ATP-Dependent Mechanism for Coordination of Intercellular Ca\(^{2+}\) Signaling and Renin Secretion in Rat Juxtaglomerular Cells

Jian Yao, Michihiro Suwa, Bing Li, Kazuko Kawamura, Tetsuo Morioka, Takashi Oite

**Abstract**—A change in intracellular Ca\(^{2+}\) is considered to be the common final signaling pathway through which renin secretion is governed. Therefore, information relating to the generation, control, and processing of Ca\(^{2+}\) signaling in juxtaglomerular cells (JG) will be critical for understanding JG cell behavior. In this study, we investigated the means by which JG cells harmonize their intracellular Ca\(^{2+}\) signals and explored the potential role of these mechanisms in renin secretion. Mechanical stimulation of a single JG cell initiated propagation of an intercellular Ca\(^{2+}\) wave to up to 11.9±4.1 surrounding cells, and this was prevented in the presence of the ATP-degrading enzyme, apyrase (1.7±0.7 cells), or by desensitization of purinergic receptors via pretreatment of cells with ATP (1.8±0.9 cells), thus implicating ATP as a mediator responsible for the propagation of intercellular Ca\(^{2+}\) signaling. Consistent with this, JG cells were demonstrated not to express the gap junction protein connexin43, and neither did they possess functional gap junction communication. Furthermore, massive mechanical stretching of JG cells elicited a 3-fold increase in ATP release. Administration of ATP into isolated perfused rat kidneys induced a rapid, potent, and persistent inhibition of renin secretion, together with a transient elevation of renal vascular resistance. ATP (1 mmol/L) caused up to 79% reduction of the renin secretion activated by lowering the renal perfusion flow (P<0.01). Taken together, our results indicate that under mechanical stimulation, ATP functions as a paracellular mediator to regulate renin secretion, possibly through modulating intra- and intercellular Ca\(^{2+}\) signals. (Circ Res. 2003;93:338-345.)

**Key Words:** juxtaglomerular cells ■ calcium ■ ATP ■ renin ■ mechanical strain

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) cells of the afferent arteriole. Renin secretion is controlled by a number of factors, such as blood pressure, sodium chloride load, sympathetic nerves, hormones, cytokines, and vasoactive materials.\(^1\)\(^-\)\(^3\) Although the signal pathways underlying the actions of these factors differ, it is generally accepted that a change in intracellular Ca\(^{2+}\) is the common final pathway through which renin secretion from JG cells is governed. In contrast to other secretory cells in which an increase of Ca\(^{2+}\) usually promotes secretion, renin secretion is inversely related to the Ca\(^{2+}\) concentration in JG cells. This view is based on the following evidence: Ca\(^{2+}\)-mobilizing agents inhibit renin secretion;\(^1\) Ca\(^{2+}\) influx via store-operated Ca\(^{2+}\) channels suppresses renin secretion;\(^4\) and lowering the extracellular Ca\(^{2+}\) stimulates renin secretion.\(^5\) Therefore, information relating to the generation, control, and processing of Ca\(^{2+}\) signals in JG cells is essential for understanding the behaviors of these cells under various pathophysiological conditions.

Recent studies have indicated that Ca\(^{2+}\), besides acting as an intracellular signal, is also able to act as an intercellular signal. Ca\(^{2+}\) can spread out in a population of cells as an intercellular Ca\(^{2+}\) wave, and groups of cells can coordinate their oscillations, thereby generating synchronous oscillatory Ca\(^{2+}\) signals.\(^6\)\(^-\)\(^8\) Intercellular Ca\(^{2+}\) waves have been demonstrated in a variety of cell types and are presumably responsible for integrated multicellular behaviors. The mechanisms underlying the propagation of intercellular Ca\(^{2+}\) waves are thought to be either diffusion of an intracellular messenger through gap junctions or paracrine effects of extracellular nucleotides, such as ATP or UTP.\(^6\)\(^-\)\(^11\) Synchronization of Ca\(^{2+}\) fluctuations is especially important in secretory units (such as the liver, pancreas, and salivary and lacrimal glands), where Ca\(^{2+}\) plays a central role in the initiation, facilitation, and maintenance of cellular secretory functions.\(^12\) Indeed, participation of intercellular communication in the coordination and amplification of insulin secretion in the pancreas,\(^13\)\(^,\)\(^14\) bile release in the liver,\(^14\) and catecholamine production in adrenal tissues,\(^15\) has been documented. Because JG cells share many of the properties of secretory cells, it is reason-
able to speculate that intercellular communication between these cells may provide them with a pathway for signal transduction as well as for coordination of multicellular secretory functions. The purpose of this study was to address the above hypothesis. Specifically, we asked (1) whether JG cells could communicate with each other to harmonize their intracellular Ca$^{2+}$ signals; and (2) whether the mediator implicated in the coordination of Ca$^{2+}$ transients could alter renin secretion.

