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. cGMP 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

The main rate-limiting step controlling the activity of the circulating renin-angiotensin system is the release of active renin from juxtaglomerular (JG) cells of the afferent glomerular arterioles in the kidney. Cyclic nucleotides are critical second messengers that determine renin secretory rate. Hormones, neurotransmitters, and autacoids that raise the intracellular production of cAMP stimulate renin secretion and renin mRNA levels.[1,2] Agonists coupled to cGMP formation have been reported both to stimulate and inhibit renin release.[3–7] At present, the response to cGMP is considered to be determined by the predominance of one of several targets for cGMP in JG cells.[7,8]

The concentrations of cyclic nucleotides in cells are determined by the rate of synthesis by cyclases and by the rate of degradation by specific cyclic nucleotide phosphodiesterases (PDEs).[9] Drugs like theophylline, which inhibit PDEs unspecifically, increase renin secretion in vivo and potentiate the renin-secretory response to β-adrenoceptor stimulation.[10] This suggests a basal phosphodiesterase activity in JG cells. Presently, at least 11 different isoforms of PDEs are recognized. They are encoded by different genes and have different substrate specificity (cAMP or cGMP or both) and regulatory mechanisms (cAMP, cGMP, Ca^{2+}, and others).[9] With regard to the control of renin release, the PDE3 and PDE4 subtypes have attracted special attention. Thus, PDE3- and PDE4-selective inhibitors increase renin release in conscious rabbits and humans.[11–14] Similar findings have been obtained with the isolated perfused rat kidney.[8] Recent data suggest presence also of functional cGMP-specific PDE5 in JG cells.[15] PDE3 and PDE4 use primarily cAMP as a substrate, and PDE3 is endogenously inhibited by cGMP.[9] This raises the intriguing possibility of an interaction between hormones acting through cGMP production and the cAMP pathway in the control of renin release. Recent data from whole animal studies[11] and from the isolated kidney[16] have indeed supported the concept that cGMP-dependent agonists might enhance renin release through inhibition of PDE3. The primary aim of this study was to test this latter hypothesis at the cellular level, where effects on renal hemodynamics, renal nerves, and signals from the macula densa are excluded. In addition, we tested the possible involvement of PDE4 in cellular control of JG-cell function. To address the question directly, we applied the patch-clamp technique to single isolated rat JG cells for measurements of whole-cell currents and cell capacitance (C_m) in response to manipulations of the cAMP and cGMP pathways. The C_m tracks were compared

Circulation Research is available at http://www.circresaha.org   DOI: 10.1161/01.RES.0000017622.25365.71

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[2,3] 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 000g for 30 minutes), resuspended in RPMI-1640 medium, and either seeded in 96-multiwell plates or loaded to superfusion chambers.

Renin Secretion From JG-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.[17] Renin concentration was determined by radioimmunoassay (RIA) for angiotensin I.[18] 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.[19] 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-Biotrak 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/H_2O_2. 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.[19]

Polymerase Chain Reactions

PCRs were performed as described,[17] and primer sequences for renin and β-actin were identical to those previously used.[17] Primers for PDE3A were sense 5′-TGCTTTCCTGGTTGC-3′, antisense 5′-AGCCAACTTTATACACA-3′, 316 bp; and for PDE3B, sense 5′-GC-AGTGGTGTAAACCTCA-3′, antisense 5′-AATCCAGGAAATG-GTGTG-3′, 216 bp.[20] Primers for PDE4C were sense 5′-CTACCACT-CCAACGTGG-3′, antisense 5′-CTCAGTTTCTGCTTGGTGC-3′, 321 bp.[20]

