Responses of Juxtaglomerular Cell Suspensions to Various Stimuli

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SUMMARY Cell suspensions were prepared from rat renal cortical tissue by dispersion with 0.1% collagenase. Unit gravity sedimentation in a 1%-4% Ficoll gradient resulted in a single-cell suspension enriched in juxtaglomerular (JG) cells. Both the cellular renin activity and the amount of renin released into the supernatant increased with time when the suspensions were incubated for 1 hour at 37°C in tissue culture medium. These cells responded to epinephrine and norepinephrine by increasing both synthesis and release of renin. The response was blocked by timolol but not by phenoxybenzamine. Cell suspensions prepared in the same manner but using 0.25% trypsin as the dispersing enzyme neither synthesized nor released renin into the tissue culture medium when similarly incubated. Trypsin-dispersed cells did not respond to catecholamine stimulation. Renin synthesis and release in collagenase-dispersed JG cells were unaltered by changes in Na, K, or Ca ion concentrations. Angiotensin II inhibited release, while saline extracts of clipped kidney from renal hypertensive rats stimulated renin release by these cells. (Hypertension 3: 157-167, 1981)

KEY WORDS • juxtaglomerular cells • renin release • renin synthesis • rat kidney • angiotensin II

RENIN release in vivo is postulated to be regulated by any or all of three mechanisms: 1) an adrenergic receptor and the sympathetic nervous system; 2) two intrarenal receptors, the macula densa and the renal baroreceptor; and 3) agents such as sodium, potassium, calcium; and angiotensin II. The question of how these mechanisms interact and to what extent the juxtaglomerular (JG) cell responds directly to stimuli have been extensively investigated in vivo. However, in vitro studies of renin release from JG cells have generally suffered from the inability to isolate the JG cell response from effects of the macula densa and other influential structures, and from an inability to assess released renin as being either preformed or newly synthesized. A number of in vitro systems have been used to study renin release, including kidney cortical slices, isolated glomeruli, and renal cortical cell suspensions.

Studies using renal cortical slices have several difficulties. Renin may passively leak from cut surfaces of such slices, and even though there is no tubular filtration, the macula densa cells may still be capable of influencing the function of the JG cells. In addition, although the composition of the incubation medium is known, the concentration of nutrients and ions bathing secretory cells within the slice may vary. Isolated glomeruli include only about 12% of renin-containing cells still attached. Since the majority of JG cells are found in the media of the afferent arteriole, experimental results would be affected by the presence and length of those vessels remaining attached to the glomeruli. These preparations may also suffer from incomplete bathing of all cells by the nutrient fluid.

Both Lyons and Churchill and Michelakis et al. have reported some success in using collagenase-dispersed renal cortical cell suspensions for examination of renin activity. Michelakis et al. incubated dog renal cortical cell suspensions with epinephrine, norepinephrine, or cAMP, and measured the total renin activity of each sample after homogenizing the entire incubation mixture. They reported an increase in total renin in response to both catecholamine and cAMP stimulation but did not distinguish between renin synthesis and renin release.
Studies of the role of sodium chloride in renin release in vitro have provided conflicting results. According to the generally accepted theory of Vander and Miller, increases in tubular sodium chloride concentration or load at the macula densa would produce a decrease in release of renin. Using rat or dog kidney slices, Michelakis and Morimoto et al. have found the expected inverse relationship, while others have shown a directly proportional response. Lyons and Churchill also reported an inverse response with decreasing sodium ion concentration using cell suspensions. Isolated rat kidney glomeruli were also shown to respond directly to increases in sodium chloride concentrations. Similarly varied results have been found with calcium. In the isolated glomeruli, decreasing the superfusate calcium from 2 mM to 0 led to a threefold increase in renin release. Conversely, rat kidney slices were shown to release renin: 1) in direct proportion to the calcium concentration; 2) after prior calcium depletion; or 3) not at all. It is therefore difficult to reach a conclusion on whether ions can produce a direct response by JG cells.

This report describes the preparation of a suspension of single renal cortical cells enriched in JG cells and suitable for measuring cellular renin synthesis as well as renin release. Effects of preparing these cells with both trypsin and collagenase were investigated, their response to catecholamines was evaluated, and we examined the direct effects of sodium, potassium, calcium, angiotensin II, and several kidney extracts on the synthesis and release of renin. In addition, samples of supernatant containing the released renin were acidified in an attempt to activate any inactive renin present.

Methods
Preparation of Cell Suspensions
The medium used throughout was prepared from powdered Leibovitz's medium L-15 (Grand Island Biologicals), which maintains a constant pH of 7.4 in room air without using 5% CO2. This medium was supplemented with 2 g/liter of glucose. Kidneys were removed from five decapitated female Sprague-Dawley rats (175-200 g each), and placed in cold (4°C) medium. The animals had been maintained on a normal rat diet containing 0.4% sodium (Ralston-Purina, Lab Chow Checkers) and were not sodium-depleted. After thorough washing, the kidneys were decapsulated, the cortical tissue removed, finely minced, and incubated with 20 volumes of either 0.1% collagenase (type I, Worthington Biochemicals), or 0.25% trypsin (Sigma Chemicals) in tissue culture medium for three 10-minute intervals with gentle agitation. At the end of each interval, the tissue was allowed to settle, and the supernatant containing the cells was poured through sieves of 150 and 200 mesh (U.S. Standard, Tyler Screens). Tubular fragments left on the 200 mesh screen were discarded, and glomeruli left on the 150 mesh screen were returned to the incubation flask for further digestion with fresh enzyme solution. Cells that passed through the 200 mesh screen were collected by centrifugation at 50 g for 7-10 minutes, followed by three washings with medium L-15. Softened tissue remaining after the last enzyme treatment was poured onto the 150 mesh screen and pressed through the mesh with a spatula while being washed with medium. The cells were collected by centrifugation with medium L-15. The washed cell pellet was resuspended in L-15 medium and poured through a column of glass wool to remove cell clumps.

