Control of Renin Secretion From Rat Juxtaglomerular Cells by cAMP-Specific Phosphodiesterases

Ulla G. Friis, Boye L. Jensen, Shala Sethi, Ditte Andreasen, Pernille B. Hansen, Ole Skøtt

Abstract—We tested the hypothesis that cGMP stimulates renin release through inhibition of the cAMP-specific phosphodiesterase 3 (PDE3) in isolated rat juxtaglomerular (JG) cells. In addition, we assessed the involvement of PDE4 in JG-cell function. JG cells expressed PDE3A and PDE3B, and the PDE3 inhibitor trequinsin increased cellular cAMP content, enhanced forskolin-induced cAMP formation, and stimulated renin release from incubated and superfused JG cells. Trequinsin-mediated stimulation of renin release was inhibited by the permeable protein kinase A antagonist Rp-8-CPT-cAMPS. PDE4C was also expressed, and the PDE4 inhibitor rolipram enhanced cellular cAMP content. Dialysis of single JG cells with cAMP in whole-cell patch-clamp experiments led to concentration-dependent, biphasic changes in cell membrane capacitance (C_m) with a marked increase in C_m at 1 μmol/L, no net change at 10 μmol/L, and a decrease at 100 μmol/L. cAMP also had a dual effect on C_m at 10-fold higher concentration compared with cAMP. Trequinsin, milrinone, and rolipram mimicked the effect of cAMP on C_m. Trequinsin, cAMP, and cGMP enhanced outward current 2- to 3-fold at positive membrane potentials. The effects of cAMP, cGMP, and trequinsin on C_m and cell currents were abolished by inhibition of protein kinase A with Rp-cAMPS. We conclude that degradation of cAMP by PDE3 and PDE4 contributes to regulation of renin release from JG cells. Our data provide evidence at the cellular level that stimulation of renin release by cGMP involves inhibition of PDE3 resulting in enhanced cAMP formation and activation of the cAMP sensitive protein kinase. (Circ Res. 2002;90:996-1003.)

Key Words: juxtaglomerular apparatus ■ renin ■ exocytosis ■ cGMP ■ phosphodiesterase
with parallel renin release studies and cAMP measurements in isolated JG cells.

**Materials and Methods**

**Isolation of Rat JG Cells**
Rat (male Sprague-Dawley, 80 g) JG cells were isolated as for mouse JG cells by enzymatic digestion of renal cortex with collagenase A (Roche Diagnostics, Denmark) and trypsin (Sigma, Denmark), followed by filtration and wash. For the patch-clamp studies, the cells were transferred directly to cover slips, and for the renin secretion and cAMP studies, the cells were separated on a Percoll density gradient (25% Percoll at 27,000 g for 30 minutes), resuspended in RPMI-1640 medium, and either seeded in 96-multwell plates or loaded to superfusion chambers.

**Renin Secretion From JC-Cell Cultures**
Cultured cells were incubated for 20 hours, and then the cells were washed and experimental agents added. After 20 hours, the medium was removed, and the remaining cells were harvested as described. Renin concentration was determined by radioimmunoassay (RIA) for angiotensin I. Renin secretion rates were calculated as fractional release of total renin content.

**Renin Secretion From Superfused JG Cells**
The superfusion chambers (Bakerbond spe Columns) contained 50 mg preswollen Biogel P-2 (45 to 90 mm, BioRad) layered on top and a filter at the bottom. The cells were superfused with RPMI-1640 medium at a rate of 230 μL/min from 1 of 2 pumps. Superfusate was collected at 2-minute intervals. Four parallel experiments were run simultaneously. Renin activity was determined by RIA. Renin is expressed in terms of Goldblatt units (GU) compared with standards from the National Institute for Biological Standards and Control (Hertfordshire, UK).

**Measurement of cAMP**
JG-cell suspensions were seeded in 1-mL aliquots and incubated with agents for 10 minutes. The cells were harvested in cold ethanol with 20 mmol/L HCl. After evaporation, cAMP was measured by RIA (Amersham-Biokart RPA509). Each experiment represents the mean of duplicate culture wells.

