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### Research Article

The renin-angiotensin system is a major regulatory system controlling extracellular fluid volume and blood pressure. The rate-limiting enzyme in this hormonal cascade is renin, which is synthesized and secreted into the circulation by renal juxtaglomerular (JG) cells. The renal baroreceptor is a key physiologic regulator of renin secretion, whereby a change in renal perfusion pressure is sensed by these cells and results in a change in renin release. However, the mechanism, direct or indirect, underlying pressure transduction is unknown. We studied the direct application of mechanical stretch to rat JG cells and human renin-expressing (CaLu-6) cells on the release of renin. JG cells released a low level of baseline renin, comprising < 5% of their total renin content. By contrast, renin secretion from CaLu-6 cells comprised approximately 30% of cellular stores, yet was also stimulated twofold by 10 microM forskolin ( $P \leq 0.001$ ). In JG cells, mechanical stretch inhibited basal renin release by 42% ( $P < 0.01$ ) and forskolin-stimulated renin release by 25% ( $P < 0.05$ ). In CaLu-6 cells, stretch inhibited basal- and forskolin-stimulated renin release by 30 and 26%, respectively (both  $P < 0.01$ ). Northern blot analysis demonstrated a stretch-induced reduction in baseline renin mRNA accumulation of 26% ( $P < 0.05$ ) in JG and 46% ( $P < 0.05$ ) in CaLu-6 cells. The data demonstrate that mechanical stretch in renin-releasing [...]

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# Biomechanical Coupling in Renin-releasing Cells

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## Abstract

The renin–angiotensin system is a major regulatory system controlling extracellular fluid volume and blood pressure. The rate-limiting enzyme in this hormonal cascade is renin, which is synthesized and secreted into the circulation by renal juxtaglomerular (JG) cells. The renal baroreceptor is a key physiologic regulator of renin secretion, whereby a change in renal perfusion pressure is sensed by these cells and results in a change in renin release. However, the mechanism, direct or indirect, underlying pressure transduction is unknown.

We studied the direct application of mechanical stretch to rat JG cells and human renin-expressing (CaLu-6) cells on the release of renin. JG cells released a low level of baseline renin, comprising < 5% of their total renin content. By contrast, renin secretion from CaLu-6 cells comprised ~ 30% of cellular stores, yet was also stimulated twofold by 10  $\mu$ M forskolin ( $P \leq 0.001$ ).

In JG cells, mechanical stretch inhibited basal renin release by 42% ( $P < 0.01$ ) and forskolin-stimulated renin release by 25% ( $P < 0.05$ ). In CaLu-6 cells, stretch inhibited basal- and forskolin-stimulated renin release by 30 and 26%, respectively (both  $P < 0.01$ ). Northern blot analysis demonstrated a stretch-induced reduction in baseline renin mRNA accumulation of 26% ( $P < 0.05$ ) in JG and 46% ( $P < 0.05$ ) in CaLu-6 cells.

The data demonstrate that mechanical stretch in renin-releasing cells inhibits basal and stimulated renin release accompanied by a decrease in renin mRNA accumulation. Further studies will be necessary to characterize the intracellular events mediating biomechanical coupling in renin-expressing cells and the relationship of this signaling pathway to the in vivo baroreceptor control of renin secretion. (*J. Clin. Invest.* 1997; 100:1566–1574.) Key words: renin • secretion • gene expression • stretch • baroreceptor

## Introduction

The renin–angiotensin system is a major regulatory system controlling extracellular fluid volume and blood pressure. The

rate-limiting enzyme in this hormonal cascade is renin, which is secreted into the circulation by renal juxtaglomerular (JG)<sup>1</sup> cells, modified vascular smooth muscle cells of the renal afferent arteriole.

A major control mechanism for renin secretion is the intrarenal baroreceptor. According to the baroreceptor theory, renin secretion is inversely related to transmural pressure across the afferent arteriole. This was first demonstrated in the late 1950s, when Tobian et al. (1) showed an inverse relationship of JG cell granularity to renal perfusion pressure. In 1971, Blaine et al. (2) demonstrated in the denervated nonfiltering kidney that renin secretion could be altered by changes in renal arterial pressure in the absence of glomerular filtration and tubular function, thereby identifying the baroreceptor as distinct from the macula densa mechanism. Many recent studies have confirmed the baroreceptor theory. Among them is the finding by Kirchheim et al. (3) that stepwise reductions in renal arterial perfusion pressure led to increased renin secretion before detectable changes in renal blood flow or glomerular filtration rate occurred. In 1990, studies by Nobiling et al. (4) using isolated perfused rat kidneys showed that pulsatile changes in renal perfusion pressure inhibited renin secretion, and the degree of inhibition correlated with the amplitude of pulse pressure. At the single nephron level, the inverse relationship of renin secretion to perfusion pressure was demonstrated by Bock et al. in 1992 (5).

Despite ample evidence supporting the existence of intrarenal baroreceptor control of renin secretion, the mechanism (direct or indirect) responsible for transducing pressure is unknown. Several investigators have hypothesized that the baroreceptor mechanism is mediated indirectly by autocooids such as nitric oxide, eicosanoids, kinins, or endothelin released from vascular endothelial cells in response to a change in pressure (6, 7). Others have hypothesized that the JG cells may be directly sensitive to transmural pressure across the arteriole (8).

Theoretically, the renal baroreceptor mechanism could be related to transmural pressure, vascular smooth muscle relaxation, and/or autocoid or endothelial factors. We selected mechanical stretch of renin-secreting cells as the most likely possibility, because Nobiling et al. (4) showed that pulsatile changes in renal perfusion pressure inhibited renin secretion in vivo. In this study, we investigated the effect of cyclic mechanical stretch on renin release in cultured rat JG cells and in a human pulmonary tumor cell line, CaLu-6, which expresses the human renin gene endogenously (9). The study was designed to determine if cell stretch decreases renin release in renin-expressing cells and if a reduction in renin release is accompanied by a change in renin mRNA accumulation.

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1. Abbreviations used in this paper: Ang I, angiotensin I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JG, juxtaglomerular; SAC, stretch-activated ion channel.