Cell Separation by Unit Gravity Sedimentation
A 4.5 x 15 cm density gradient column was prepared by dropwise addition of 175 ml of 4% Ficoll (type 400, Sigma Chemical) in L-15 medium from a 500 ml separatory flask into a stoppered 500 ml Erlenmeyer flask containing 175 ml of 1% Ficoll in L-15 medium. A siphon from the Erlenmeyer flask was attached to the bottom of the column, which filled by gravity. After approximately 300 ml of Ficoll solution entered the column, 10% Ficoll solution was added to the bottom of the column to raise the gradient to within 5 cm of the top of the column. Ficoll was added to a cell suspension prepared as described above to make the medium 1% (w/v) in Ficoll. This suspension was then rapidly applied through a fine nylon mesh sieve onto the top of the density gradient column. The cells were allowed to settle for 2 hours at unit gravity in the cold (4°C); then 15 ml fractions were collected by allowing the Ficoll solution to flow slowly out of the column. Those fractions containing the renin-releasing cells, as identified by angiotensin I generation when incubated with renin substrate, or by Bowie stain, and/or thioflavin T stain, were pooled and used for experimentation. Viability of the cells was tested by their refractility and ability to exclude Trypan blue. One drop of 0.4% Trypan blue in 0.9% saline was added to one drop of cell suspension. After 5 minutes the cells were examined by phase microscopy. The cells that were refractile and excluded Trypan blue were then enumerated and expressed as percentage of the total number of cells.

Preparation of Substrate
Plasma was prepared from rats bilaterally nephrectomized 48 hours earlier. Blood was collected using a syringe rinsed either with heparin (4000 units/ml) or 15% ammonium EDTA as anticoagulant and processed according to the method of Boucher et al. For renin determinations, 1.5 g of lyophilized substrate was dissolved in 29 ml of 0.05 M phosphate buffer, pH 6.5. Then 1% NaN3 (0.4 ml) and 15% ammonium EDTA (0.4 ml) were added and the pH re-adjusted to 6.5.

Determination of Renin Activity
First, 0.5 ml of each cell fraction from the gradient column was centrifuged at 50 X g for 10 minutes. The cells were then resuspended in 0.5 ml of 0.9% saline.
containing 5 mM N-ethyl maleimide. The suspension was frozen and thawed three times and centrifuged at 100 × g for 10 minutes. A 10 μl aliquot of the supernatant was incubated with 100 μl of rat renin substrate solution at pH 6.5, at 37°C for 15 minutes. One drop of 5% diisopropylfluorophosphate (DFP) in isopropanol was added to inhibit angiotensinases. The amount of angiotensin I generated was determined by radioimmunoassay (New England Nuclear). With this amount of substrate (5 mg) the reaction will be linear until about 6 ng of angiotensin I has been generated.18 In these assays, the amount of renin-containing sample was adjusted, if necessary, so that less than 5 ng of angiotensin I was generated for each sample. A sample of the incubation medium was assayed using the same procedure.

Staining Procedures

A 0.5 ml aliquot of each fraction was removed, centrifuged at 50 × g for 10 minutes, and resuspended in 100–500 μl of 10% formalin. After 10 minutes, the cells were collected again by centrifugation and most of the supernatant was removed, leaving just enough to make a heavy cell suspension. A drop of this suspension was spread on a glass microscope slide and allowed to air dry.

The stock Bowie stain was prepared from Biebrich Scarlet (Matheson, Coleman, and Bell) and ethyl violet (Allied Chemical) as described by Smith.40 The staining procedure was a modification of that developed by Smith. The slides were placed in 2.5% potassium dichromate for 2 hours, washed in running tap water for 30 minutes, and placed overnight in 20% ethanol containing 1–3 drops of stock Bowie stain. The slides were blotted, dipped in acetone to remove excess stain, and the cells were then differentiated in 1:1 xylene and clove oil. After washing in xylene, the slides were mounted and dried.

The Thioflavin T stain (Chroma-Gesellschaft, Stuttgart-Unterturkheim) was used according to the method of Harada.*1 Slides were incubated in Delafield hematoxylin for 0.5 minutes and then washed in tap water. This was followed by 3 minutes in 0.1% Thioflavin T stain. Differentiation was in 0.1% acetic acid for 1 minute, followed by washing and air drying. Screening of slides was done on a Zeiss Model G1-140 Photomicroscope.

Incubation and Collection of Samples

Fractions from the Ficoll column containing renin-releasing cells were pooled, counted on a Bright-line hemocytometer, washed in medium L-15, and diluted with medium to give 200,000 cells/ml. Tubes containing 3 ml of this cell suspension were preincubated for 15–20 minutes of 37°C with gentle agitation to allow the cells to equilibrate. The cells were again centrifuged at 50 g for 10 minutes, resuspended in 3 ml of fresh medium, and incubated at 37°C in air for 60 minutes with gentle agitation. They were not bubbled with gas during the incubation period. Triplicate samples (0.2 ml) were taken for renin assay before incubation and at 15, 30, and 60 minutes during the incubation. These samples were placed in test tubes containing one drop of 5% N-ethyl maleimide and immediately centrifuged, separated into supernatant and pellet fractions, and frozen. The renin activity in the supernatant fraction would represent renin released by the cells and the renin activity in the pellet fraction would represent cellular renin. The pellet was prepared for assay by reconstitution in a volume of PBS equivalent to the original supernatant volume, frozen and thawed three times to rupture cells, and centrifuged to remove particulate matter. An aliquot of the extract was diluted 1:10 for renin assay. Renin activity was determined as described above within 24 hours. Other samples were also taken during the incubation to determine cell viability by Trypan blue exclusion and refractility, and for cell counts on a hemocytometer.