**Immunocytochemical Labeling for Renin**
The JG cells were rinsed with TRIS-Tween Buffer solution, subsequently fixed, permeabilized, and peroxidase-blocked with methanol/H2O2. The cells were then sequentially incubated with goat serum/BSA, with rabbit anti-renin antibody, with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (DAKO, Glostrup, Denmark), and finally developed with DAB+ substrate chromogen.

**Identification of Cells Used for Patch Clamp**
Presumed JG cells were sampled through patch pipettes and mRNA was isolated and reverse transcribed by oligo dT-coated magnetic beads (Dynal micro RNA kit) according to manufacturers instructions. Renin activity in sampled cells was determined by RIA.

**Polymerase Chain Reactions**
PCRs were performed as described, and primer sequences for renin and β-actin were identical to those previously used. Primers for PDE3A were sense 5'-TGGTTTCTGGTGTCG-3', antisense 5'-AGCCAACTTATACACA-3', 316 bp; and for PDE3B, sense 5'-GC-AGTGGTGAACCTCA-3', antisense 5'-AATCCAGGAAAATG-GTGTGC-3', 216 bp. Primers for PDE4C were sense 5'-CTACCTC- CCAACGTGG-3', antisense 5'-CTCAGTTTCTGCTGGTGTCG-3', 321 bp.

**Patch-Clamp Experiments**
The patch-clamp experiments on single rat JG cells were performed as for mouse JG cells, with the exception that membrane capacitance, Cm, was measured with the “sine+dc” method using the Lock-in extension of the PULSE v8.11 software. These measurements started maximally 30 seconds after the initial current-voltage (I-V) recording.

**Solutions**
“Internal” control solution for patch clamp was as follows (in mmol/L): K-glutamate 55; NaCl 10; KCl 90; MgCl2 1; HEPES 10; Mg-ATP 0.5; and Na2GTP 0.3. osmolality was 307 mOsm/kg; pH 7.00 (22°C). “External” (bath) solution for patch clamp was as follows (in mmol/L): NaCl 140; KCl 2.8; MgCl2 1; CaCl2 2; glucose 11; and sucrose 10; osmolality was 296 to 314 (range) mOsm/kg; pH 7.25 (25°C).

**Reagents**
The following reagents were used: 4,2-(hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES); Tris-HCl: glucose, sucrose, insulin, penicillin, streptomycin, K-glutamate, Mg-ATP, trequinsin, forskolin, isoproterenol, dithiothreitol, milrinone, rolipram, and trypsin were from Sigma Chemical (USA). RPMI-1640 and FCS were from GIBCO Life Technologies. Rp-cAMPs and Sp-cAMPS were from Research Biochemicals International. The 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphorothioate Rp-isomer (Rp-8-cPT-cAMPS) was from Biolog GmbH. Collagenase A, Na2-GTP, cGMP, and cAMP were from Roche. Percoll was from Pharmacia. All other chemicals were of analytical grade.

**Statistics**
All values are given as mean±SEM. Paired Student’s t test was used to calculate statistical difference from zero in experiments where Cm was measured. The change in Cm was calculated as the difference (in %) in Cm (at t=0 minutes and t=10 minutes). ANOVA was used to calculate statistical significance among several groups (Figures 3D and 4E) and Dunnett’s t test was used to compare several groups with a control (Figure 3C). A value of P<0.05 was considered statistically significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

**Characterization of Isolated JG cells**
The whole-cell recording was obtained in 192 single cells isolated from 74 preparations. These cells showed a current-voltage relation typical both for isolated and in situ JG cells (Figure 1E). Thus, the cells displayed outward current rectification at positive membrane potentials and very limited net currents between -30 to 0 mV. The JG cells had an average Cm value of 2.82±0.06 pF (n=192). Evidence for correct cell identity was obtained by demonstration of prorenin mRNA expression in serial dilution of cDNA from 20 sampled JG cells. PCR-amplification products for renin were seen with cDNA corresponding to 2 JG cells (Figure 1A). Renin enzyme content was measured in single sampled rat JG cells (Figure 1B). There was a linear relation between the number of cells sampled and the renin concentration. Moreover, immunocytochemical labeling for renin with a specific rabbit renin antibody showed strong positive immunostaining of a subset of granular cells (Figure 1C). Original recordings of the whole-cell currents after applying 11 pulses from -110 mV to +130 mV in 30-mV steps for 60 ms from a holding potential of -30 mV are shown in Figure 1D with the resulting I-V curves in Figure 1E. The current-voltage
Whole-cell currents were measured in response to 11 pulses of +130 mV in 30-mV steps for 60 ms from a holding potential of −30 mV. E, Steady-state average I-V relationships were measured immediately after the whole-cell configuration was obtained (circle) and after 10 minutes of capacitance recording (square). The figure shows that the two I-V curves are identical under control conditions. Figure 1F shows a 10-minute recording of Cm in a single JG cell, in which the pipette contained control internal solution and the cell was bathed in control external solution. During the recording time (up to 20 minutes), basal Cm did not change significantly (+3.8±1.7%, n=7, P>0.05) (Figure 1G).