## Methods

**Preparation of rat JG cells.** Male Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottsdale, PA) weighing 100–120 g were used. Renal cortical cells were harvested using a modification of a method previously described by our laboratory (10). For each cell preparation, one rat was killed by decapitation, the abdominal or descending aorta was cannulated, and both kidneys were perfused with RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 0.07% collagenase type A (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.12 mg/ml elastase (type III), 0.25 mg/ml soybean trypsin inhibitor, 0.16 mg/ml deoxyribonuclease I, and 0.3% crystallized BSA (all from Sigma Chemical Co., St. Louis, MO), and 100 µg/ml penicillin and 100 µg/ml streptomycin (Pen/Strep; GIBCO BRL) (enzyme solution).

Both kidneys were excised, decapsulated, hemisected, and demedulated. The cortices were minced and transferred to a spinner flask (Bellco Glass, Inc., Vineland, NJ), brought to 25 cm<sup>3</sup> with enzyme solution, and incubated at 37°C with spinning for three consecutive 30-min periods. Between periods, cells were triturated and gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. After 90 min, the enzymatic dispersion was stopped by adding 5 ml FCS and further diluting the solution three- to fourfold in RPMI 1640 media containing 0.1% BSA and Pen/Strep (RPMI-BSA-P/S). Cells were collected by centrifugation at 200 g for 10 min and resuspended in 100 ml RPMI-BSA-P/S. The cells then were centrifuged in a Percoll density gradient; eight tubes containing 20 ml of an isotonic 40% (vol/vol) Percoll solution (Sigma Chemical Co.) were prepared and centrifuged at 31,000 g for 20 min. Cells which sediment at a density of 1.067 g/ml [previously documented as the renin-rich fraction (references 10–12)] were aspirated carefully from the gradient and washed free of Percoll by centrifugation (200 g) in 5 vol RPMI-BSA-P/S.

Cells were resuspended in culture medium (RPMI 1640, 25 mM Hepes, 0.66 U/ml insulin, 2% FCS, Pen/Strep), and diluted to a concentration of  $1.25\text{--}2 \times 10^5$  cells/ml. The cells were seeded at  $3\text{--}4 \times 10^4$  cells/cm<sup>2</sup> in plastic tissue culture dishes or flexible collagen I-coated culture plates (Flexcell Corp., McKeesport, PA) for stretch experiments. The cells were incubated at 37°C in 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 24–72 h.

**Immunoperoxidase immunocytochemistry for rat renin.** Cells were cultured for 48 h on Permax plastic-coated multi-well chamber slides (Nunc, Inc., Naperville, IL). Immunoperoxidase staining was performed using the avidin–biotin peroxidase complex (ABC) kit from Vector Laboratories, Inc. (Burlingame, CA), as described previously (10, 11). Cells were fixed in methanol (4°C) for 10 min, followed by a 10-s exposure to acetone (4°C), and then air dried. For renin staining, rabbit polyclonal antibody to rat renin (kindly provided by Dr. Tadashi Inagami, Vanderbilt University, Nashville, TN) was used at a 1:750 dilution. This antibody has been documented previously as specific for rat prorenin and renin (13). Controls included normal rabbit serum and renin antibody which had been preadsorbed overnight at 4°C with a 10-fold molar excess of pure rat renin.

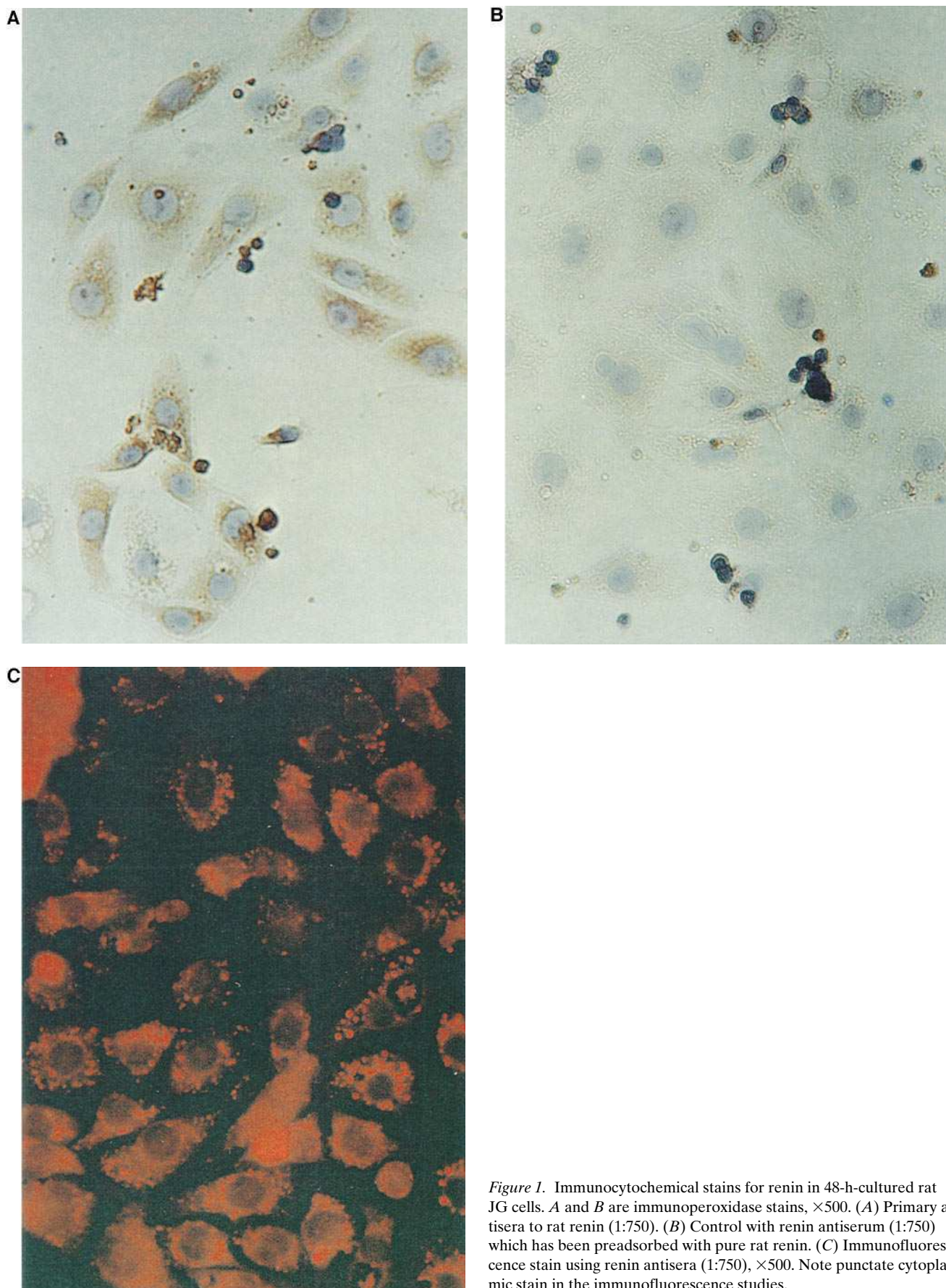
**Immunofluorescence immunocytochemistry for rat renin.** Immunofluorescence staining was performed after 48 h of culture on the Flexcell stretch membranes. Cells were fixed as above and rehydrated in FA buffer (Difco Labs Inc., Detroit, MI) with 3% BSA for 5 min. Blocking sera (normal goat sera at a 1:1,000 dilution in FA/BSA buffer) were added for 30 min. Cells were then incubated with the primary antibody diluted in FA/BSA buffer for 1 h. Primary antibodies included the renin antibody and controls described above, Thy-1 mAb (Serotec Ltd., Kidlington, Oxford, UK), rabbit anti-human Factor VIII antibody (DAKO Corp., Carpinteria, CA), and  $\alpha$ -smooth muscle actin mAb (Sigma Chemical Co.). Cells were washed three to four times and then incubated for 1 h with a TRITC-conjugated secondary antibody [goat anti-rabbit IgG for polyclonal primary antibodies, and goat anti-mouse IgG for primary mAbs (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA)]. Cells were washed again and mounted with Gelmount (Biomed, Foster City, CA) for fluorescence microscopy with an Axiophot photomicroscope (Carl Zeiss, Inc., Thornwood, NY).