Patch-Clamp Experiments

The patch-clamp experiments on single rat JG cells were performed as for mouse JG cells,[17] with the exception that membrane capacitance, C_m, was measured with the "sine+dc" method using the LockIn 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; MgCl$_2$ 1; HEPES 10; Mg-ATP 0.5; and Na$_2$GTP 0.3; osmolality was 307 mOsm/kg; pH 7.00 (22°C). "External" (bath) solution for patch clamp was as follows (in mmol/L): HEPES 10; NaCl 140; KCl 2.8; MgCl$_2$ 1; CaCl$_2$ 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, Na$_2$-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 C_m was measured. The change in C_m was calculated as the difference (in %) in C_m (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 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.[17,21] The JG cells had an average C_m value of 2.82±0.06 pF (n=192). Evidence for correct cell identity was obtained by demonstration of pre-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

A

B

C

D

E

F

G
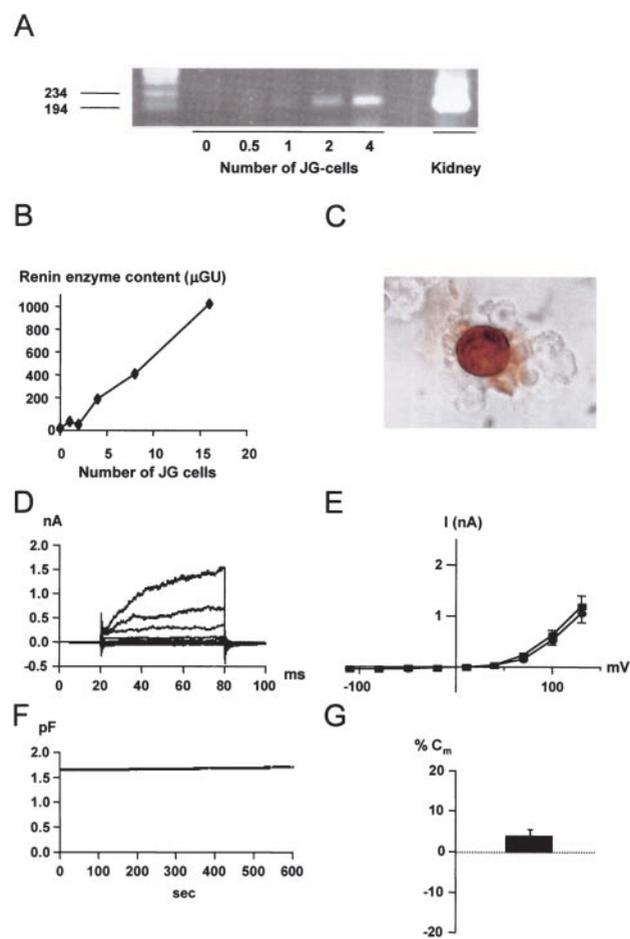
Figure 1. Validation of method. A, Expression of preprorenin mRNA in cells used for patch clamp. Serial diluted cDNA from sampled JG cells was amplified for renin by reverse transcription-PCR for 34 cycles. Lane 1, molecular weight marker (ϕX174DNA/*Hae*III); lane 2 ("0"), cell-free fluid from the JG-cell preparation; lanes 3 to 6, PCR amplification products for renin with cDNA as indicated; lane 7, negative control for the PCR with water replacing cDNA; and lane 8, positive control with RT-PCR amplification of 50 ng kidney cortex RNA. Agarose gel (2%) was stained with ethidium bromide. B, Renin activity in sampled JG cells. JG cells were sampled with modified patch pipettes from the same preparation as used for single-cell elec-trophysiology. C, Immunolabeling of renin protein in a typical JG cell with rabbit anti-mouse renin antibody. D, Recording of whole-cell current from a JG cell dialyzed with control solution. Whole-cell currents were measured in response to 11 pulses from -110 mV to $+130$ mV in 30-mV steps for 60 ms from a holding potential of -30 mV. E, Steady-state average *I*-V rela-tionships from 6 independent control experiments before ($\bullet$) and after ($\blacksquare$) 10 minutes of capacitance measurements. The maximal outward current at $+130$ mV amounted to 1.1 ± 0.3 nA (at the beginning of the experiment) and 1.2 ± 0.3 nA (after 10 minutes of measurements). F, Typical time course of cell capacitance (C_m) in a single rat JG cell dialyzed with control solution. G, Rel-ative average change of C_m using control solutions in the pipette and bath (n=6). There was no significant change in C_m during the recording time.