Thus, the present protocol for cell isolation yields single JG cells suited for patch-clamp experiments. Under control conditions, Cm and current-voltage relations remain stable at least within the recording time used in the present study. The cells maintain all typical features of fully differentiated renin-producing JG cells.

### Role of Protein Kinase A for cAMP-Mediated Effects on Current and Cm in JG Cells

Similarly to previous studies using single mouse JG cells,17 cAMP leads to biphasic concentration-dependent changes in Cm, ie, increase in Cm (exocytosis) at 1 μmol/L cAMP, and decrease in Cm (endocytosis) at 100 μmol/L cAMP (Table). The increase of Cm in response to 1 μmol/L cAMP was completely abolished by the PKA-blocker Rp-cAMPS (25 μmol/L) (Table). Otherwise stimulated by cAMP-dependent agonists (forskolin 24.9±1.1%, n=4, P<0.05; isoproterenol 16.8±1.5%, n=4, P<0.05).

During 20 hours of primary culture, rat JG cells released 11.3±1.0% of active renin. Renin release rate was significantly stimulated by cAMP-dependent agonists (forskolin 24.9±1.1%, n=4, P<0.05; isoproterenol 16.8±1.5%, n=4, P<0.05).

### Effect of cGMP on Current and Cm in JG Cells

Figure 2A shows the current-voltage relationship before (circle) and after (square) 10 minutes of capacitance recording. These cells were bathed with cGMP (10 μmol/L), which, like cAMP, resulted in a significant 2.4-fold increase in the outward current at +130 mV (P<0.05, n=9). This increase was abolished by Rp-cAMPS (25 μmol/L) (data not shown).

Original traces from 2 cells dialyzed with 10 μmol/L (upper trace) or 100 μmol/L cGMP (lower trace) are shown in Figure 2C. Thus, addition of 10 μmol/L cGMP to the patch pipette solution led to a modest but significant increase in Cm (+5.4±1.1%, n=7, P<0.05; Figure 2D). Cyclic GMP at 50 μmol/L did not significantly change Cm (−1.3±2.5%, n=5), whereas 100 μmol/L cGMP significantly decreased Cm (−5.0±0.7%, P<0.05, n=4) (Figure 2D). Thus, cGMP mimicked the biphasic effect of cAMP on Cm, although at a higher concentration. The increase of Cm in response to 10
ml/L cGMP was abolished by Rp-cAMPs (25 ml/L) (Figure 2D), suggesting that PKA mediates the effect of cGMP on Cm in rat JG cells.

Effect of PDE3 and PDE4 Blockers on cAMP Level, Whole-Cell Current, Cm, and Renin Release in JG Cells

The expression profile for PDE3 isoenzyme mRNAs was analyzed by RT-PCR on serial dilutions of cDNA from cells recovered from the Percoll gradient (Figure 3A). Based on serial dilution, there was a more marked PDE3A-expression compared with PDE3B in freshly isolated cells. Furthermore, PDE3A and PDE4C were also expressed in single JG cells sampled with the patch pipette (Figure 3B).
Inhibition of cAMP-specific PDE3 and PDE4 with trequinsin and rolipram for 10 minutes significantly increased cAMP levels in isolated JG cells (n=6, with 2 wells assigned per condition in 1 experiment; Figure 3C). Trequinsin and rolipram both stimulated cellular cAMP accumulation after 10 minutes of incubation (Figure 3D). Preincubation with trequinsin (100 μmol/L) for 1 hour stimulated cAMP formation significantly, and furthermore, trequinsin markedly potentiated the stimulatory effect of forskolin after 10 minutes of incubation (Figure 3D).