Using an in vitro model of mechanical stimulation, our study demonstrated that ATP, but not gap junction channels, was responsible for synchronizing intercellular Ca$^{2+}$ signals in JG cells. Furthermore, ATP was identified as a potent inhibitor of renin secretion in isolated perfused kidneys. It is therefore proposed that ATP released by JG cells under mechanical stimulation might be an important mediator implicated in the control of renin secretion.

**Materials and Methods**

**Preparation of Rat JG Cells**

Rat JG cells were cultured according to the method described by Carey et al. Briefly, the cortical tissues of male Wistar rats were taken, minced, and digested with enzymatic solution. Cells were separated on a Percoll density gradient, resuspended in culture medium, and seeded onto collagen-precoated plastic tissue culture dishes or special glass-bottom microwell dishes for primary culture. The JG cells within 96 hours of culture were used for experiments.

**Immunocytochemistry**

Immunocytochemical staining for Cx43 and renin in cultured JG cells was performed as described previously. For double-label staining, cells were incubated simultaneously with both goat anti-renin and rabbit anti-Cx43 antibodies, followed by sequential incubation with secondary antibodies, CY2-conjugated donkey anti-goat IgG, and tetramethyl rhodamine B isothiocyanate-conjugated goat anti-rabbit IgG, respectively.

**Measurement of Gap Junctional Intercellular Communication (GJIC)**

GJIC was assessed by transfer of the membrane-impermeable fluorescent dye, Lucifer yellow (LY), after single-cell microinjection with an automated microinjection system (Zeiss), using a method described previously. For double-label staining, cells were incubated simultaneously with both goat anti-renin and rabbit anti-Cx43 antibodies, followed by sequential incubation with secondary antibodies, CY2-conjugated donkey anti-goat IgG, and tetramethyl rhodamine B isothiocyanate-conjugated goat anti-rabbit IgG, respectively.

**Single-Cell Mechanical Stimulation**

Mechanical stimulation of a single JG cell was achieved by briefly distorting the apical surface of the cells with a blunt prepulled Eppendorf micropipette.

**Measurement of Ca$^{2+}$**

Cultured JG cells were loaded with fura-2 by incubation with 5 μmol/L fura-2 acetoxyethyl ester (Fura-2 AM) in Hanks’ balanced salt solution (HBSS) containing 2.0 mmol/L CaCl$_2$ and 1.0 mmol/L MgCl$_2$ at room temperature. Ca$^{2+}$ was determined by the ratio method as reported previously.

**Mass Mechanical Stimulation and Collection of Extracellular Samples**

Mass mechanical stimulation and collection of extracellular samples were performed as previously published with minor modifications. Briefly, primary cultures of JG cells in 35-mm culture plates were washed three times with HBSS, and cultured in 0.5 mL of HBSS with or without 10 mg/dish glass microbeads (30 to 50 μm; Polysciences). The dishes were gently tilted to 45 degrees and then returned to a normal position to initiate mechanical stimulation. Twenty seconds after mechanical stimulation, the HBSS supernatants were collected and immediately used for ATP determination.

**ATP Measurement**

ATP was measured using a luciferin/luciferase bioluminescence assay kit (Molecular Probes) and a luminometer (Luminosenser-PSN.AB-2200).

**Isolated Kidney Perfusion**

Male Wistar rats weighing 350 to 400 g were purchased from Charles River Japan (Yokohama, Kanagawa, Japan), and the study was approved by the Committee on the Guidelines for Animal Experiments at Niigata University. The kidney perfusion system was established as previously described.

**Renin Activity Assays**

To determine the renin activity, perfusate samples were incubated with an excess of rat renin substrate (plasma from 36-hour bilaterally nephrectomized rats). The amount of Ang I generated was determined by a radioimmunoassay using a Renin Ria bead kit (Dainabot Co Ltd).