relationship was 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 C_m in a single JG cell, in which the pipette contained control internal solution and the

Effect of cAMP on Membrane Capacitance (C_m) in Single Rat Juxtaglomerular Cells

Compound	C_m (%)	n	P
cAMP (1 μmol/L)	15.3 ± 5.2	7	<0.05
cAMP (10 μmol/L)	-4.1 ± 1.8	4	NS
cAMP (100 μmol/L)	-15.8 ± 6.4	6	<0.05
Sp-cAMPs (10 μmol/L)	15.2 ± 3.4	4	<0.05
Rp-cAMPs (25 μmol/L)	-2.9 ± 2.8	4	NS
cAMP (1 μmol/L)+Rp-cAMPs (25 μmol/L)	-1.8 ± 1.6	4	NS

cells were bathed in control external solution. During the recording time (up to 20 minutes), basal C_m did not change significantly ($+3.8\pm1.7\%$, 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, C_m 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 C_m in JG Cells

Similarly to previous studies using single mouse JG cells,[17] cAMP leads to biphasic concentration-dependent changes in C_m, ie, increase in C_m (exocytosis) at 1 μmol/L cAMP, and decrease in C_m (endocytosis) at 100 μmol/L cAMP (Table). The increase of C_m in response to 1 μmol/L cAMP was completely abolished by the PKA-blocker Rp-cAMPs (25 μmol/L) (Table). The stable cAMP analogue, Sp-cAMPs (10 μmol/L), also increased C_m significantly (Table). Further-more, cAMP (1 μmol/L) increased outward current 3.3-fold (at $+130$ mV) from 0.7 ± 0.4 to 2.3 ± 0.6 pA ($P<0.05$, n=4). This increase was abolished by Rp-cAMPs (25 μmol/L) (data not shown).

During 20 hours of primary culture, rat JG cells released $11.3\pm1.0\%$ of active renin. Renin release rate was signifi-cantly stimulated by cAMP-dependent agonists (forskolin $24.9\pm1.1\%$, n=4, $P<0.05$; isoproterenol $16.8\pm1.5\%$, n=4, $P<0.05$).

Effect of cGMP on Current and C_m in JG Cells

Figure 2A shows the current-voltage relationship before (circle) and after (square) 10 minutes of capacitance recording. These cells were dialyzed 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=5). This increase was abolished by Rp-cAMPs (25 μmol/L) (Figure 2B).