Figure 4A shows the current-voltage relationship before (circle) and after (square) 10 minutes of Cm recording. These cells were dialyzed with trequinsin (2 mmol/L), which resulted in a 3.3-fold increase in the outward current at +130 mV (P<0.05, n=4). This increase in outward current was abolished by the PKA-blocker Rp-cAMPS (Figure 4B). Similar results were obtained with 200 nmol/L trequinsin (data not shown).

The original Cm trace from a cell dialyzed with trequinsin (2 mmol/L) is shown in Figure 4C (left panel). Capacitance measurements of single JG cells represent cumulative changes in membrane surface area over time. In order to compare such data with renin release rates, the cell capacitance measurements can be converted to a rate by differentiation with respect to time. Figure 4C (right panel) shows the differentiated data from the experiment shown in the left panel.

Trequinsin (2 mmol/L) led to a marked increase in Cm (+11.4±3.1%, P<0.05, n=5) (Figure 4D), whereas a 100-fold higher concentration of trequinsin decreased Cm (−19.5±6.6%, P<0.05, n=7) (Figure 4D). Both effects of trequinsin on Cm were blocked by Rp-cAMPS (Figure 4D), indicating that trequinsin alters the Cm of JG cells through a cAMP/PKA-dependent pathway. A different (albeit less potent) PDE3 inhibitor, milrinone was also tested. Milrinone (2 μmol/L) increased Cm significantly (8.7±0.7%; P<0.05, n=4) (Figure 4D). Furthermore, the PDE4 inhibitor rolipram (1 μmol/L) increased Cm of JG cells significantly (9.2±2.6%; P<0.05, n=4) (Figure 4D).

The additivity of cGMP and trequinsin was also tested. When the cell was dialyzed with trequinsin (2 mmol/L) and cGMP (10 μmol/L), Cm (amounting to 5.2±0.5%, P<0.05, n=6) was identical to the Cm value obtained with cGMP alone (5.4%). Outward current at +130 mV increased 1.8-fold (from 1.2±0.4 to 2.1±0.3 pA; P<0.05, n=6). This finding supports the concept of a common target for cGMP and trequinsin in the sequence of events leading to Cm changes.

The effect of trequinsin and forskolin on renin secretory activity was assessed on the same preparations of JG cells as used for single cell patch clamp in 2 different experimental setups. First, isolated JG cells were superfused and the effluent was collected with a time resolution of 2 minutes. As shown in Figure 4E, addition of 10 μmol/L forskolin, 1 μmol/L trequinsin, and 10 μmol/L rolipram all resulted in rapid and transient stimulations of renin release to levels significantly above time controls.

When the time course of the renin release from the superfused juxtaglomerular cells stimulated by trequinsin (Figure 4E) is compared with the time course of the (differentiated) membrane capacitance data shown in Figure 4C (right panel), it can be seen that the time courses in the 2 experimental situations are very similar.

Second, renin release was studied over prolonged time in primary cultures of JG cells. Under control conditions, these cells released 8.7±1.3% of total content (n=4) (Figure 4F). Forskolin (10 μmol/L) significantly increased renin release to 23.4±2.2% of total renin content. Similarly, 10 μmol/L trequinsin led to a significant stimulation of renin release (19.5±1.5% of total content, P<0.05). In a separate series of experiments, the effect of the membrane permeable PKA antagonist Rp-8-CPT-cAMPS on trequinsin-mediated renin secretion was tested in the primary cultures of JG cells. In this series of experiments, 10 μmol/L trequinsin stimulated renin secretion 2.6-fold (n=5, with 4 wells assigned per condition in 1 experiment, P<0.05) (Figure 4G). Rp-8-CPT-cAMPS (10−4 mol/L) significantly reduced this effect of trequinsin (29.3±2.5% versus 21.1±1.7%, P<0.05; Figure 4G).

Discussion

We used isolated rat juxtaglomerular granular (JG) cells as a model to study renin secretion at the cellular level with electrophysiological and biochemical methods. The results demonstrate that renin release from JG cells is significantly regulated at the level of degradation of cAMP by the cGMP-inhibited PDE3 and by PDE4 and that the target for cAMP is PKA.