**Statistics**

Values are expressed as either the mean±SEM or mean±SD. Statistical analyses were performed by unpaired, two-tailed Student’s t test. A value of P<0.05 was considered statistically significant.

**Results**

**Characterization of the Cultured Rat JG Cells**

All the experiments in this study were performed on JG cells within 72 to 96 hours of primary culture. During this period, the adherent layer was nearly confluent and composed of cells with prominent cytoplasmic granules (Figure 1A). Immunofluorescent staining of the cultures using a goat anti-renin antibody demonstrated that >90% of the adherent cells exhibited a granular cytoplasmic pattern characteristic of renin particles (Figure 1B).
ATP, but not Gap Junctions, Mediates the Mechanically Elicited Ca\textsuperscript{2+} Wave Propagation in JG Cells

To assess the synchronizing mechanisms of Ca\textsuperscript{2+} signaling in JG cells, we studied the mechanically induced propagation of intercellular Ca\textsuperscript{2+} waves in cell monolayers. Mechanical stimulation of a single JG cell using a micropipette induced an immediate elevation of Ca\textsuperscript{2+} in the targeted cell, followed by propagation of the Ca\textsuperscript{2+} signal to the surrounding cells. This wave was able to spread between clusters of cells that did not appear to have physical contact (Figure 2C, top). By analyzing the data from 22 separate Ca\textsuperscript{2+} wave experiments, it was found that the mechanical stress caused a 7-fold increase in the Ca\textsuperscript{2+} concentration in the targeted JG cells, from 30 nmol/L at the basal level to 205 nmol/L after stimulation (Figure 2A). To quantify the number of cells involved in the Ca\textsuperscript{2+} wave propagation, cells with an increase in intracellular Ca\textsuperscript{2+} to >100 nmol/L were arbitrarily designated as positive. According to this criterion, 11.9±4.1 JG cells (mean±SEM; n=22) participated in the Ca\textsuperscript{2+} wave propagation in the control (Figure 2B).

Previous studies in various cell types have demonstrated that propagation of Ca\textsuperscript{2+} waves between cells can be mediated by either intercellular diffusion of messengers via GJIC, extracellular diffusion of cell-released ATP via activation of purinergic receptors, or simultaneously by both mechanisms.\textsuperscript{6–8,10,11,18} Because stimulation of JG cells could result in a Ca\textsuperscript{2+} wave that was able to spread between clusters of cells that did not appear to have physical contact, a mechanism involving diffusion of extracellular mediators is likely to be operating. We therefore focused on the potential role of ATP in the transmission of intercellular Ca\textsuperscript{2+} signaling. For this purpose, we manipulated the JG cell Ca\textsuperscript{2+} responses to extracellular ATP by either pretreatment of cells with high doses of ATP (100 \mu mol/L) 15 minutes before mechanical stimulation (to desensitize purinergic receptors) or addition of the ATP-degrading enzyme apyrase (50 U/mL) directly into the assay system. Both procedures were shown to be effective in abolishing the JG cell Ca\textsuperscript{2+} responses to the subsequent challenge with extracellular ATP, but did not interfere with the Ca\textsuperscript{2+} release from internal stores, as reflected by an increase in cytosolic Ca\textsuperscript{2+} after subsequent addition of thapsigargin (Figure 3). When mechanical stimulation was used to evoke intercellular Ca\textsuperscript{2+} wave propagation under the above conditions, the increases in Ca\textsuperscript{2+} in the targeted cells were not altered (Figure 2A); however, propagation of the Ca\textsuperscript{2+} wave from the stimulated cell to the surrounding cells was completely eliminated (control, 11.9±4.1, n=22; ATP-pretreated, 1.8±0.9, n=10; Apyrase addition, 1.7±0.7, mean±SEM, n=10) (Figure 2B). Typical sequences of Ca\textsuperscript{2+} ratio images obtained before and after stimulation of a single cell in the differently treated JG cells are depicted in Figure 2C.

These results suggest that the changes in Ca\textsuperscript{2+} in JG cells occurring during mechanically stimulated Ca\textsuperscript{2+} wave propagation are mediated by an ATP-dependent pathway. Consistent with this conclusion, the gap junction inhibitor heptanol (1 mmol/L) had no influence on the propagation of Ca\textsuperscript{2+} waves (data not shown). In addition, the major gap junction protein, connexin43, was not detected in the primary culture of JG cells by immunohistochemical staining (Figure 4). Furthermore, the dye transfer assay using a single-cell microinjection of Lucifer yellow also did not support the existence of
functional gap junctional communication between these cells. In 10 separate experiments, no dye diffusion from the injected cell was observed (dyecoupled cell number = 1 ± 0; mean ± SEM; n = 10) (Figure 5). As a positive control for these experiments, cultured mesangial cells, which anatomically adjoin JG cells in vivo, possessed abundant Cx43 in the regions of cell-to-cell contacts and were coupled with each other via gap junctions (Figures 4 and 5).