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 C_m ($+5.4\pm1.1\%$, n=7, $P<0.05$; Figure 2D). Cyclic GMP at 50 μmol/L did not significantly change C_m ($-1.3\pm2.5\%$, n=5), whereas 100 μmol/L cGMP significantly decreased C_m ($-5.0\pm0.7\%$, $P<0.05$, n=4) (Figure 2D). Thus, cGMP mimicked the biphasic effect of cAMP on C_m, although at a higher concentration. The increase of C_m in response to 10
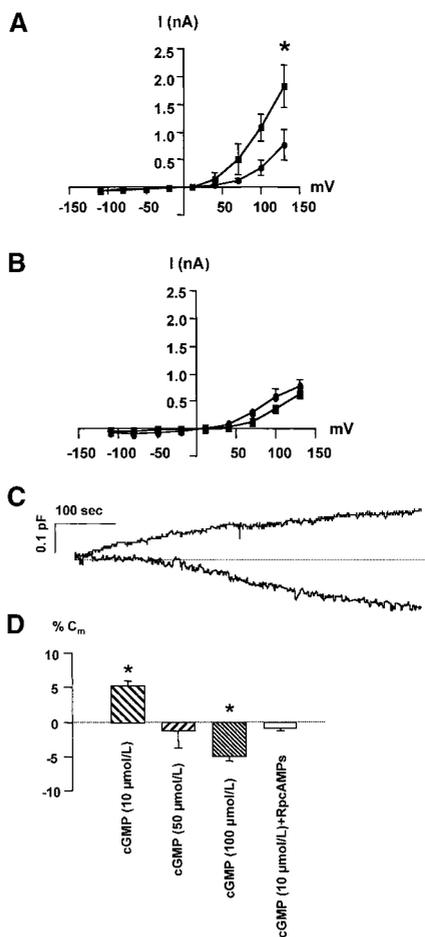
Figure 2. Effects of cGMP on whole-cell currents and C_m of isolated JG cells. A, Steady-state average I-V relationships before (●) and after (■) 10 minutes of capacitance measurements. When the cells were dialyzed with 10 μmol/L cGMP, the maximal outward current at +130 mV was 0.8 ± 0.3 nA at the beginning of the experiment (●) and 1.8 ± 0.4 nA after 10 minutes of measurement (■) (n=5). B, Steady-state average I-V relationships before (●) and after (■) 10 minutes of capacitance measurements. When the cells were dialyzed with cGMP (10 μmol/L) together with the PKA blocker Rp-cAMP (25 μmol/L), the maximal outward current amounted to 0.8 ± 0.1 nA at the beginning of the experiment (●) and 0.6 ± 0.1 nA after 10 minutes of measurement (■) (n=3). C, Typical time courses of C_m in single rat JG cells dialyzed with 10 μmol/L cGMP (top trace) and 100 μmol/L cGMP (bottom trace). D, Relative changes of C_m in response to cGMP alone (10, 50, and 100 μmol/L) or with the PKA-blocker Rp-cAMPs (25 μmol/L).

μmol/L cGMP was abolished by Rp-cAMPs (25 μmol/L) (Figure 2D), suggesting that PKA mediates the effect of cGMP on C_m in rat JG cells.

Effect of PDE3 and PDE4 Blockers on cAMP Level, Whole-Cell Current, C_m, 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).
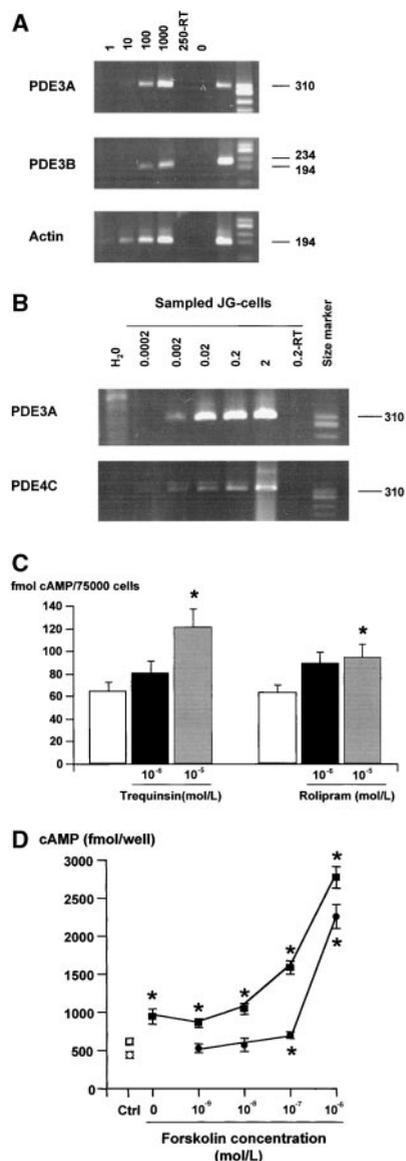
Figure 3. Expression and functional significance of phosphodiesterase 3 and 4. A, PCR amplification (32 cycles) of cDNA from isolated JG cells for PDE3A (top), PDE3B (middle), and β-actin (bottom). JG cells were allowed to recover from the Percoll gradient, counted, and total RNA was isolated and used for RT-PCR amplification as indicated. A negative control with omission of reverse transcriptase was included ($-$RT). "0" is negative control for PCR without cDNA. As a positive control, cDNA from renal cortex was amplified. Size marker is ϕX174DNA/*Hae*III. B, PCR-amplification of cDNA from JG cells sampled with the patch pipette for PDE3 and PDE4. Messenger RNA was isolated from single JG cells by magnetic beads, mRNA was reverse transcribed, and cDNA was PCR amplified for 30 cycles. A dilution series of this cDNA was used as template for a second round of PCR amplification as indicated. Size marker is ϕX174DNA/*Hae*III. PDE3: 313 bp; PDE4: 303 bp. C, Enhancement of cAMP concentration by trequinsin and rolipram in isolated JG cells. JG cells were exposed to 1 and 10 μmol/L of the drugs for 10 minutes (n=6). D, Enhancement of forskolin-induced cAMP production in JG cells by trequinsin. Ctrl indicates control values in the absence of drugs (open symbols). Lower curve (●), Forskolin was added for 10 minutes in concentrations as indicated (n=6). Upper curve (■), JG cells were preincubated with 100 μmol/L trequinsin for 1 hour, and then forskolin was added for 10 minutes in concentrations as indicated (n=5). *Significant difference from control in same series.