Electron Microscopy

Fractions containing suspensions of granulated cells were pooled and applied to a Millipore filter (24 mm diameter and 0.45 μm pore size) with gentle vacuum filtration. After washing with medium L-15, freshly prepared fixative solution was added for 3- to 10-minute periods and removed with gentle vacuum. Fixative solution consisted of 1% osmium tetroxide, 1% acrolein, and 1.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) containing 2.5% sucrose. (The composition of this fixative solution was kindly given to us by Dr. Richard Briggs, Harvard Medical School, Department of Pathology, Boston, Massachusetts.) The Millipore membrane filter containing fixed cells was cut into small pieces, dehydrated in alcohol, followed by two 10-minute exposures to propylene oxide, and embedded in Epon. Thin sections were poststained with uranyl acetate and lead citrate and examined in a Zeiss AR-10 transmission electron microscope.

Effect of Catecholamines on Renin Release

A 0.1 ml aliquot of either norepinephrine or epinephrine in 0.1% ascorbic acid was added to 2.9 ml of cell suspension in L-15 medium to give a final agonist concentration of 10-4 M. Suspensions were incubated for 1 hour at 37°C with gentle shaking, and samples were collected as described under incubation and collection of samples. To test the specificity of the drug response, an alpha-adrenergic antagonist, phenoxybenzamine (final concentration 1.65 × 10-4 M), or a beta-antagonist, timolol (final concentration 1.8 × 10-4 M), was added 2–3 minutes prior to the addition of the agonist. Sampling was done as described before.

Effect of Cations on Renin Release

A modified Krebs' buffer with the following composition was used: Na+ 146 mM, K+ 4.7 mM, Ca++ 2.5 mM, Mg++ 1.2 mM, tris 0.01 M, phosphate 1.3 mM, glucose 2 mg/ml, and bovine serum albumin...
0.5%, at pH 7.4. For each experiment, the concentration of each cation being tested varied; all other constituents were constant. Initially, sodium concentrations of 25, 50, 100, and 150 mM were used; later, 110, 130, and 150 mM were used. In both cases, the cells were preincubated in L-15 medium as previously described. After centrifugation the cells were resuspended in the Krebs' buffer containing the desired cation concentration. Samples were assayed as described in the previous experiments. The calcium ion concentrations examined were 1.5, 3.0, and 6.0 mM.

Preparation of Tissue Extracts

Rats were made hypertensive by placing a 0.2 mm silver clip on the left renal artery. After 4 weeks, animals with a consistent blood pressure over 170 mm Hg, as measured by the tail pressure cuff, were killed. The clipped and contralateral kidneys were used to prepare extracts. Cortical tissue was also removed from the kidney tissue of normal rats for extract preparation. The tissue weights were recorded and then separately homogenized in Krebs' buffer. The homogenate was centrifuged at 50,000 g for 20 minutes. The pellet was washed twice with Krebs' buffer, and all the supernatants were pooled. The final volume was adjusted so that each ml of extract corresponded to 1 mg of cortical tissue.

Effect of Kidney Extracts on Renin Release

Extract (0.5 ml) from either normal, contralateral, or clipped kidneys was added to 2.9 ml of medium L-15 containing 200,000 cells per ml. Samples were taken as previously described. Renin activity of the extracts and samples was measured simultaneously, and the activity of the extracts was subtracted from each sample. Extracts were also incubated alone to determine possible changes in the renin activity of the extract itself during incubation. To determine if the renin release in response to the clipped kidney could be inhibited by a protein synthesis inhibitor, cycloheximide (0.5-2.0 mg/ml final concentration) was added to the cell suspension prior to the start of incubation. Samples were collected and assayed as above.

The clipped kidney extract was dialyzed for 24 hours against distilled water to determine whether the stimulatory factor could be dialyzed. The dialysate was corrected for volume change and added to the incubation mixture as described. Sampling and assaying were carried out as for the clipped kidney extract.

To ensure that the clipped kidney extract stimulation was not a result of catecholamines present in the extract, adrenergic antagonists, phenoxybenzamine (1.65 x 10^-4 M final concentration) and timolol (1.8 x 10^-4 M final concentration), were added to the incubation medium. Indomethacin (1 mg/ml final concentration) was added to eliminate an effect due to an increase in prostaglandin synthesis. Samples were assayed as for the clipped kidney extract. All drugs were dissolved in 0.1% ascorbic acid and diluted with L-15 tissue culture medium.

Activation of Inactive Renin

The supernatant fluid remaining after 60 minutes of incubation was dialyzed for 24 hours against a pH 3.3 buffer containing 0.05 M glycine-HCl, 0.1 M NaCl, 3 mM Na,EDTA, and further dialyzed back to pH 7.4 against 0.01 M sodium phosphate containing 0.05 M NaCl and 3 mM Na, EDTA. This procedure produces activation of inactive renin in human plasma and amniotic fluid. The dialysate was corrected for volume, and the renin activity was measured at the same time as the undialyzed sample.

Effect of Angiotensin II on Renin Release

Angiotensin II, synthesized by Dr. M.C. Khosla in our laboratory, was added to the cell suspensions at final concentrations of 10^-6, 10^-7, 10^-8, and 10^-4 M. Incubations, sampling, and assaying were done as above.