**In situ hybridization histochemistry for rat renin.** JG cells were cultured on glass slides for 48 h. Cells were rinsed with PBS, fixed with 4% paraformaldehyde, and processed for in situ hybridization as previously described (14). The oligonucleotide probe used was a 28-residue oligonucleotide complementary to nucleotides 313–340 of rat renin mRNA. The oligonucleotide was labeled with  $\alpha$ -thio [<sup>35</sup>S]dATP. A sense nucleotide was used as control.

**Application of mechanical stretch to cultured rat JG cells.** Cells were cultured in six-well plates with flexible collagen I-coated bottoms (Flex Plate; Flexcell Corp.). After 24 h of culture, the wells were washed twice and replaced with fresh culture media. At this point, one-half of the plates were subjected to a 20 h period of cyclic stretch at 3 cycles/min (i.e., repetitive cycles of 10 s, 20% elongation, and 10 s relaxation) in a stretch unit similar to that initially described by Baner et al. (15). The remainder of the plates were maintained in a static environment under identical conditions (control group). For release experiments, media were removed after 20 h of stretch, and the cells were incubated with or without isoproterenol (10 µM) or forskolin ( $10^{-7}$ – $10^{-5}$  M) for an additional 2 h under the same stretch regimen. In the release experiments, forskolin, a direct stimulator of adenylyl cyclase, and isoproterenol, an indirect stimulator of adenylyl cyclase through the beta-adrenergic receptor, were used to validate the ability of the cultured JG cells to respond to standard secretagogues. For the stretch experiments, forskolin was used as a standard secretagogue which is independent of the baroreceptor mechanism. Cell supernatants were then collected for renin activity assays, and cell lysates were prepared in order to measure total protein levels and intracellular renin content. Stretch is achieved by placing the culture plates on top of a rubber gasket which is centered over a vacuum port. When vacuum is applied, the bottom of each well is deformed, thereby stretching the cells attached to its surface. When the vacuum is released, the plate bottoms return to their original flat position. The computer-assisted strain unit (Flexcell Corp.) provides precisely timed pressure cycles of controlled magnitude. Vacuum intensity set at 20% elongation is achieved only at the outer annulus of the well membrane. The amount of strain decreases toward zero at the center of the membrane. The average elongation is 8–9% over the entire culture plate surface.

**Renin activity assays.** To quantitate renin secretion from the rat JG cells, the media were removed from the culture wells and centrifuged at 400 g for 10 min to pellet any residual nonadherent cells. The supernatant was collected and assayed for renin activity. Cell lysates were prepared by washing the adherent cells twice in PBS and lysing them in a buffer containing 150 mM NaCl, 0.5 mM EDTA, 25 mM Hepes, 1% Triton X-100, 0.5% deoxycholic acid, and 0.5 mM PMSF. The cells were scraped off of the culture wells and sonicated for 15 s (ultrasonic processor; Heat Systems Inc., Farmingdale, NY). The renin activity assay used to quantitate both renin secretion and total intracellular renin content consisted of an angiotensin I (Ang I) generation assay followed by an Ang I RIA. Cell supernatants or lysates were incubated with an excess of rat renin substrate (plasma from 48-h bilaterally nephrectomized rats) for 60 min at 37°C. The Ang I generation was carried out in 50 mM PO<sub>4</sub> buffer (pH 6.2) containing 4 mM EDTA and 1.4 mM PMSF. The reaction was stopped by immersing the tubes in ice, and the amount of Ang I generated was determined by RIA (16). Renin activity is expressed as nanograms of Ang I generated per milligram of cellular protein per hour of incubation. Protein concentration was measured in the cell lysates using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

**Application of mechanical stretch to human CaLu-6 cells.** CaLu-6 cells were grown in MEM with nonessential amino acids, sodium pyruvate 1 mM, and 10% FCS. Cells were seeded into the stretch plates at a concentration of  $2.5 \times 10^5$  cells per ml in a 1.5-ml volume per well. They were cultured at 37°C in 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 48 h until confluent. Fresh medium was added with and without addition of forskolin 10 µM (used as a standard secretagogue), and cells were subjected to 3-cpm stretch (20% elongation). After 20 h of stretch, cell supernatants were collected for the human renin assay, and the cells were lysed in order to measure total protein levels.