Release of ATP by Mass Mechanical Stimulation of JG Cells

To confirm that the mechanical strain induced the release of ATP from JG cells, a method involving massive mechanical stimulation of JG cells with microbeads was used, and the amount of ATP in the supernatants after stimulation was measured using a sensitive luciferase bioluminescence assay. As expected, the mechanical strain caused a 3-fold increase in ATP (Figure 6) in samples collected from JG cultures (control = 42 ± 14 nmol/L; mechanically stimulated = 125 ± 17 nmol/L; mean ± SEM; n = 10; P < 0.01). It should be noted that the actual concentration of ATP in the proximity of ATP-releasing JG cells was likely to have been much higher, because the released ATP would not have had time to equilibrate fully with the overlying medium. In addition, extracellular ATP is rapidly hydrolyzed by membrane-bound ectoenzymes. These results support the idea that ATP is the extracellular messenger mediating the propagation of intercellular Ca\textsuperscript{2+} signaling in mechanically stimulated JG cells.

Inhibition of Renin Secretion by ATP in Isolated Kidneys

ATP can both induce intracellular Ca\textsuperscript{2+} and mediate synchronization of intercellular Ca\textsuperscript{2+} signaling, and this led us to address the role of ATP in the control of renin secretion. To this end, we examined the effect of ATP on renin secretion in the isolated perfused kidney. We chose this experimental model instead of cell culture, because it is difficult to observe acute changes in renin in culture systems.

Isolated rat kidneys were perfused at a stable basal flow rate of 18 mL/min, and the perfusion pressure was around 70 to 90 mm Hg (80 ± 15 mm Hg; mean ± SEM; n = 11). The renin secretion rates from the isolated kidneys reached stable plateaus 10 to 20 minutes after the onset of perfusion. Because the low renin secretion rate seen under basal conditions may hinder observation of small decreases in renin secretion after administration of ATP, we chose this experimental model instead of cell culture, because it is difficult to observe acute changes in renin in culture systems.

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Figure 3. Modulation of JG cell Ca\textsuperscript{2+} responses to externally added ATP by pretreatment of JG cells, ATP or addition of apyrase into the assay system. A, JG cells were preincubated with 100 μmol/L ATP for approximately 15 minutes before being sequentially challenged with 100 μmol/L ATP and 200 nmol/L thapsigargin. B, JG cells were exposed to 100 μmol/L ATP and 200 nmol/L thapsigargin in the presence of 50 U/mL apyrase. Results are presented as both the ratiometric imaging (top) and dynamic traces of Ca\textsuperscript{2+} over time (bottom), representing the average level of Ca\textsuperscript{2+} in 25 cells in a single study. Similar results were obtained in 4 additional separate experiments.

Figure 4. Negative staining of Cx43 in cultured JG cells. Double-label immunofluorescence studies of JG cells with anti-renin and anti-Cx43 antibodies. Note the positive staining of renin particles (green, A) in these cells but no staining for Cx43 (red, B). As a positive control for Cx43, the characteristic punctate staining of Cx43 was found in both the cell-cell contact and perinuclear regions in cultured rat mesangial cells (red, C) under the same staining conditions. Magnification ×400. Similar results were obtained in 4 additional separate experiments. No labeled cells were found after reactions with irrelevant primary or secondary antibodies (data not shown).
contrast to the short-term increase in renal vascular resistance seen, the inhibition of renin secretion by ATP was persistent and was sustained over the whole observation period of 30 minutes (Figure 7B).