Calculations

For each cell preparation, the initial renin activity of the cells was determined by taking triplicate cell samples immediately prior to the start of incubation. These triplicate samples varied by 10% in renin activity when taken from a single cell pool and served to establish the initial renin activity for the cell pool used for each experiment. Each experiment was conducted with a single cell pool. The assumption involved in this measurement is that all cell types are equally distributed at the time of sampling. Since the initial renin activity varied from one cell preparation to another (about 60%), the data were normalized by conversion to percent change from the time 0 determination to permit comparison of the results from several different cell preparations used for replicate experiments. This normalization was used only to permit comparison of findings among different cell preparations and not to reduce variance. Statistical comparisons were made by paired t test to compare a control sample to an experimental (drug or extract-treated) sample derived from the same initial cell pool and incubated for the same length of time. Differences were considered significant when p < 0.05.

Results

Separation of Cells by Unit Gravity Sedimentation

Two hours of unit gravity sedimentation resulted in a separation of renin-releasing cells from the bulk of cells (fig. 1). Approximately 10% of the cells (Fractions 6-8 from the top of the gradient column, which is Fraction 1) contained over 60% of the renin activity. These fractions contained two main cell types as observed by light microscopy. One, a relatively small cell (about 7 to 10 μm in diameter), exhibited heavy granulation when stained with either Bowie stain or Thioflavin T stain. The other larger cell (about 15 to 18 μm in diameter) was covered with long microvilli with some showing a brush border, suggesting proximal tubule cells. The cells that stained with Bowie...
stain in the high renin fraction (Fractions 6–8) represented 15% ± 3.2% of the total number of cells from six trials. Viability after sedimentation ranged from 74% to 81% in all fractions from three trials. Renin activity of the high renin fractions used for experimentation ranged from 400 to 1000 ng AI/10^8 cells/hr for collagenase-prepared cells and from 0 to 31 ng AI/10^8 cells/hr for trypsin-treated cells. Because of this variability from one cell preparation to another, most of the results of individual experiments are presented as percent change from time 0.

By electron microscopy, however, three different cell types were observed in these high renin cell fractions from collagenase-digested tissue. Figures 2–4 are representative of the cell types observed. As seen in figure 2 (left), the cytoplasm was characterized by the presence of numerous granules (690 ± 360 nm in diameter) with a homogeneous appearing matrix. Present were numerous mitochondria and a small amount of rough endoplasmic reticulum (RER) that was slightly distended and filled with a material of moderate electron density. The golgi apparatus was well developed and appeared active (fig. 2 right).

**FIGURE 1.** Representative separation of collagenase-dispersed rat renal cortical cells on 1 to 4% Ficoll gradient column.

**FIGURE 2.** Electron micrographs of representative cell types from Fractions 6 to 8 (see fig. 1) showing high renin activity. Cells of this type contain numerous mitochondria and large moderately electron dense granules. A well developed and active golgi apparatus is present. Abbreviations: g = golgi apparatus; gr = granules; mt = mitochondria; mv = microvilli; v = vacuoles; mf = microfilaments; rer = rough endoplasmic reticulum. × 13,000 (left) and × 20,500 (right).
The second type of cell (fig. 3 left) was characterized by the presence of numerous, heavily-stained cytoplasmic granules (470 ± 130 nm), and a prominent, distended RER filled with a flocculent, moderately electron-dense material. Numerous 6.0 to 6.5 nm microfilaments appeared within the cytoplasm. Vacuoles containing electron-dense material appeared to be forming from both the RER and the golgi (fig. 3 right). These characteristics suggest a modified smooth muscle cell and are consistent with those described for JG cells. Figure 4 shows a vacuolated cell containing a few granules and numerous mitochondria. The presence of long microvilli is suggestive of a proximal tubule cell.

In Vitro Release of Renin

The effect of collagenase and trypsin as cell-dispersing enzymes on renin release is shown in table 1. Collagenase-dispersed cells released renin into the supernatant medium when incubated at 37°C in tissue culture medium L-15 (table 1). The amount released after 60 minutes was 153 ng AI/10⁸ cells/hr, a significant increase from the start of incubation (p < 0.01). Cellular renin activity (table 1) increased after 1 hour from 708 ng AI/10⁸ cells/hr to 1513 ng AI/10⁸ cells/hr, also a significant increase of 114% (p < 0.02).

Trypsinized cells released 1.40 ng AI/10⁸ cells/hr after 60 minutes incubation (table 1), and this was not significantly different from that released by nonincubated control cells. The cellular renin content of these cells after 60 minutes of incubation (table 1) was not different from that at the start of incubation (12.2 ± 8.5 ng AI/10⁸ cells/hr vs 12.7 ± 18 ng AI/10⁸ cells/hr).

Effect of Catecholamines on Renin Release

Collagenase-prepared cells released more renin into the supernatant in response to added norepinephrine (NE) (5.4 × 10⁻⁴ M) than did cells incubated without NE (table 2). The addition of timolol prior to the addition of NE to the incubation medium not only abolished the NE response, but also inhibited renin release, i.e., there was less of an increase than with control cells. Addition of phenoxybenzamine (PBZ) to the incubation mixture prior to addition of NE gave the same results as NE alone.