*Figure 1.* Immunocytochemical stains for renin in 48-h-cultured rat JG cells. *A* and *B* are immunoperoxidase stains,  $\times 500$ . (*A*) Primary antisera to rat renin (1:750). (*B*) Control with renin antiserum (1:750) which has been preadsorbed with pure rat renin. (*C*) Immunofluorescence stain using renin antisera (1:750),  $\times 500$ . Note punctate cytoplasmic stain in the immunofluorescence studies.



**Direct immunoradiometric assay for human renin and prorenin.** Active human renin and prorenin were measured in the CaLu-6 cell supernatants using a commercially available immunoradiometric kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). The results of this immunoradiometric assay are correlated with renin activity measurements, with a correlation coefficient of 0.91 ( $P < 0.001$ ). For the human immunoradiometric assay, 100  $\mu\text{U/ml}$  renin is equivalent to 8 ng/ml/h renin enzyme activity.

**Northern blot analysis.** RNA was harvested from rat JG or human CaLu-6 cells that had been exposed to 20 h of cyclic stretch at 3 cpm and 20% elongation or from the nonstretched control group. Total cellular RNA was isolated using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) and pooled from one stretch plate (6 wells) per group. Northern blots were made by denaturation of the RNA in 8% formaldehyde/64% formamide, and size fractionation through a 1.2% agarose, 0.4 M formaldehyde gel, followed by capillary transfer to a zeta-probe membrane (Bio-Rad Laboratories, Hercules, CA). The blots were hybridized overnight at 65°C in Church hybridization buffer (17) to a full-length rat renin cDNA (18) (gift of Dr. Kevin Lynch, University of Virginia) or to a 555-bp fragment of human renin cDNA obtained by reverse transcription PCR of CaLu-6 cell RNA, after cloning and sequencing to confirm the correct insert. The fragment was cut out of the vector, gel-purified, and labeled by random primers. The DNA was labeled with  $^{32}\text{P}$  using a random primer DNA labeling kit (Boehringer Mannheim Biochemicals). Renin mRNA was detected by autoradiography and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or by an Image Quant densitometric analysis program. To control for RNA loading and specificity of changes, the same RNA blots were

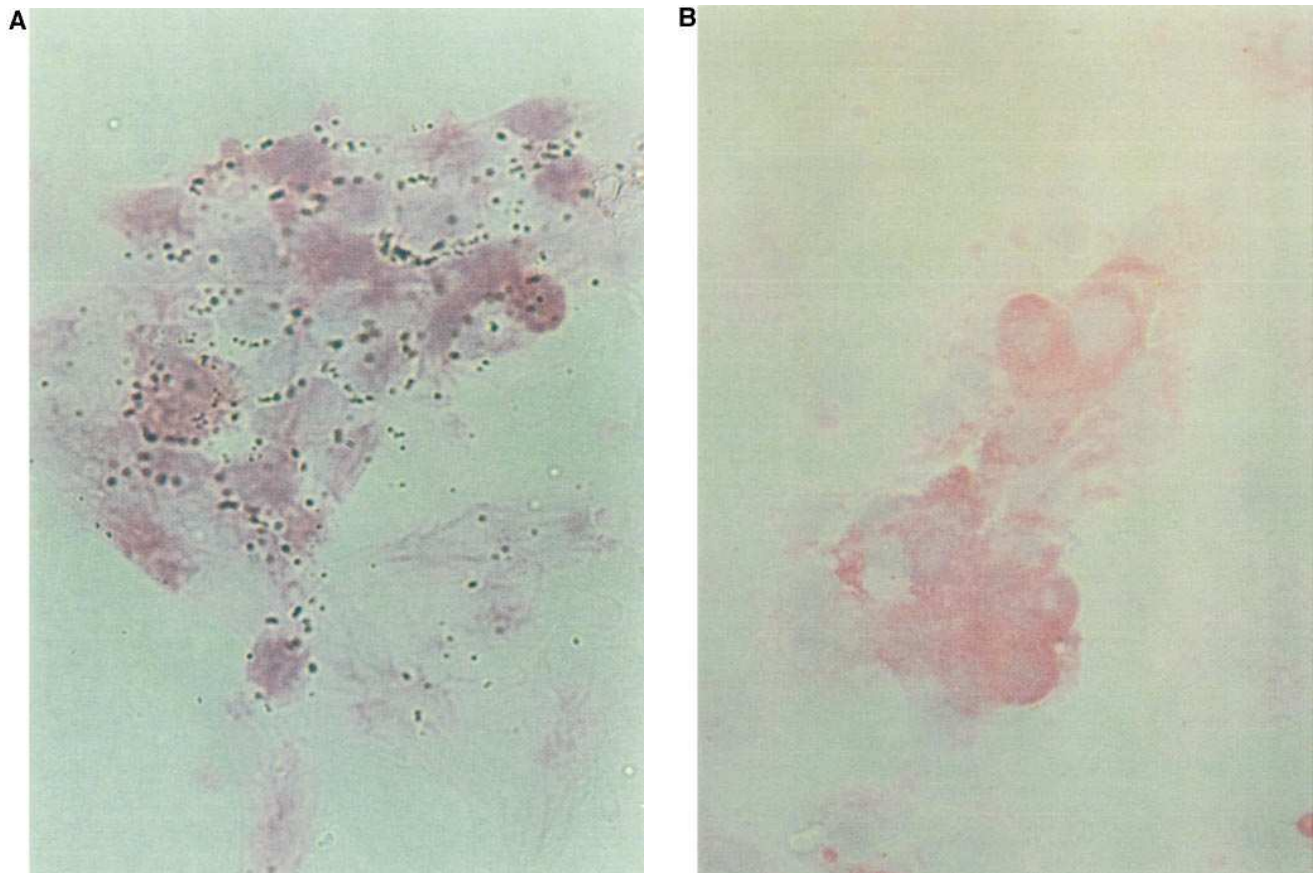
**Table I. Specific Stains for Other Kidney Cell Types**

Stain	Cell type	Results
$\alpha$ -Actin	Smooth muscle	Positive
Alkaline phosphatase	Proximal tubule	Negative
Thy-1	Glomerular mesangial	Negative*
Factor VIII	Endothelial	Negative

\*1–2% of cells in the adherent layer stained positively, indicating the presence of contaminating mesangial cells in the cultures. These cells were very distinct morphologically from the granular renin-staining cells.

hybridized to a  $^{32}\text{P}$ -labeled 780-bp cDNA fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (American Type Culture Collection, Rockville, MD).