Discussion

Increases in intracellular Ca\textsuperscript{2+} concentration that spread from cell to cell provide a mechanism for cells to coordinate many cellular activities. In this study, we investigated the mechanisms implicated in the transmission of intercellular Ca\textsuperscript{2+} signals in JG cells, using an in vitro model of single-cell mechanical stimulation. Our study demonstrated that ATP, rather than gap junction channels, mediates the transmission of mechanically elicited intercellular Ca\textsuperscript{2+} signaling in JG cells. The evidence supporting this conclusion is as follows: (1) the mechanically elicited Ca\textsuperscript{2+} wave was able to spread between clusters of cells that did not appear to be in physical contact; (2) desensitization of purinergic receptors with ATP or addition of an ATP-degrading enzyme directly into the assay system could completely eliminate the mechanically elicited propagation of the intercellular Ca\textsuperscript{2+} wave; (3) a mechanical stretch-dependent release of ATP from JG cells was detected; and (4) the gap junction inhibitor heptanol, at a concentration (1 mmol/L) that proved to be effective in blocking Ca\textsuperscript{2+} wave propagation in mesangial cells,\textsuperscript{18} did not exhibit any effects in JG cells (data not shown). Furthermore, our data support neither the presence of the major gap junction protein Cx43, nor functional gap junction communication in cultured JG cells. Thus, it seems unlikely that the Ca\textsuperscript{2+} waves in these cells were propagated by a gap junction-dependent mechanism.

The lack of gap junction-mediated cell-to-cell communication in JG cells is unexpected. Ultrastructural observations revealed the presence of gap junctions among renin-secreting cells.\textsuperscript{23} Furthermore, histochemical data from our own studies,\textsuperscript{18,19} as well as those from other investigators,\textsuperscript{24–26} have shown the presence of abundant gap junction proteins, Cx43 and Cx40, in the juxtaglomerular region, although characterization of the proteins on a cellular basis has not been performed. The reason for the lack of functional gap junction communication in cultured JG cells is unclear. Loss of the differentiated cell phenotype in culture or the presence of gap junction proteins other than Cx43, which are less permeable to Lucifer yellow or Ca\textsuperscript{2+}, might be possible explanations. The selective permeabilities of different connexin proteins to Ca\textsuperscript{2+} and dyes have recently been reported.\textsuperscript{27,28} In this context, participation of gap junction channels in intercellular communication in vivo is still likely. A detailed structural and functional analysis of gap junctions in JG cells in vivo is needed for conclusive evidence.

Ca\textsuperscript{2+} waves are considered to coordinate multicellular processes, such as ciliary beating in tracheal epithelial cells,\textsuperscript{29} bile expulsion in the liver,\textsuperscript{30} hormone secretion in the pan-

![Figure 5](https://example.com/fig5.png)

Figure 5. No diffusion of Lucifer yellow (LY) dye from microinjected JG. LY was pressure-injected into a single JG (top) or mesangial (bottom) cell. LY diffusion into adjacent cells was monitored within a 3-minute time period. A and C. Phase-contrast micrographs of cultured cells. B and D, LY diffusion. Magnification ×320.

![Figure 6](https://example.com/fig6.png)

Figure 6. Induction of ATP release from JG cells by massive mechanical stimulation. Primary cultures of JG cells in 35-mm culture plates were washed 3 times with HBSS and cultured in 0.5 mL of HBSS with or without glass microbeads. Dishes were gently tilted to 45 degrees and then returned to a normal position to initiate mechanical stimulation. Twenty seconds after mechanical stimulation, the HBSS supernatants were collected and immediately used for ATP determination. Values shown represent the mean±SEM from 10 separate experiments. *P<0.01 vs the control.
creas, information processing in neural cells, and mesangial cell contraction in the kidney, and we therefore tried to elucidate the physiological relevance of the intercellular Ca\(^{2+}\) waves in JG cells.

Because ATP was identified as mediating the propagation of Ca\(^{2+}\) waves in JG cells, the question equates to addressing the role of ATP in renin secretion. For this purpose, we examined renin release in isolated perfused kidneys after administration of exogenous ATP. We chose the perfusion system instead of a cell culture model for this study because it is impossible to detect acute changes in renin in culture with the assays that are presently available. As a signaling mediator implicated in the regulation of renin secretion, ATP should be able to affect renin secretion quickly. As expected, ATP induced a rapid and potent inhibition of renin release. This result is consistent with the generally accepted notion that Ca\(^{2+}\)-mobilizing agents suppress renin secretion. Because all the cells in the JGA are considered to possess purinergic receptors, it can be envisaged that the activation of these receptors by ATP would elicit a rise in intracellular Ca\(^{2+}\) in these cells, thus leading to contraction of smooth muscle cells and reduced secretion of renin in JG cells. However, it is noteworthy that the factors and mechanisms actually contributing to the ATP-induced inhibition of renin secretion in this system might be multiple and complicated. For example, ATP can be rapidly and extensively degraded in vivo by membrane-bound ectoenzymes, and the metabolized product, adenosine, was reported to be capable of influencing renin secretion; furthermore, ATP-inducible endothelial-derived autacoids, such as NO and PGE\(_2\), were proven to be potent regulators of renin secretion. In addition, an increase in perfusion pressure after ATP administration is also able to cause a subsequent reduction in renin secretion via the baroreceptor mechanism. Therefore, the suppression of renin secretion by ATP observed in our study may reflect the overall actions of many known or unknown factors. Nevertheless, it does not alter our conclusion that ATP is able to modulate renin secretion.

