There were other cell layers at a variety of densities, but we concentrated on well-defined upper and lower cell layers (Layers A and B, respectively) that could be identified consistently. For the most part, the other layers tended to be composed of single cells, doublets, triplets, and some clumps of cells. Also, a layer of high renin activity was identified as a granular layer, as it was not sedimentable at low speeds but did sediment at high speeds (100,000 g). As can be seen from Figure 1, renin activity was greater in the lower layer, Layer B, than in the upper layer, Layer A (77.4 ± 31 vs 11.3 ± 4.0 ng ANG I/mg protein/hr; n = 10, p < 0.05). Comparison of renin-specific activity in Layer B with that of the cortical extract placed on the Percoll gradient revealed an approximately 36-fold increase in renin-specific activity in Percoll Layer B. The amount of renin activity placed on each Percoll tube varied (range, 1–17 ng ANG I/mg protein/hr) but was usually about 3.0 ng ANG I/mg protein/hr. The renin activity recovered in Layers A and B averaged 27% of the total renin activity (cortical extract) loaded onto the Percoll gradients (approximately 33% of protein was recovered in Layers A and B). Protein and renin activity were distributed throughout the Percoll tube, but nowhere as concentrated as in Layers A and B. The remainder of the protein and renin activity probably can be accounted for by cell doublets, triplets, and perhaps, free granules. Comparison with a simultaneously spun tube containing standard density beads revealed that the renin-rich Layer B sedimented at approximately 1.067 g/ml. Thus, the renin-rich layer (n = 7, p < 0.05) corresponded to a density of 1.067 g/ml on Percoll. When cultured rat aortic smooth muscle cells (gift of Dr. Gary Owen), aortic-derived endothelial cells (gift of Dr. Michael J. Peach), or renal mesangial cells (gift of Dr. Jeffrey Kreisberg) were spun on a 40% standard isosmotic Percoll continuous gradient sedimentation densities were 1.017, 1.033, and 1.048 g/ml, respectively.

Figure 2 illustrates the effect of pH on the generation of ANG I by rat renin with homologous substrate in five experiments. As can be seen in Figure 2, the pH optimum was between 6.0 and 7.0 in the presence of 5 mM EDTA and 2 mM PMSF. Addition of 5 mM EGTA or 0.8 mM CaCl₂ decreased renin activity. The effect of pH was neutralized by the addition of 0.1 M Tris buffer, pH 7.0. As can be seen from Figure 2, the pH optimum was between 6.0 and 7.0 in the presence of 5 mM EDTA and 2 mM PMSF. Addition of 5 mM EGTA or 0.8 mM CaCl₂ decreased renin activity. The effect of pH was neutralized by the addition of 0.1 M Tris buffer, pH 7.0.

Figure 1. Rat renal cortical cells were dispersed (enzymatic treatment) and separated according to density by ultracentrifugation (29,000 g for 20 minutes) on 40% isosmotic Percoll. The tube on the left depicts the location of standard density beads (g/ml) spun, in 40% isosmotic Percoll, simultaneously with the tube on the right containing dispersed kidney cortical cells. Comparison with the tube on the left reveals that cells in Layer B, which exhibited most of the renin activity (n = 10, p < 0.05), sedimented at approximately 1.067 g/ml. Thus, the renin-rich layer is Layer B and corresponds to a density of 1.067 g/ml on Percoll.

TABLE 1

<table>
<thead>
<tr>
<th>Layer</th>
<th>Renin Activity (ng ANG I/mg protein/hr)</th>
<th>P (g/ml)</th>
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<tbody>
<tr>
<td>Layer A</td>
<td>11.3 ± 4.0</td>
<td>1.033</td>
</tr>
<tr>
<td>Layer B</td>
<td>77.4 ± 31</td>
<td>1.067</td>
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EFFECT OF pH ON RENIN ACTIVITY

FIGURE 2. Incubation of rat renin (obtained from cells in Percoll Layer B, shown in Figure 1) with homologous substrate in the presence of 3 mM EDTA and 2 mM PMSF revealed a pH optimum for ANG I generation of approximately 6.0 ng/hr/mg protein (p < 0.05). The pH curve was not altered by addition of 5 mM captopril, 5 mM British anti-Lewisite, or 1 mM 8-OH-quinoline. Values are means ± SE of five experiments.

captopril, 5 mM British anti-Lewisite, or 1 mM 8-hydroxyquinoline did not alter the shape of the pH curve (three experiments, results not shown).

Figure 3 depicts the inhibition of renin activity by prior incubation with rabbit antirat renin antibody. Rat renin (cell lysate obtained from Percoll Layer B) and rabbit antirat renin antibody (1:30 dilution) were incubated at 4°C for 18 to 22 hours prior to generation of ANG I from homologous substrate. Renin activity fell from 54.8 ± 4.0 to 2.4 ± 1.9 ng ANG I/mg protein/hr (95% inhibition; n = 8, p < 0.05).

Immunocytochemical staining with the immunoperoxidase technique revealed renin-positive cells (brown cytoplasm) in the renin-rich Layer B, as can be seen in Figure 4. Controls, including omission of the rabbit antirat renin antibody and substitution of rabbit serum for antirat renin antibody, were negative. The rabbit antirat renin antibody recognized inactive prorenin and the active renin proteins.

Figure 5A is an electron photomicrograph of an isolated renal cortical cell containing numerous granules and protogranules. Figure 5B, a higher magnification, reveals the ultrastructure of the protogranule and Golgi body apparatus.