Cellular renin activity also increased after 60 minutes when NE was added to the incubation medium (table 2). The total increase in cellular renin activity was 229% with NE and 138% when it was omitted. When timolol was added along with NE, the increase in cellular renin activity after 60 minutes was...
TABLE 1. Renin Production by Collagenase and Trypsin-Dispersed Rat Renal Cortical Cells at the End of 60 Minutes of Incubation (n = 5)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Collagenase-dispersed cells†</th>
<th>Trypsin-dispersed cells†</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Cells</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>63 ± 31</td>
<td>708 ± 756</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>92 ± 33</td>
<td>830 ± 98</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>120 ± 32*</td>
<td>1200 ± 457</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>153 ± 32†</td>
<td>1513 ± 419*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supremenat*</td>
<td>Cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.4</td>
<td>12.7 ± 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 ± 2.4</td>
<td>13.3 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 ± 0.2</td>
<td>11.7 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.4 ± 0.2</td>
<td>12.2 ± 9</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.02 when compared to time 0 sample.
†P < 0.01 when compared to time 0 sample.
‡Units = ng AI produced/10^6 cells/hr.

not significantly different from that of the control cells. When PBZ was added with NE, however, the difference after 60 minutes was significantly higher than with the control cells and similar to values obtained with NE alone.

The addition of epinephrine (Epi) resulted in effects similar to those of NE addition (table 2). At 60 minutes, the increase in renin release into the medium in response to Epi was 220% as compared to 108% for the control cells. Addition of timolol along with Epi suppressed this response to Epi, and at 60 minutes, renin release was significantly lower than with Epi alone or with control cells. Epi together with phenoxybenzamine produced a significant increase in renin release into the medium over control cells at 30 minutes, but this was not significantly higher than the increase observed with Epi alone. After 60 minutes of incubation with both Epi and PBZ, renin release was 32% higher than with the control, which was not statistically significant.

Greater cellular renin activity was observed in the presence of Epi over that of control cells, and this was not altered by the addition of timolol. However, these increases were not statistically significant. Addition of PBZ with Epi resulted in an increase in total cellular renin activity of 320% after 60 minutes. This was significantly higher than the control cell increase of 113%, but not significantly higher than the value of 203% obtained with Epi alone.

The number of viable cells did not change during any of the incubations presented in this report. To illustrate this point, the data for cell viability during the above experiments are presented in table 3 as an example of a typical experiment.

TABLE 2. Renin Production by Collagenase-Dispersed Rat Renal Cortical Cells at the End of 60 Minutes of Incubation With Catecholamines (n = 3)

<table>
<thead>
<tr>
<th>Additive in medium</th>
<th>Supernatant*</th>
<th>Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>108 ± 12</td>
<td>138 ± 11</td>
</tr>
<tr>
<td>Norepinephrine (5.4 × 10^-5 M)</td>
<td>150 ± 31†</td>
<td>229 ± 45†</td>
</tr>
<tr>
<td>Norepinephrine (5.4 × 10^-5 M) plus timolol (1.8 × 10^-4 M)</td>
<td>50 ± 26†</td>
<td>173 ± 27</td>
</tr>
<tr>
<td>Norepinephrine (5.4 × 10^-5 M) plus phenoxybenzamine (1.7 × 10^-4 M)</td>
<td>187 ± 34†</td>
<td>256 ± 22§</td>
</tr>
<tr>
<td>Epinephrine (5.4 × 10^-6 M)</td>
<td>220 ± 55§</td>
<td>203 ± 49</td>
</tr>
<tr>
<td>Epinephrine (5.4 × 10^-6 M) plus timolol (1.8 × 10^-4 M)</td>
<td>55 ± 13§</td>
<td>158 ± 32</td>
</tr>
<tr>
<td>Epinephrine (5.4 × 10^-6 M) plus phenoxybenzamine (1.7 × 10^-4 M)</td>
<td>140 ± 20</td>
<td>320 ± 106¶</td>
</tr>
</tbody>
</table>

*All results expressed as percent change from time 0 sample.
†P < 0.05, as compared to control cells for the same time interval.
‡P < 0.02.
§P < 0.01.
¶P < 0.01.
TABLE 3.  
Number of Viable Cells \( \times 10^3 \) as Determined by Trypan Blue Exclusion

<table>
<thead>
<tr>
<th>Medium</th>
<th>0 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>191 ± 10</td>
<td>219 ± 10</td>
<td>195 ± 21</td>
<td>200 ± 4</td>
</tr>
<tr>
<td>Epinephrine (1 ( \times 10^{-6} ) M)</td>
<td>202 ± 15</td>
<td>203 ± 4</td>
<td>194 ± 4</td>
<td>194 ± 4</td>
</tr>
<tr>
<td>Epi + timolol</td>
<td>200 ± 5</td>
<td>196 ± 3</td>
<td>210 ± 16</td>
<td>207 ± 12</td>
</tr>
<tr>
<td>Epi + PBZ</td>
<td>207 ± 7</td>
<td>204 ± 10</td>
<td>197 ± 4</td>
<td>186 ± 11</td>
</tr>
<tr>
<td>Noepsi</td>
<td>192 ± 6</td>
<td>195 ± 8</td>
<td>214 ± 22</td>
<td>199 ± 3</td>
</tr>
<tr>
<td>NE + timolol</td>
<td>207 ± 9</td>
<td>198 ± 10</td>
<td>195 ± 7</td>
<td>201 ± 4</td>
</tr>
<tr>
<td>NE + PBZ</td>
<td>212 ± 4</td>
<td>204 ± 5</td>
<td>202 ± 16</td>
<td>212 ± 10</td>
</tr>
</tbody>
</table>

When trypsin-prepared cells were incubated with either Epi or NE, the amount of renin released into the supernatant decreased with time, changing by \(-20.1\% ± 21.1\%\) after 60 minutes for Epi, and \(-15.0\% ± 14.1\%\) after 60 minutes for NE incubation. Neither value was significantly lower than the control value of \(11.6\% ± 23.2\%\). Cellular extracts of cells incubated with either NE or Epi showed a net increase in cellular renin activity after 60 minutes, but this increase was not different from the increase observed with control cell extracts.