It must be stressed that previous studies regarding the effect of ATP on renin secretion have produced inconsistent results. Using cultured mouse juxtaglomerular cells, Kurtz et al reported that ATP had no effect on the basal renin secretory rate; however, it antagonized forskolin- and isoproterenol-stimulated renin release. Churchill and Ellis reported that ATP stimulated renin secretion from rat renal cortical slices via a NO-dependent mechanism. These discrepancies could be due to the different experimental systems used. The exposure time to ATP, the presence or absence of heterocellular interactions, and the extent of ATP degradation are all potential factors influencing the results.

Our findings may have significant physiological implications. (1) The results provide a coordinating mechanism for renin secretion. In contrast to other secretory cells, which are usually tightly packed in a well-organized structure, JG cells exhibit an irregular and variable arrangement in the wall of afferent arterioles. This structure has been suggested to be not ideal for sensing changes in the hydrostatic pressure. It is therefore unlikely that all the granular cells would be affected by a pressure increase to the same extent. The question arises as to how the coordinated response of JG cells is achieved under this condition, and our study appears to provide an acceptable explanation for this situation. Hydrostatic pressure on afferent arterioles would activate the most sensitive JG cells (just like the mechanically stimulated cells in our system), causing a rise in intracellular Ca\(^{2+}\) and a simultaneous release of ATP into the paracellular milieu. ATP-dependent Ca\(^{2+}\) propagation mechanisms would allow Ca\(^{2+}\) to spread from the most responsive cells to the other cells in the JGA and thereby coordinate their secretory function. Even if the cells are homogeneously affected by the stimulus, the autocrine and/or paracrine action of the released ATP would further amplify and integrate individual Ca\(^{2+}\) response. (2) The results provide a potential mechanism for the mechanical strain–elicited inhibition of renin secretion. In this study, the Ca\(^{2+}\) wave was elicited by mechanical strain via deformation of a JG cell by an external device. This is somewhat similar to the mechanical deformation anticipated in vivo where increased renal perfusion pressure would lead to cellular elongation or stretching. Therefore, demonstration of the release of ATP induced by mechanical stress, as well as the inhibition of renin secretion by ATP, in this study may have significant relevance for the mechanically elicited inhibition of renin secretion. Indeed, the implication of ATP as a
signaling mediator in the regulation of vasoconstriction of the afferent arteriole during myogenic responses is now being recognized.\textsuperscript{32-34,42} Direct evidence regarding the effects of ATP on renin secretion during renal autoregulatory responses is still lacking. As two major reactions after elevated transmural pressure in the afferent arteriole, vascular constriction, and renin secretion may share the same controlling mechanisms, or at least, be somehow related to each other. Besides that produced by JG cells, ATP from other cell types may also participate in the control of renin secretion. Shear stress–dependent release of ATP has been demonstrated in a variety of cell types, including cells situated adjacent to JG cells, such as endothelial cells and smooth muscle cells.\textsuperscript{43-45} Recently, a positive relationship between renal hydrostatic pressure and interstitial ATP concentrations has been established.\textsuperscript{42,46} Taken together, these data support the critical role of ATP in mediating the inhibition of renin secretion under mechanical stimulation. Of note, a recent study by Bell et al\textsuperscript{47} has demonstrated that ATP was released from macula densa cells in response to increased luminal NaCl concentration via a maxi anion channel at the basolateral membrane. The released ATP mediated the transmission of intercellular Ca\textsuperscript{2+} signal between macula densa cells and mesangial cells. It is therefore conceivable that a similar paradigm of interaction may exist between macula densa cells and renin-secreting cells, which might be involved in the inhibition of renin secretion seen in tubuloglomerular feedback (TGF).