**Statistical analysis.** For the stretch experiments, renin release was determined for each individual well of the stretch plates. There were 6 wells per stretch plate. One stretch plate was subjected to cyclic stretch, and the other was not. There were 16 such experiments for rat JG cells and 9 for human CaLu-6 cells. Thus, the mean of the 6 values for each experiment was used as the value for one experiment, and the statistics were calculated for the 16 or 9 experiments, respectively. The paired design in cell culture (JG and CaLu-6 cells) was used because we compared stretch and nonstretch in samples from the same rat under similar conditions. Data are presented as



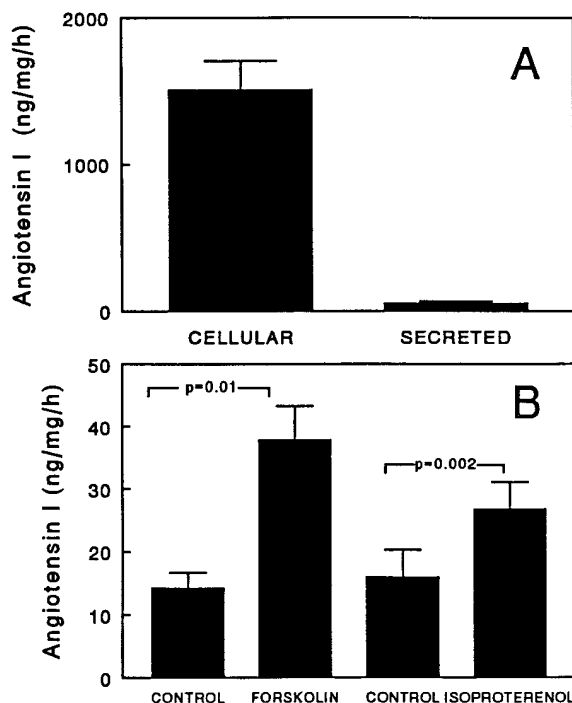
**Figure 2.** Renin mRNA in situ hybridization in 48-h-cultured rat JG cells. (A) Antisense probe. (B) Control (sense) probe. Black grains, Specific mRNA hybridization signals.

means  $\pm$  SE. Results were analyzed by Student's two-tailed *t* test for paired samples. *P* values  $< 0.05$  were considered statistically significant.

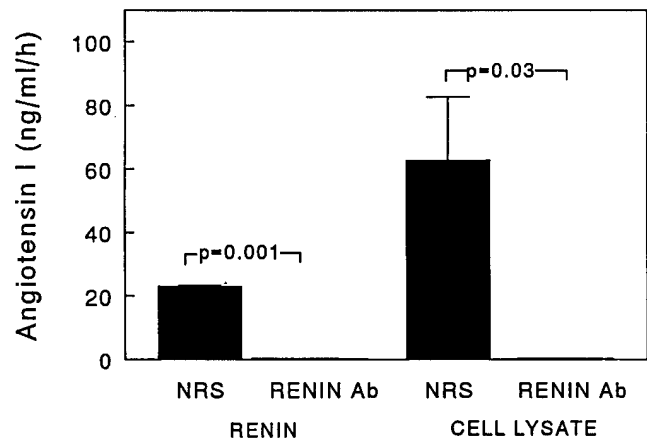
## Results

**Morphological characterization of rat JG cell culture.** Experiments were performed on JG cells after 48 h of primary culture. At this time, the adherent layer was 50–60% confluent and composed of round patches of cells with prominent cytoplasmic granules. Immunoperoxidase stains of the 48-h cultures demonstrated that  $> 95\%$  of the adherent cells contained renin. Fig. 1 *A* is an immunoperoxidase stain using polyclonal renin antibody. All cells pictured are positive with brown cytoplasmic staining. Fig. 1 *B* is a control in which the renin antiserum was preadsorbed with a 10-fold molar excess of purified rat renin. No immunostaining was present, nor was there any positive staining in the controls in which normal rabbit serum was used (data not shown). Similar results were obtained when the staining was performed using immunofluorescent techniques. Using immunofluorescence, the granular cytoplasmic pattern of renin immunostaining was localized to JG cell granules (Fig. 1 *C*), and was absent in the preadsorption control. Further immunocytochemical staining revealed that the 48-h-cultured cells were negative for other kidney cell-specific markers (Table I), except for  $\alpha$ -smooth muscle actin, which was used to verify their smooth muscle origin.

Fig. 2 shows in situ hybridization histochemistry for renin



**Figure 3.** Renin activity in 48-h-cultured rat JG cells. (*A*) Comparison of baseline intracellular and extracellular (secreted) renin. Extracellular renin was measured from supernatants collected over a 24-h time period. (*B*) Increase in renin secretion from 72-h-cultured JG cells in response to a 2-h stimulation with forskolin (10  $\mu$ M) or isoproterenol (10  $\mu$ M). Values are expressed as mean  $\pm$  1 SE from five separate experiments (*A* and *B*).



**Figure 4.** Effect of renin antibody on renin activity from 6.25 pg pure rat renin (*left*) or cultured rat JG cell lysates (*right*). Normal rabbit sera (NRS) or renin antibody (Ab) was added to pure renin or cell lysates at a 1:100 dilution overnight at 4°C. The next day, the Ang I generation assay and RIA were performed. Data are expressed as mean  $\pm$  1 SE from three separate experiments.

mRNA in 48-h-cultured JG cells. Fig. 2 *A* shows the hybridization signal when the antisense probe was used. Greater than 90% of the adherent cells exhibited grains localized specifically in the cytoplasm, indicating renin mRNA accumulation. Controls using a sense oligonucleotide probe (Fig. 2 *B*) were negative. The demonstration of renin mRNA accumulation identifies these cells conclusively as JG cells.