Effect of Cations on Renin Release

Sodium chloride at concentrations of 150, 130, and 110 mEq/liter was examined for an effect on renin release (table 4). At concentrations less than 110 mEq/liter there was a loss of cell viability and so these results will not be included. At all time intervals tested, similar increases in renin release were observed with sodium chloride at concentrations lower than the normal 150 mEq/liter. Renin activity of the cell extracts showed a similar pattern (table 4). Since no correction was made for differences in osmolality, the results indicate that neither renin release nor cellular renin activity was affected by changes in osmolality. Similarly, neither low (2.35 mM) nor high (9.4 mM) concentrations of potassium ion altered the amount of renin released by these cells. However, these alterations in potassium concentrations did significantly reduce the amount of cellular renin after 60 minutes of incubation. Cells incubated in medium containing calcium ion at several concentrations showed no difference from normal control values either in renin release or intracellular renin activity.

Effect of Angiotensin II on Renin Release

Cells incubated with angiotensin II showed significant differences in renin released after 30 minutes incubation with all concentrations of angiotensin II tested (table 5). At that time interval, renin release was markedly suppressed as compared to control cells incubated in medium without angiotensin II. After 60 minutes, however, both control and treated cells had comparable amounts of renin in the media. Angiotensin II appears to have no effect on the cellular renin activity as evidenced by comparable increases in activity of cell extracts from treated and control cells (table 5).

Kidney Renin Activity of Extracts

Renin activity of the clipped kidney extract was 2032 ± 263 ng Al/mg/hr (n = 10), significantly higher than the value of 802 ± 72 ng Al/mg/hr (n = 10) observed for normal kidney extract (p < 0.001). The pooled contralateral kidney extract had a renin activity of 125 ± 57 ng Al/mg/hr (n = 8), which is significantly lower than that of the normal kidney (p < 0.001). After dialysis against distilled water for 24 hours, the clipped kidney extract had a renin activity of 1657 ± 168 ng Al/mg/hr (n = 10). If the extract was boiled, the renin activity was 4.2 ng Al/mg/hr (n = 2). When these extracts were diluted with tissue culture medium and incubated for 60 minutes, the percent change in renin activity was 9.3% ± 6.1%.

Effect of Kidney Extracts on Renin Release

When 0.1 ml (0.1 mg tissue) of extract from a normal kidney was added to cells in 2.9 ml medium (200,000 cells/ml) and incubated for up to 60 minutes, the amount of renin released did not differ from the amount released by the control cells incubated in medium alone (table 6). In addition, there was no difference in renin activity of cells incubated with normal kidney extract as compared to cells incubated without the extract. Renin release from cells incubated with extract from the contralateral kidney of hypertensive rats decreased, but this difference was not statistically significant (table 6). However, renin activity of cells incubated with the contralateral kidney extract did show a significant decrease from the control cells after 60 minutes incubation (114% vs 68%; p < 0.05).

Addition of the clipped kidney extract to the incubation medium caused a significant increase in renin

TABLE 4.  
Renin Production by Collagenase-Dispersed Rat Renal Cortical Cells at the End of 60 Minutes of Incubation With Medium Modified in Either Sodium or Potassium Concentration (n = 3)

<table>
<thead>
<tr>
<th>Ionic alteration</th>
<th>Supernatant*</th>
<th>Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium, 150 mEq/liter (normal)</td>
<td>97 ± 7</td>
<td>69 ± 13</td>
</tr>
<tr>
<td>Sodium, 130 mEq/liter</td>
<td>109 ± 6</td>
<td>57 ± 9</td>
</tr>
<tr>
<td>Sodium, 110 mEq/liter</td>
<td>81 ± 10</td>
<td>66 ± 15</td>
</tr>
<tr>
<td>Potassium, 4.7 mEq/liter (normal)</td>
<td>113 ± 38</td>
<td>94 ± 14</td>
</tr>
<tr>
<td>Potassium, 2.35 mEq/liter (low)</td>
<td>189 ± 20</td>
<td>47 ± 16†</td>
</tr>
<tr>
<td>Potassium, 9.4 mEq/liter (high)</td>
<td>116 ± 25</td>
<td>32 ± 5‡</td>
</tr>
</tbody>
</table>

*All results expressed as percent change from time 0 sample.
†p < 0.05, as compared to normal (Na, 150 mEq/liter and K, 4.7 mEq/liter) values for the same time.
‡p < 0.01.
release of 287 ± 72 for treated cells compared to an increase of 126 ± 37 for control cells (p < 0.05). Cells incubated with the clipped kidney extract showed the same renin activity as control cells incubated without any extract. Dialyzing the clipped kidney extract did not change its effect on renin release, but boiling the clipped kidney extract destroyed its effectiveness (table 6).

**Activation of Renin Released into the Medium**

The ratio of total renin activity after acid activation to renin activity before activation was 2.10 and 2.40 for two control cell samples; 1.83, 2.12, and 2.21 for three samples from cells incubated with clipped kidney extract; and 1.11, 1.13, and 1.16 for three samples from cells incubated with angiotensin II.

**Discussion**

With most of the previously utilized methods for examining JG cell function in vitro, it is difficult to quantitatively evaluate both renin released into the medium and the renin content of the cell. Both measurements are required to permit assessment of renin synthesis during the experiment as well as cellular release. Furthermore, the assessment of cell viability before and after the experiment is important for in vitro studies of renin release to exclude passive release of renin due to cellular damage or death. In addition, retention of cellular renin may be viewed as added evidence for retention of cell viability. Suspensions of single cells are required to permit examination of the direct action of various stimuli on the JG cell itself.