Individual JG cells from rat kidneys were chosen for patch clamp based on visible granularity and were included if they displayed the characteristic current-voltage relation previously reported from mouse JG cells. Cell identity was assured by detection of renin mRNA expression, presence of renin activity, and immunoreactive renin protein. Average cell membrane capacitance, Cm, was 2.82 pF, which corresponds to a cell surface area of 2.82 μm² if the specific capacitance is 1 μF/cm². This number is similar to values obtained from mouse JG cells (3.13 pF). In the control situation, Cm was stable, and the current-voltage relationship was not affected by the whole-cell mode within the time of recording.

An increase in Cm, which indicates net addition of membrane to the cell surface area, is an accepted measure of exocytosis at the level of the single cell. After moderate stimulation of renin secretion, Cm increased and achieved a stable level in about 10 minutes. The time courses of changes in Cm (differentiated to show rates) and changes in renin release after stimulation of secretion were very similar (cf, Figures 4C and 4E). The transient increase in renin release rate probably reflects the existence of a pool of rapidly releasable renin granules. Further release must await recruitment of renin granules from deeper within the cytoplasm. Therefore, the cellular mechanisms responsible for the more persistent increase in renin secretion observed in 20-hour incubated JG cells are likely to include effects on intracellular granule trafficking.

A decrease in Cm indicates net loss of cell surface area and may occur after endocytosis or after shedding of cell mem-
brane material. The sudden decreases in \( C_m \) (and thereby membrane surface area) observed after dialyzing JG cells with forskolin were associated with enhanced release of renin whenever this parameter was measured. Thus, increases in \( C_m \) correlates to exocytosis, whereas a decrease in \( C_m \) reflects a net loss of surface membrane area, but does not exclude simultaneous exocytosis. Conclusions about secretion in the present study are therefore only drawn based on observed increases in \( C_m \).

The view of cAMP as a stimulator of renin release through PKA was confirmed by the observation that dialysis of single JG cells with 1 \( \mu \)mol/L cAMP caused an increase in \( C_m \), which was blocked by a PKA inhibitor. Furthermore, \( C_m \) was enhanced by treatment with a PKA activator. In addition to the stimulation of secretion, cAMP also increased outward currents at positive potentials similarly to results from mouse JG cells.17 This is in accordance with previous data showing no hyperpolarizing response of the membrane potential of JG cells to cAMP-dependent stimulators of renin release.

cGMP had effects on \( C_m \) and current that were qualitatively similar to the effects of cAMP, although at 10-fold higher concentrations. The effects of cGMP on \( C_m \) and current were abolished by PKA inhibition, thus providing evidence that the cAMP-PKA pathway mediates these cGMP-mediated responses in JG cells. In agreement with this, cGMP-dependent NO donors enhance cAMP levels.