**Measurements of renin activity from rat JG cells.** Cultured JG cell lysates and supernatants were assayed to quantify intracellular and released renin. Fig. 3 *A* shows that the secreted renin released into the supernatant over a 24-h time period was  $< 5\%$  of the total renin activity in 48-h cultured cells. Fig. 3 *B* compares basal and stimulated renin release after a 2-h incubation of the cells with forskolin and isoproterenol (10  $\mu$ M). In these studies, JG cells had been cultured for 72 h before the addition of fresh media with the secretagogues or their vehicle control. Forskolin stimulated renin release 2.5-fold, and isoproterenol stimulated release by 75% over baseline renin release. No change in cellular renin activity was observed in these experiments.

In the JG cell experiments, renin activity was measured using an enzymatic assay to generate a product, Ang I. To confirm that the Ang I activity being measured was generated specifically by renin and not by another renin-like enzyme such as a cathepsin, antibody-blocking experiments were performed (Fig. 4). Rat renin substrate was added to cell lysates which had been preincubated with a 1:100 dilution of rabbit antisera to rat renin or normal rabbit sera at 4°C for 18 h. Ang I was generated and measured by RIA. The generation of Ang I by the JG cell lysates was blocked completely by the renin antibody. In addition, the renin activity generated by 6.25 pg pure rat renin (kindly provided by Dr. S. Kim, Osaka, Japan), used as a positive control, was likewise abolished. Cathepsin D (Sigma Chemical Co.) did not generate any measurable Ang I under these assay conditions (pH = 6.2; data not shown).

**Measurements of renin and prorenin from human CaLu-6 cells.** Fig. 5 depicts cellular (*A*) and spontaneously released (*B*) renin and prorenin from cultured human CaLu-6 cells. The

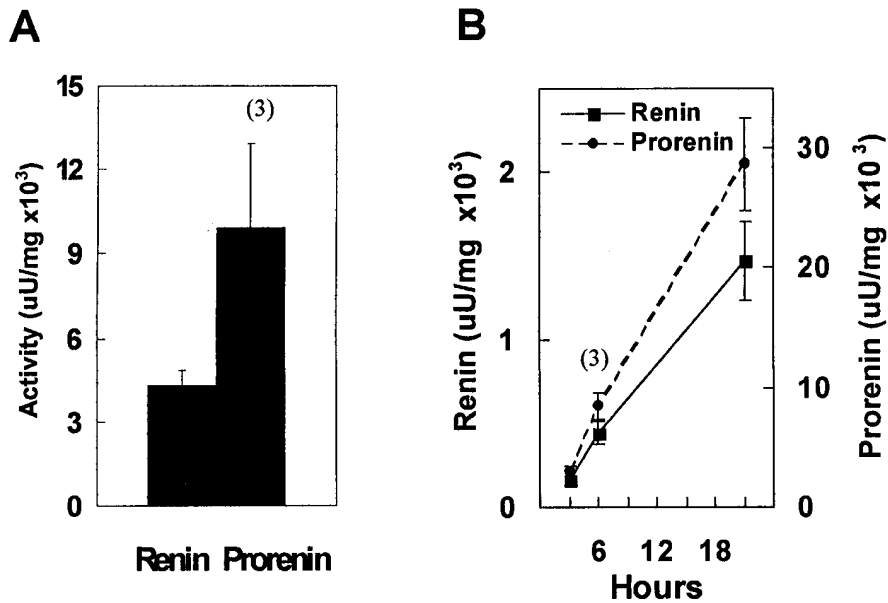


Figure 5. Relationship among cellular and spontaneously released renin and prorenin in cultured human CaLu-6 cells. (A) Cellular renin and prorenin content. (B) Renin and prorenin released spontaneously over a 3–20-h period. Data are expressed as mean  $\pm$  1 SE from six experiments except where (n) indicated.

CaLu-6 cells contained approximately twice the amount of prorenin as renin (A). Basal prorenin release was 10-fold higher than renin release at 3 h of incubation. At 20 h, prorenin release was 20-fold higher than renin release ( $P < 0.001$ ).

**Effect of stretch on renin release from rat JG and human CaLu-6 cells.** Fig. 6 A demonstrates the inhibition of rat JG cell renin release in response to stretch. Cells cultured for 24 h on collagen I-coated flexible-bottom plates were subjected to a 3-cpm, 20% elongation stretch regimen. After 20 h of stretch, cells were stimulated (with stretch) for an additional 2 h with or without 10  $\mu$ M forskolin. The control group consisted of cells that were grown under identical culture conditions on the stretch plates that remained stationary. Basal renin release from JG cells was  $24 \pm 4$  ng Ang I/mg protein/h. In 16 experiments, stretch inhibited basal renin release by 42%, to  $15 \pm 3$

ng Ang I/mg protein/h ( $P < 0.01$ ). Forskolin (10  $\mu$ M) stimulated renin activity from  $24 \pm 4$  to  $63 \pm 10$  ng Ang I/mg protein/h ( $P < 0.001$ ). Stretch inhibited forskolin-stimulated renin activity by 25% (from  $63 \pm 10$  to  $47 \pm 6$  ng Ang I/mg protein/h;  $n = 15$ ,  $P < 0.05$ ). There was no depletion of cellular renin in these experiments.

Fig. 6 B summarizes the effect of cyclic stretch on basal and forskolin-stimulated renin release from human CaLu-6 cells. In nine experiments, the CaLu-6 cells exhibited a high basal rate of renin release ( $3,718 \pm 845$   $\mu$ U/mg protein) that was stimulated approximately twofold (to  $6,786 \pm 1,157$   $\mu$ U/mg protein) by 10  $\mu$ M forskolin ( $P < 0.001$ ). In response to stretch, basal renin release was inhibited by 30%, from  $3,718 \pm 845$  to  $2,582 \pm 604$   $\mu$ U/mg protein ( $P < 0.01$ ). Stretch also inhibited forskolin-stimulated renin release by 26%, from  $6,786 \pm 1,157$