Preparation of cell suspensions by purely mechanical means provides poor cell yield and low viability.** Alternatively, enzymatic dispersion has the potential of good cell yield, but alters the cell membrane and interrupts normal function. For comparative purposes in our study, cells were dispersed with two commonly used enzymes, trypsin and collagenase. We have studied the effect of these two dispersing enzymes on renal cortical tissue and have shown that collagenase-dispersed cells synthesize and release renin into the supernatant medium and respond as expected to pharmacological stimuli, while cells dispersed with trypsin fail in both regards. No obvious differences were noted in cell viability or in gross cellular morphology under phase microscopy following treatment with either enzyme.

Trypsin yielded fewer cells from the same amount of cortical tissue, however. The amount of renin contained in the trypsinized cells after gradient sedimentation was markedly less than the amount found in collagenase-treated cells. This indicated that renin-containing cells were either more susceptible to trypsin or that trypsin was causing them to lose renin by impairing their ability for further renin synthesis. The collagenase-prepared cell not only contained vastly greater amounts of renin, but also demonstrated greater ability to synthesize and release renin when incubated in medium L-15 at 37°C for 1 hour. Incubated under identical conditions, the trypsinized cells showed no change in intracellular renin or in renin released into the medium. Lack of response to catecholamines by trypsin-dispersed cells compared to the positive response by collagenase-dispersed cells confirmed that trypsin was acting upon the cell in a detrimental way, as has been observed in other types of studies.**

Collagenase (type I, Worthington) is an enzyme preparation that contains proteases other than collagenase. Purified collagenase did not work well for preparation of cell suspension. This collagenase preparation has been used successfully to prepare suspensions of spermatogonic cells, adrenal glomerulosa cells, and smooth muscle cells.

---

**Table 5. Inhibition of Renin Release by Angiotensin II Using Suspensions of Rat Cortical Cells (n = 2)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control Cells</th>
<th>10^(-9)M Ang II</th>
<th>10^(-7)M Ang II</th>
<th>10^(-5)M Ang II</th>
<th>10^(-3)M Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant renin activity (expressed as percent change from time 0 sample):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>21.8 ± 14.0</td>
<td>46.3 ± 6.9</td>
<td>1.0 ± 11.7</td>
<td>13.5 ± 36.0</td>
<td>56.1 ± 19.8</td>
</tr>
<tr>
<td>30</td>
<td>59.3 ± 12.2</td>
<td>-0.6 ± 14.9*</td>
<td>18.6 ± 5.8*</td>
<td>12.0 ± 2.7*</td>
<td>12.8 ± 9.0*</td>
</tr>
<tr>
<td>60</td>
<td>100.9 ± 27.6</td>
<td>86.4 ± 17.8</td>
<td>126.7 ± 27.7</td>
<td>77.3 ± 12.0</td>
<td>103.6 ± 25.0</td>
</tr>
</tbody>
</table>

| Cellular renin activity (expressed as percent change from time 0 sample): | | | | | |
| 15 | 13.8 ± 10.9 | 8.0 ± 7.2 | 6.4 ± 10.7 | 8.0 ± 8.0 | 7.4 ± 10.5 |
| 30 | 28.3 ± 22.9 | 28.8 ± 10.8 | 19.8 ± 12.0 | 30.7 ± 20.0 | 24.1 ± 32.5 |
| 60 | 80.3 ± 39.8 | 56.0 ± 44.0 | 82.4 ± 49.3 | 60.9 ± 39.7 | 75.1 ± 47.9 |

*p < 0.01 as compared to control cell value for the same time interval.

---

**Table 6. Effect of Kidney Extracts on Renin Production by Collagenase-Dispersed Rat Kidney Cortical Cells at the End of 60 Minutes of Incubation (n = 3)**

<table>
<thead>
<tr>
<th>Extract added</th>
<th>Supernatant*</th>
<th>Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>126 ± 37</td>
<td>114 ± 28</td>
</tr>
<tr>
<td>Normal kidney</td>
<td>194 ± 66</td>
<td>88 ± 10</td>
</tr>
<tr>
<td>Contralateral kidney</td>
<td>86 ± 27</td>
<td>68 ± 13†</td>
</tr>
<tr>
<td>Clipped kidney</td>
<td>287 ± 72†</td>
<td>90 ± 21</td>
</tr>
<tr>
<td>Dialyzed clipped kidney</td>
<td>275 ± 50†</td>
<td>120 ± 29</td>
</tr>
<tr>
<td>Boiled clipped kidney</td>
<td>139 ± 54</td>
<td>88 ± 23</td>
</tr>
</tbody>
</table>

*All results expressed as percent change from time 0 sample.

†p < 0.05 as compared with control cells at the same time interval.
Collagenase-treated JG cells when incubated in L-15 tissue culture medium for 1 hour released substantial amounts of renin into the supernatant and the cell extract also showed an increase in renin activity (table 1). This net increase in renin indicated de novo synthesis of the enzyme. The amount of renin released into the medium at each time interval was approximately 9% of the total renin. This is much higher than the 2% to 3% reported by DeJong2 for the release of renin from rat kidney slices but is in close agreement with the 10% release from isolated dog renin granules reported by Funakawa et al.5 Funakawa's figure represents the maximum release by isolated granules when incubated in buffer of physiological pH at 37°C. This may be equivalent to the maximum basal release of renin by the intact cell.