Figure 4. Effects of trequinsin on whole-cell current, \( C_m \), and renin release. A. Steady-state average I-V relationships before (●) and after (▲) 10 minutes of capacitance measurements. When the cells were dialyzed with trequinsin (2 nmol/L), the maximal outward current at +130 mV amounted to 1.0±0.4 nA at the beginning of the experiment (●) and 3.2±0.9 nA after 10 minutes of measurement (▲) \((n=4)\). B. Steady-state average I-V relationships before (●) and after (▲) 10 minutes of capacitance measurements. When the cells were dialyzed with trequinsin (2 nmol/L) together with the PKA blocker Rp-cAMP (25 \( \mu \)mol/L), the maximal outward current amounted to 1.5±0.4 nA at the beginning of the experiment (●) and 1.0±0.4 nA after 10 minutes of measurement (▲) \((n=4)\). C. Recording of \( C_m \) from a single rat JG cell dialyzed with 2 nmol/L trequinsin. Left shows original trace with averages per 2 minutes shown as triangles (△); Right, same data after differentiation to allow comparison of time course with secretion data shown in E. D. Relative changes of JG-cell \( C_m \) in response to trequinsin (2 or 200 nmol/L with or without addition of the PKA-blocker Rp-cAMPs [25 \( \mu \)mol/L]). miirinone (2 \( \mu \)mol/L), and rolipram (1 \( \mu \)mol/L). E. Effect of trequinsin on renin release from superfused juxtaglomerular cells. Isolated rat JG cells were superfused with RPMI-medium in all experiments. After 60 minutes, the superfusion medium was changed and drugs were added: trequinsin 1 \( \mu \)mol/L (●) \((n=7)\); trequinsin 10 \( \mu \)mol/L (▲) \((n=7)\); forskolin 10 \( \mu \)mol/L (○) \((n=7)\); and control (□) \((n=7)\). *Significant difference between control and forskolin. F. Effect of the PKA-inhibitor Rp-8-CPT-cAMPS on trequinsin-induced stimulation of renin secretion. Isolated rat JG cells were incubated with 10\(^{-5}\) mol/L trequinsin alone or in combination with increasing concentrations of Rp-8-CPT-cAMPS. The response to 10\(^{-5}\) mol/L forskolin is shown for comparison. (n=4; 4 wells assigned per condition in 1 experiment). \( P<0.001 \), when compared with control. †Significant difference between control and forskolin. G. Effect of the PKA-inhibitor Rp-8-CPT-cAMPS on trequinsin-induced stimulation of renin secretion. Isolated rat JG cells were incubated with 10\(^{-5}\) mol/L trequinsin alone or in combination with increasing concentrations of Rp-8-CPT-cAMPS. The response to 10\(^{-5}\) mol/L forskolin is shown for comparison (n=5). *Significant difference \((P<0.01)\) between trequinsin and trequinsin with Rp-8-CPT-cAMPS. †Significant differences between control and trequinsin or forskolin, respectively.
and renin release in JG-cell cultures. Likewise, renin secretion is promptly stimulated in a PKA-dependent fashion by activation of the NO-cGMP pathway in the isolated perfused kidney. In conclusion, our data provide evidence that the cellular level that PKA mediates stimulatory cGMP responses in JG cells.

PDE3B has been suggested to mediate cross talk between cAMP and cGMP pathways in JG cells. The PDE3 enzymes are cAMP-specific PDEs that are inhibited by cGMP and comprise the 3A and 3B subtypes, which are encoded by two separate genes. cGMP inhibits PDE3A activity in the micromolar range, whereas PDE3B is less sensitive. PDE3B was cloned from rat adipocytes, whereas PDE3A is mainly expressed in myocardium and vasculature. There has been some confusion with respect to the nomenclature of PDE3A and B. We used the corrected nomenclature (PDE3A, GenBank accession No. 97-2-2-9-22527, 01-1-2-30-22896, 99-2-2-36-sels,20,26,27 we found more marked PDE3A expression (PDE3A, GenBank accession No. GI 8567397). As previously observed in kidney ves-
tory cGMP responses in JG cells.

Corrections to the nomenclature of PDE3A and B. 20,26 We used the corrected nomenclature (PDE3A, GenBank accession No. GI 8567397). As previously observed in kidney ves-

cells in a PKA-dependent way. Further evidence that PKA mediates the cellular responses through PDE3 was provided by the observation that the effects of cGMP and trequinsin were not additive. The patch-clamped cells were more sensitive to the blockers compared with incubated JG cells. This is probably due to the difference in the route of administration with direct intracellular dialysis versus diffusion across the cell membrane from the medium. Taken together, the results show that inhibition of PDE3 has a significant impact on renin release and cell currents, and that these effects are mediated through the cAMP-PKA pathway. In addition to PDE3, PDE4 also influences cAMP-PKA pathway function. We conclude from this that although cGMP stimulates renin release through the PDE3 cAMP-PKA pathway, PDE3 is not the only determinant of cAMP breakdown rate.

Taken together, our data strongly indicate that phosphodiesterase 3 and 4 are significant determinants of membrane trafficking and renin secretion at the level of single JG cells, and that the target for cAMP in the exocytotic pathway in JG cells is PKA. The data show that cGMP changes JG-cell membrane turnover and cell currents in a PKA-dependent way. Our results therefore provide direct cellular evidence for the hypothesis that cGMP-dependent agonists enhance renin release through a PDE3-mediated change in cAMP metabolism in JG cells.

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