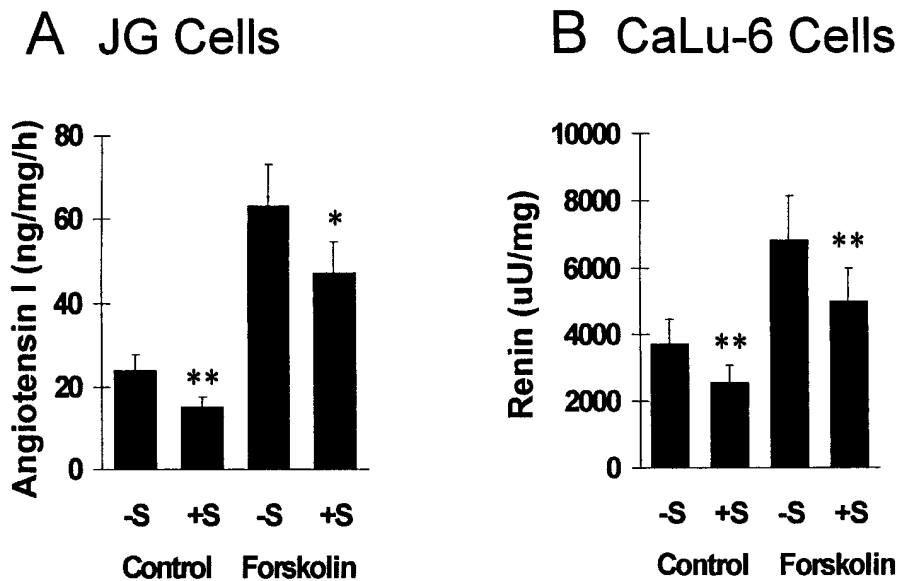
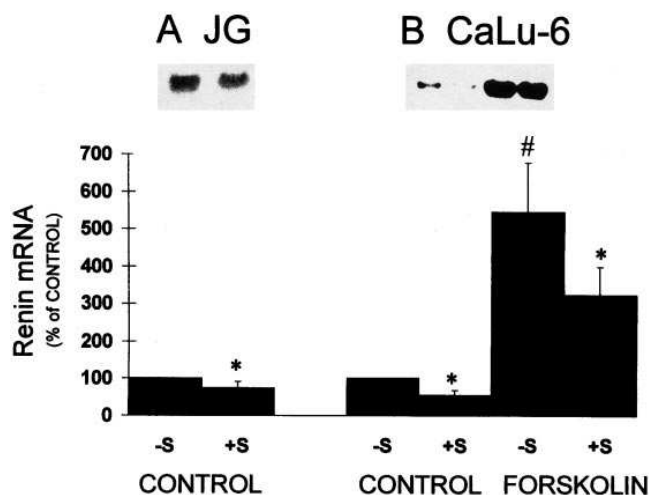


Figure 6. Effect of direct mechanical stretch (20 h, 3 cpm, 20% elongation) on renin secretion from cultured rat JG (A,  $n = 16$ ) and human CaLu-6 (B,  $n = 9$ ) cells. Stretch inhibition of basal renin (control) and forskolin-stimulated renin secretion. +S, Presence of cell stretch. -S, Absence of cell stretch. Data are expressed as mean  $\pm$  1 SE. \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Figure 7.** Northern blot analysis for renin RNA of rat renal JG and human CaLu-6 cells in response to mechanical stretch (20 h, 3 cpm, 20% elongation). Densitometric analysis is depicted below each representative Northern blot. -S, Control. +S, Stretch. CONTROL, Vehicle for forskolin. FORSKOLIN, Forskolin 10  $\mu$ M. Data are expressed as mean  $\pm$  1 SE for seven experiments in the JG cell group and five experiments in the CaLu-6 cell group.

to  $5,000 \pm 916$   $\mu$ U/mg protein ( $P < 0.01$ ,  $n = 7$ ). Cell protein levels were not affected by mechanical stretch in any of the rat JG or human CaLu-6 cell experiments.

**Effect of stretch on renin mRNA in rat JG and human CaLu-6 cells.** Stretch-induced inhibition of renin release was accompanied by a corresponding decrease in renin mRNA accumulation. In seven experiments with Northern blot analysis, rat JG cell mRNA levels were decreased by 26% ( $P < 0.05$ ) in response to 20 h of cyclic stretch (3 cpm, 20% elongation) (Fig. 7). The GAPDH mRNA loading control was unchanged by stretch. Bands were analyzed densitometrically and corrected by GAPDH. The ratios of the corrected values (stretch/control) were  $0.74 \pm 0.1$  ( $n = 7$ ,  $P < 0.05$ ). In human CaLu-6 cells (Fig. 7), forskolin increased renin mRNA by Northern blot analysis ( $P < 0.05$ ). In the CaLu-6 cells, stretch decreased the level of basal ( $n = 5$ ) human renin mRNA by 46% ( $P < 0.05$ ) in control cells and by 38% ( $P < 0.05$ ;  $n = 5$ ) in response to forskolin. GAPDH mRNA loading controls were similar in control, forskolin-treated, or stretched CaLu-6 cells. Densitometric ratios (stretch/control) were  $0.54 \pm 0.1$  ( $n = 5$ ,  $P < 0.05$ ) without forskolin and  $0.62 \pm 0.1$  ( $n = 5$ ,  $P < 0.05$ ) in the presence of forskolin.

## Discussion

The major new findings of this study include: (a) the development of a highly purified population of renal JG cells, as demonstrated by immunocytochemistry for renin protein and in situ hybridization for renin mRNA; (b) inhibition of renin release by cyclic stretch directly at the renin-expressing JG or CaLu-6 cell; and (c) stretch-induced inhibition of renin mRNA accumulation. We were also able to demonstrate for the first time the relationships among cellular and secreted renin and prorenin in a renin-expressing human pulmonary tumor cell line (CaLu-6).

The JG cell preparation used for these studies demonstrated a high degree of cell viability,  $< 5\%$  contamination with other cell types, and increased renin release with isoproterenol and forskolin, indicating intact beta-adrenergic receptors and AMP-dependent mechanisms of renin release. Virtually every cell contained the renin mRNA signal, confirming conclusively that these are JG cells at a high level of purity. Positive renin immunocytochemistry was also present in  $\sim 100\%$  of these cells, but immunostaining could theoretically be due to renin uptake from the extracellular environment. We demonstrated a low level of baseline renin release from these JG cells that comprised  $< 5\%$  of their total renin content.