When either NE or Epi was added to the incubation medium, the collagenase-prepared cells responded by increasing both the rate of renin synthesis and the rate of release. The specificity of this effect was shown by the ability of timolol, a beta antagonist, to suppress this response. PBZ neither inhibited nor enhanced the release of renin in response to catecholamine. After 60 minutes of incubation, the amount of renin activity in the cell was not significantly higher than that of cells incubated with Epi alone. However, the increase in synthesis as measured by cellular renin activity occurred more rapidly when PBZ was added than when it was absent. It is clear from these data that catecholamines have a specific direct effect on the JG cell in promoting synthesis and release. This is in agreement with in vitro results reported by Michelakis et al.10 with renal cell suspensions and by Weinberger et al. with cortical slices. Other workers6,11 have observed inhibition of renin release by an α-adrenergic receptor mechanism using cortical slices. In these studies, renin release was stimulated by placing the rats on a sodium deficient diet 2 weeks prior to removal of the kidneys. We do not know at this time whether this difference in diet can explain these differing in vitro responses. This evidence does not exclude the possibility that catecholamines may also act indirectly in vivo through arteriolar vasoconstriction to inhibit renin release.

The direct effect of ions on the release of renin by JG cells can be readily investigated by the use of these single cells suspensions since they consist of single cells devoid of clumps and tubular fragments, and the anatomical relationship between the JG cell and all other cell structures has been destroyed. To evoke a response, the ionic effect must necessarily be direct on the JG cell. The osmolarity was not corrected to account for changes in sodium chloride concentration, so any osmotic effect on the isolated cell would also be apparent. When the concentrations of sodium were below 100 mEq/liter, the amount of renin released into the medium appeared to be inversely proportional to the concentration. However, examination of the cells revealed that most of the cells were dead and many had ruptured, particularly at the lower sodium concentrations (25 and 30 mEq/liter). With fewer intact cells, the renin activity measured the highest. For that reason, results obtained at those concentrations were disregarded, and experiments were done at sodium concentrations closer to the physiological range. An inverse relationship between renin release and medium sodium concentration were observed in vitro only with sodium concentrations below 100 mM.6,12 Since these authors did not evaluate the viability of JG cells at the conclusion of their incubations, these observations may be subject to some question. Also, in the experiments of Lyons and Churchill,13 the cell suspensions were not evaluated in terms of morphology, and they were not cleared of tissue fragments or clumps. Thus, these preparations may have contained JG cells that remained attached to tissue fragments.

As evidenced by results summarized in Table 4, changes in sodium chloride concentrations had no appreciable effect on either renin release or renin synthesis. Such results lend support to the theories of both Vander and Miller14 and Thurau et al.15 who have postulated an involvement of the macula densa cell in the mediation of the sodium influence on renin release. Likewise, changes in potassium ion concentration did not alter renin release from these cell suspensions. However, there may have been a reduction in renin synthesis by cells exposed to either low potassium (2.35 mM) concentration or high potassium (9.4 mM). It has previously been observed that perfusion of either intact kidneys or isolated glomeruli with high potassium concentrations inhibited the release of renin.16 However, these observed decreases in "release" may have been caused by a decrease in renin synthesis without affecting the release process.

Angiotensin II inhibited renin release when added to the incubation medium, and this suppression was strongly evident at 30 minutes. However, no effect was observed at 60 minutes, suggesting that the cells possibly destroyed the angiotensin II. Angiotensin II is known to have a negative feedback on renin release in vivo, and these data would suggest a direct effect on the JG cell.

Addition of tissue extract from clipped kidneys of hypertensive rats caused a marked increase in renin release, and an equivalent increase in synthesis. Extracts of either contralateral kidneys or normal kidneys did not appear to have such an effect on renin release. This suggests that there was a soluble factor in the clipped kidney extract that would influence renin synthesis and release. When the clipped kidney extract was dialyzed, the activity remained unchanged, but when the extract was boiled prior to addition to the incubation, the effect was destroyed. These results indicate that the active factor was a nondialyzable, heat-labile molecule.

Addition of extract from the contralateral kidney to the cell suspension significantly suppressed renin synthesis as measured by cellular renin activity (p < 0.05). Renin release tended to be lower than control values, but the depression was not significant (table 6). It is possible that this extract contained a factor capable of acting in opposition to the extract from the clipped kidney.
Acid activation was used to determine if the renin released by the isolated JG cell was all of the active form or if there was some combination of active and inactive forms. The average increase in renin activity following acid dialysis for 10 samples was 78%, indicating that at least part of the renin being released by these cells in vitro was in an inactive form. The samples from cells incubated with angiotensin II showed less of an increase by acid activation than all other samples, with an average increase of only 13.3%. These data suggest that angiotensin II may suppress the release of the inactive form of renin as well as the release of active renin. Such a possibility is only speculative, however, on the basis of these preliminary results. Speculation on the possible influence of angiotensin on the activation of inactive renin is also premature.

The method of cell preparation described here is rapid, reproducible, gentle, and as much as 30 ml of a cell suspension containing 5 X 10^6 cells/ml can be fractionated at one time. Thus, large numbers of the cells can be prepared at one time suitable for in vitro testing of many physiological and pharmacological agents on both renin release and renin synthesis. It is clear that the enzyme used for dispersing the cells is a critical factor in their ability to function physiologically. The results here have shown that crude collagenase is suitable for cell dispersion, while trypsin is not. Further studies on the effects of trypsin on the internal structure of the cells may provide information as to the nature of its action. These cells respond to catecholamines and angiotensin II as expected and may be used as an in vitro model for further examination of JG cell function and responses in regard to regulation of renin biosynthesis and release.

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doi: 10.1161/01.HYP.3.2.157

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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