Renin messenger RNA (mRNA) was identified by hybridization analysis (Northern blot technique). Figure 6 is a Northern blot analysis using the 1000 base pair Eco RI fragment of the rat renin cDNA. Total RNA was extracted from the different cell preparations, and 8 µg of total RNA was placed in each Northern gel lane as follows: cortical extract (medulla removed), Percoll Layer A, Percoll Layer B. This analysis revealed the greatest fraction of renin mRNA specific activity (renin mRNA/total RNA) in Percoll Layer B, followed by that derived from cortical extract and, finally, by that from Percoll Layer A. This rank order has been consistent in data derived from 12 separate rat preparations. Another experiment (Figure 7) using the same procedure (different nick-translation, cDNA specific activity, and film exposure time from that of Figure 6) compared the renin mRNA specific activity of whole kidney extract (total kidney) to that of cortical extract. As can be seen from Figure 7, the greatest fraction of renin mRNA was in the cortical extract. Thus, the renin mRNA signal was greatest in the following rank order: Percoll Layer B (density 1.067 g/ml), kidney cortical extract, whole kidney, Percoll Layer A.

Figure 8 illustrates a representative plaque (identified by central clearing around a single renin-containing cell) formed by complement-mediated lysis of sheep erythrocytes. Sheep erythrocytes, conjugated with staphyloccocal protein A, were mixed with dispersed renal cortical cells and allowed to attach to poly-L-lysine-coated glass slides forming a Cuningham chamber. The cells were incubated with rabbit antirat renin antibody (1:40 dilution) in culture medium containing 2.5 mM calcium or calcium-free medium for approximately 2 hours. Subsequently, hemolysis was provoked by introduction of freshly thawed
FIGURE 4. Immunocytochemical staining (immunoperoxidase technique) reveals renin-positive cells in the renin-rich layer (Percoll Layer B; magnification × 350).

FIGURE 5. A. Electron micrograph of cells obtained from rat cortex reveals numerous granules. Protogranule is identified by arrows (original magnification × 30,000). B. Protogranule enlarged (original magnification × 189,000). (Photograph courtesy of Susan I. Ramos, VSM Electron Microscopy Core.) Original magnification has been moderately reduced (see bar scale).
The renin-containing kidney cortical cells had the greatest density when compared with rat aortic-derived smooth muscle, endothelial cells, and rat renal mesangial cells. Knowledge of the sedimentation density (approximately 1.067 g/ml for the renin-containing cells) allowed us to locate the renin-containing cells, regardless of which Percoll gradient was selected for cell separation.

We sought to compare our yield of isolated renin-containing cells with those of others. Attempts to quantitate differences in the various preparations were thwarted by the use of different reagents and conditions for cell isolation, different definitions of the JG cell (e.g., use of renin activity, morphology, immunohistochemistry), and occasionally, by insufficient experimental detail to ascertain the degree of enrichment of the isolated population of cells.

Discussion

In the present study we used enzymatic dispersion and ultracentrifugation in Percoll to separate a population of renin-containing rat kidney cells according to density. Comparison with standard (colored) density beads, spun simultaneously in a similar Percoll tube, allowed determination of the sedimentation density of each cell layer. We also compared the sedimentation density of our population with those of other cell types derived from cultures of homogenous populations.
**Renin synthesis** that is quite consistent with the hypothesis that the renin-containing cells synthesize renin protein(s).

Next, we sought evidence that the isolated cells had maintained functional ability despite the experimental manipulation required to isolate the renin-containing cells. Based on enzymatic activity, calcium has been reported to regulate the release of renin from the JG cells. Using the reverse hemolytic plaque assay, we documented the release of renin (proteins) from the renin-containing cells and defined their ability to respond to a physiological regulatory signal (calcium). Moreover, the release of renin was related inversely to the calcium concentration. These results clearly exclude the possibility that the inverse relationship of calcium and renin release may result from an effect of calcium on posttranslational modification or activation of renin.

In summary, we have used enzymatic dispersion and density gradient (Percoll) sedimentation to isolate a population of renin-containing, granule-laden cells. Using immunocytochemistry (light microscopy) and electron microscopy, we defined the presence and ability of these cells to store renin protein(s). Using techniques of molecular biology and recombinant DNA, we have documented that these cells express the renin gene. The reverse hemolytic plaque assay defined the functional properties of the renin-containing cells and, in a fashion unique to date, postulated the inverse relationship between calcium concentration and release of renin. Thus, we have isolated a population of functional rat kidney cells that synthesize, store, and release renin. These properties have heretofore been ascribed to JG cells. Current work is underway to define the precise in situ location of the renin-containing cells and the regulation of posttranslational processing of renin protein(s).

**Figure 8.** Representative plaque (central clearing) formed by complement-mediated lysis of sheep erythrocytes provoked by release of renin from rat renal cortical cell (central cell). Sheep erythrocytes conjugated with staphylococcal protein A were mixed with dispersed kidney cortical cells and allowed to attach to poly-L-lysine-coated glass slides (Cunningham chamber). Renin antibody (1:40 dilution) in Roswell Park Memorial Institute culture medium containing either EGTA or calcium (2.5 mM) was introduced into the Cunningham chamber and incubated approximately 2 hours at 37°C. Freshly thawed complement (1:20 dilution) was then added to the chamber, and after 15 to 25 minutes the slides were checked for hemolysis. Once hemolysis occurred, the cells were fixed in glutaraldehyde and stained with azure II and methylene blue. The plaques were counted using a light microscope, and the plaque number and area were determined by Videoplan computer (magnification × 120).

**Figure 9.** Reduction of calcium increased renin release. Exposure of cells to low calcium medium (0.4 mM CaCl₂ + 0.8 mM EGTA) increased the number of hemolytic plaques formed (p < 0.05). Values are means ± SE of seven experiments.
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