The CaLu-6 cell is a human renin-expressing pulmonary carcinoma cell line. This cell has been shown to have abundant renin mRNA which is increased in response to forskolin (9, 19). Because of the availability of a human renin antibody, renin release from the CaLu-6 cell can be quantified directly by radioimmunometric assay, obviating the need to generate Ang I as a product and the inherent imprecision of a multistep, sequential assay. Before using these cells in mechanical stretch experiments, we demonstrated the cellular content of renin and the spontaneous release of renin and prorenin. We found that CaLu-6 cells contain approximately twice as much prorenin as renin and that they release large quantities of both prorenin and renin over time. The spontaneous release of prorenin is  $\sim 20$ -fold that of renin, similar to the ratio of prohormone to active hormone from most other tumor cell lines. In CaLu-6 cells, prorenin appears to be released constitutively without storage. On the other hand, CaLu-6 cells appear to store some renin, as they release only about one-third of their cellular content within a 20-h period. Assuming that 100  $\mu$ U of human assayable renin is equivalent to 8 ng/ml/h of renin enzyme activity, human CaLu-6 cells store approximately one-fourth as much active renin as rat JG cells. This finding for CaLu-6 cells is in contradistinction to other renin-secreting tumor cells, which are unable to store renin and which secrete prorenin almost exclusively in a constitutive fashion (20).

In this study, we demonstrated that cyclic mechanical stretch caused an inhibition of renin release from both rat JG and human CaLu-6 cells. The degree of inhibition of renin release was similar ( $\sim 35\%$ ) for both JG and CaLu-6 cells. For both cell types, stretch also inhibited forskolin-induced renin release to a similar degree ( $\sim 25\%$ ). No depletion of cellular renin was observed from either cell type. We also demonstrated that the reduction in renin release transduced by cell stretch was accompanied by a corresponding decrease in renin mRNA accumulation in both JG and CaLu-6 cells. Therefore, we conclude that mechanical stretch directly at the renin-expressing cell inhibits renin release and renin gene expression.

The effect of decreased renal perfusion pressure on renin gene expression has been studied in the rat kidney, where aortic coarctation was shown to increase renin mRNA levels (21). However, the increase in renin mRNA was mediated by recruitment of renin-expressing cells along the afferent arteriole (21). In this study, there was no opportunity for recruitment of renin-expressing cells by stretch, since virtually the entire cell population already expressed the renin gene.

In our stretch experiments, we chose a paradigm of 20 h of cyclic stretch followed by a 2-h measurement period of renin release while the cells continued to be stretched. Our reasons for selecting this paradigm were related to our desire to ob-



serve the effects of chronic cyclic stretch on renin release and mRNA, since Nobiling et al. (4) showed that pulsatile changes in renal perfusion pressure inhibited renin secretion in vivo. Our studies do not address the effects of acute, non-pulsatile stretch on renin release, because the low sensitivity of the available renin assays makes it impossible to detect small changes. One study of a population of renal cortical cells (not documented as JG) indicated a stretch-induced decrease in renin release over a 10-min period (22). However, in this study it was impossible to exclude endothelial cell-derived factors as responsible for the changes in renin secretion.

How JG cell stretch might decrease renin secretion has been a subject of speculation. Several investigators have hypothesized that JG cell stretch may be mediated by changes in intracellular calcium concentration, yet this has not been demonstrated directly at the level of the JG cell. A possible mechanism would be that cell stretch opens cation stretch-activated ion channels (SACs). The resulting  $\text{Ca}^{2+}$  influx through these channels would increase intracellular calcium concentration, resulting in an inhibition of renin secretion. Sufficient  $\text{Ca}^{2+}$  permeability of SACs has established a clear function for these channels as regulators of cytosolic  $\text{Ca}^{2+}$  in many cell types, including vascular smooth muscle cells (23). An alternative possibility is that the opening of cation SACs increases sodium flux into the cell, resulting in a depolarization of the cell membrane, a secondary opening of voltage-dependent calcium channels, and inhibition of activity in the renin secretion. Further studies will be necessary to delineate the second messenger mechanisms underlying the stretch-induced inhibition of renin secretion.

The demonstration of stretch-induced inhibition of renin release identifies a possible element in the renal baroreceptor mechanism. Mechanical stretch of renin-releasing cells in vitro is similar to the mechanical deformation anticipated in vivo where increased renal perfusion pressure would lead to cellular elongation or stretch. Other studies that have maintained the importance of a flow-sensing baroreceptor have implicated the endothelial cells lining the afferent arteriole as the likely sensor cells (6). These cells have been shown to respond to shear stress or stretch by increased production of factors such as nitric oxide and endothelin, which can inhibit renin secretion (24–27), or prostacyclin, which stimulates renin secretion (28). Neither in our JG cell preparation nor in the CaLu-6 cells were contaminating endothelial cells present. Thus, endothelial cell mediators did not play a role in stretch-induced renin release in our studies. However, although we have clearly demonstrated a stretch effect directly on renin-releasing cells, it is likely that baroreceptor control of renin secretion in vivo involves a complex integration of vascular responses, some direct and some indirect, which ultimately work together in the overall control of renin secretion.

In contrast to the biomechanical inhibition of renin secretion demonstrated in this study, stretch has been shown to stimulate the production of cellular proteins and hormones (e.g., eicosanoids, nitric oxide synthase, parathyroid hormone-related peptide, tissue plasminogen activator, and endothelin) in several cell systems (25, 29–33), including vascular smooth muscle and endothelial cells. In most of these cell systems, specific stretch-induced changes in cell morphology and orientation have been described (25, 32–34). Despite a stretch-induced inhibition of secretion, stretch engendered similar changes in JG and CaLu-6 cells. Cells at the periphery of the stretch

membranes were elongated and aligned perpendicular to the direction of stretch. In contrast, the cells receiving less stretch in the center of the membrane showed no changes in shape or alignment from their nonstretched controls. The importance and meaning of these morphological changes induced by stretch are not clear. Although increased cell proliferation has been reported during exposure to stretch (31–34), in our studies a comparison of total cellular protein levels from all of the experiments showed no significant differences among the stretched and nonstretched groups.

In summary, we have shown that mechanical deformation inhibits renin release and is accompanied by a decrease in renin mRNA accumulation in cultured rat JG and human renin-expressing CaLu-6 cells. Further studies will be necessary to identify the intracellular events mediating biomechanical coupling in renin-expressing cells and the relationship of this signaling pathway to the in vivo baroreceptor control of renin secretion.

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