In summary, our study implicated ATP as a paracellular mediator participating in the transmission of intercellular Ca\textsuperscript{2+} signaling and inhibition of renin secretion in JG cells. The findings may help us to understand the coordinating mechanisms of renin secretion, and may also have close relevance for the mechanically elicited inhibition of renin secretion observed under physiopathological conditions.

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Materials and Methods

Materials

Goat anti-rat renin antibody was a generous gift of Dr. T Inagami, Department of Biochemistry, Vanderbilt University. BSA was purchased from Calbiochem (La Jolla, CA). Fura 2-acetoxymethyl ester (Fura-2 AM) came from Dojindo Molecular Technologies, Inc. (Bethesda, MD). All other reagents were obtained from Sigma (St. Louis, MO).

Preparation of rat JG cells

Rat JG cells were cultured according to the method described by Carey et al. Male Wistar rats weighing 100 to 150 g were used. For each cell preparation, two rats were killed by decapitation and the kidneys were excised, decapsulated and hemisected. The cortical tissue was taken, minced and transferred to a spinner flask with an enzyme solution, and incubated at 37°C with spinning for three consecutive 30-min periods. Between periods, cells were gassed with 95% O₂, 5% CO₂. After the 90 min, the enzymatic digestion was stopped by adding 10 ml FCS and the solution was further diluted three- to four-fold in Hanks balanced
salt solution (HBSS) containing 0.1% BSA and penicillin/streptomycin. Cells were collected by centrifugation at 1000 rpm for 5 min and resuspended in 70 ml Hanks-BSA-P/S. The cells then were centrifuged in a Percoll density gradient; four tubes containing 25 ml of an isotonic 30% (vol/vol) Percoll solution were prepared and centrifuged at 16000 rpm for 20 min. Cells which sedimented at a density of 1.0678 g/ml (previously reported as the renin-rich fraction)\textsuperscript{2} were carefully separated from the gradient and washed free of Percoll by centrifugation (1000 rpm) in 5 vol RPMI-BSA-P/S. After washing, cells were resuspended in culture medium, adjusted to a concentration of $10^5$ cells/ml, and seeded onto collagen-precoated plastic tissue culture dishes or special glass-bottom microwell dishes for primary culture. The JG cells were used for experiments within 72 and 96 h of culture.

**Immunocytochemistry**

Immunocytochemical staining for Cx43 and renin in cultured JG cells was done as described previously.\textsuperscript{3-5} In brief, the kidney sections and confluently cultured JG cells were fixed in 2% paraformaldehyde in PBS for 15 min and permeabilized with 1% Triton X-100 before staining. Then, the materials were
incubated overnight with anti-Cx43 or anti-renin antibody (diluted 1:200 in 1% FCS in PBS, 4°C). After rinsing with PBS, the appropriate secondary antibody (diluted in 1% FCS in PBS, 37°C) was added for 2 hr before final washing. The slides and sections were covered with Tris-buffered moviol, pH 8.6 and microscopy was performed using an Olympus BX50 microscope with a 40 x Planapo and 570 nm emission filter. Immunoﬂuorescent images were photographed using Fujichrome Sensia II (100 ASA) film (Fiji, Tokyo, Japan).

Measurement of gap junctional Intercellular communication

GJIC was assessed by transfer of the membrane-impermeant fluorescent dye, Lucifer yellow (LY), after single cell microinjection with an automated microinjection system (Zeiss, Oberkochen, Germany), using a method described previously.3-5 Briefly, the JG cells in 35-mm dishes were starved in 0.5% FCS-DMEM overnight before use. Cells with characteristic granual morphology of JG cells and full, close contacts with surrounding cells were chosen for injection. A mixture of Lucifer yellow (10% dissolved in 0.33 mol/l lithium chloride) and ethidium bromide (0.5 mg/ml; for nuclear staining) was injected into cells using a Zeiss-Eppendorf automated microinjection system.
(Zeiss Oberkochen, Germany) at pressures of 500 hectopascals applied for 0.5 s. The intracellular Lucifer yellow/ethidium bromide fluorescence was examined under a fluorescence microscope immediately thereafter. The number of cells exhibiting dye labeling was counted and the cell images were captured and stored directly onto the hard disk of the attached computer.

**Single cell mechanical stimulation**

Mechanical stimulation of a single JG cell was achieved by briefly distorting the apical surface of the cells with a blunted pre-pulled Eppendorf micropipette (Eppendorf, Hamburg, Germany). The movement of the pipette was done under the control of an automated microinjection system (Zeiss, Oberkochen, Company). The tip of the pipette was positioned near the apical membrane of a single cell; it was automatically deflected downwards to give a rapid (less than 1 second) mechanical stimulation of the cell when the action command was given.

**Measurement of Ca\(^{2+}\)**

JG cells were cultured in special glass bottom microwell dishes (MatTek Corporation, Ashland MA, USA) for 3 days. Dishes with confluent cultures of JG
cells were selected for experiments. The cells were washed with HBSS three times and then loaded with fura-2 by incubation with 5 \( \mu \text{mol/L} \) fura-2 acetoxymethyl ester (Fura-2 AM) in HBSS containing 2.0 mmol/L \( \text{CaCl}_2 \) and 1 mmol/L \( \text{MgCl}_2 \) at room temperature in the dark for 60 minutes. Cells were then rinsed and incubated with fura-2 free Hanks' solution for an additional 15 minutes to allow complete de-esterification of intracellular fura-2-AM. The dish was mounted in the chamber of an inverted epifluorescence microscope (Zeiss, Oberkochen, Germany). \( \text{Ca}^{2+} \) was determined by the ratio method, based on in vitro calibration \(^6\). Fura-2 was excited alternatively at 340 and 380 nm and fluorescence emission at 510 nm was detected with an ICCD camera (Hamamatsu, Japan). Images were captured and stored directly onto the hard disk of the attached computer and processed with special \( \text{Ca}^{2+} \) image analyzing software Argus/Hisca (Hamamatsu, Japan).

**ATP measurement**

ATP was measured using a luciferin/luciferase bioluminescence assay (Molecular Probes) and a luminometer (Luminosenser-PSN.AB-2200, Atto, Tokyo). Experimental samples and positive controls containing known
concentrations of ATP were examined. The amounts of ATP in the supernatants were calculated from the standard curve generated from the known ATP control.

**Isolated kidney perfusion**

Male Wistar rats with body weight of about 350-400 g were used for isolated perfusion. Kidney perfusion system was as previously published.\(^7,^8\) Briefly, the rats were anesthetized with pentobarbital sodium (0.1 mg/100 g body weight). After opening of the abdominal cavity by a midline incision, the right kidney and mesenteric artery were exposed. A double-lumen catheter was advanced through the mesenteric artery, the perfusion flow was started, and the catheter was inserted into the right renal artery and tied in place. The aorta and vena cava were rapidly cut to free the kidney. After several seconds to permit flushing of blood, the kidney was placed into the warmed cup. The surface of the kidney was covered with oil to reduce dehydration. The kidney was perfused at a constant perfusion flow of about 18 ml/min. The renal artery pressure was monitored through the inner part of the pressure cannula. The basic perfusion solution consisted of a modified Krebs-Hanseleit solution containing (mM): all physiological amino acids at concentrations of between 0.2 and 2, 8.7 glucose,
0.3 pyruvate, 2.0 L-lactate, 1.0 α-ketoglutarate, 1.0 L-malate and 6.0 urea. The perfusate was supplemented with 6 g/100 ml bovine serum albumin. In all experiments, the perfusate was initially equilibrated with 95% air-5% CO₂ and kept in a temperature-controlled water bath (37°C). Perfusate flow rates were obtained from the revolutions of the peristaltic pump, which was calibrated before and after each experiment. Renal flow rates and perfusion pressure were continuously monitored. After establishing the perfusion loop, perfusion pressure usually stabilized within 15 min. The ATP solution was freshly prepared and infused into the arterial limb of the perfusion circuit 20 mintues after lowing the perfusate flow rate. To measure renin activiety in the perfusate, aliquots were drawn at 5-min intervals from the arterial limb of the circulation and stored at –20°C until assayed for renin activity.

**Renin activity assays**

To determine the renin activity, the perfusate samples were incubated with an excess of rat renin substrate (Plasma from 36-h bilaterally nephrectomized rats) for 60 min at 37°C. Ang I generation was allowed to proceed in 50 mM PO4 bufer 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 radioimmunoassay using a Renin Riabead kit from Dainabot Co, LTD, Tokyo,
Japan). Renin secretion rates were calculated as the product of the
arterio-venous differences of renin activity and the perfusate flow rate (ml/min/g).
References