Inhibition of cAMP-specific PDE3 and PDE4 with trequinsin[22] 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 at 10 μmol/L. In a second series of experiments, forskolin concentration-dependently increased cAMP production 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 C_m recording. These cells were dialyzed with trequinsin (2 nmol/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 (25 μmol/L) (Figure 4B). Similar results were obtained with 200 nmol/L trequinsin (data not shown).

The original C_m trace from a cell dialyzed with trequinsin (2 nmol/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 nmol/L) led to a marked increase in C_m ($+11.4\pm3.1\%$, $P<0.05$, n=5) (Figure 4D), whereas a 100-fold higher concentration of trequinsin decreased C_m ($-19.5\pm6.6\%$, $P<0.05$, n=7) (Figure 4D). Both effects of trequinsin on C_m were blocked by Rp-cAMPs (Figure 4D), indicating that trequinsin alters the C_m 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 C_m significantly ($8.7\pm0.7\%$; $P<0.05$, n=4) (Figure 4D). Furthermore, the PDE4 inhibitor rolipram (1 μmol/L) increased C_m of JG cells significantly ($9.2\pm2.6\%$; $P<0.05$, n=4) (Figure 4D).

The additivity of cGMP and trequinsin was also tested. When the cell was dialyzed with trequinsin (2 nmol/L) and cGMP (10 μmol/L), C_m (amounting to $5.2\pm0.5\%$, $P<0.05$, n=6) was identical to the C_m 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 C_m 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 trequinsin 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 $8.7\pm1.3\%$ of total content (n=4) (Figure 4F). Forskolin (10 μmol/L) significantly increased renin release to $23.4\pm0.9\%$ of total renin content. Similarly, 10 μmol/L trequinsin led to a significant stimulation of renin release ($19.5\pm1.5\%$ 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 2.5-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 ($29.3\pm2.5\%$ versus $21.1\pm1.7\%$, $P<0.05$; 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.[17,21] Cell identity was assured by detection of renin mRNA expression, presence of renin activity, and immunoreactive renin protein. Average cell membrane capacitance, C_m, was 2.82 pF, which corresponds to a cell surface area of 2.82 μm^2 if the specific capacitance is 1 μF/cm^2. This number is similar to values obtained from mouse JG cells (3.13 pF).[17] In the control situation, C_m was stable, and the current-voltage relationship was not affected by the whole-cell mode within the time of recording.

An increase in C_m, which indicates net addition of membrane to the cell surface area, is an accepted measure of exocytosis at the level of the single cell.[29] After moderate stimulation of renin secretion, C_m increased and achieved a stable level in about 10 minutes. The time courses of changes in C_m (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 C_m indicates net loss of cell surface area and may occur after endocytosis or after shedding of cell mem-
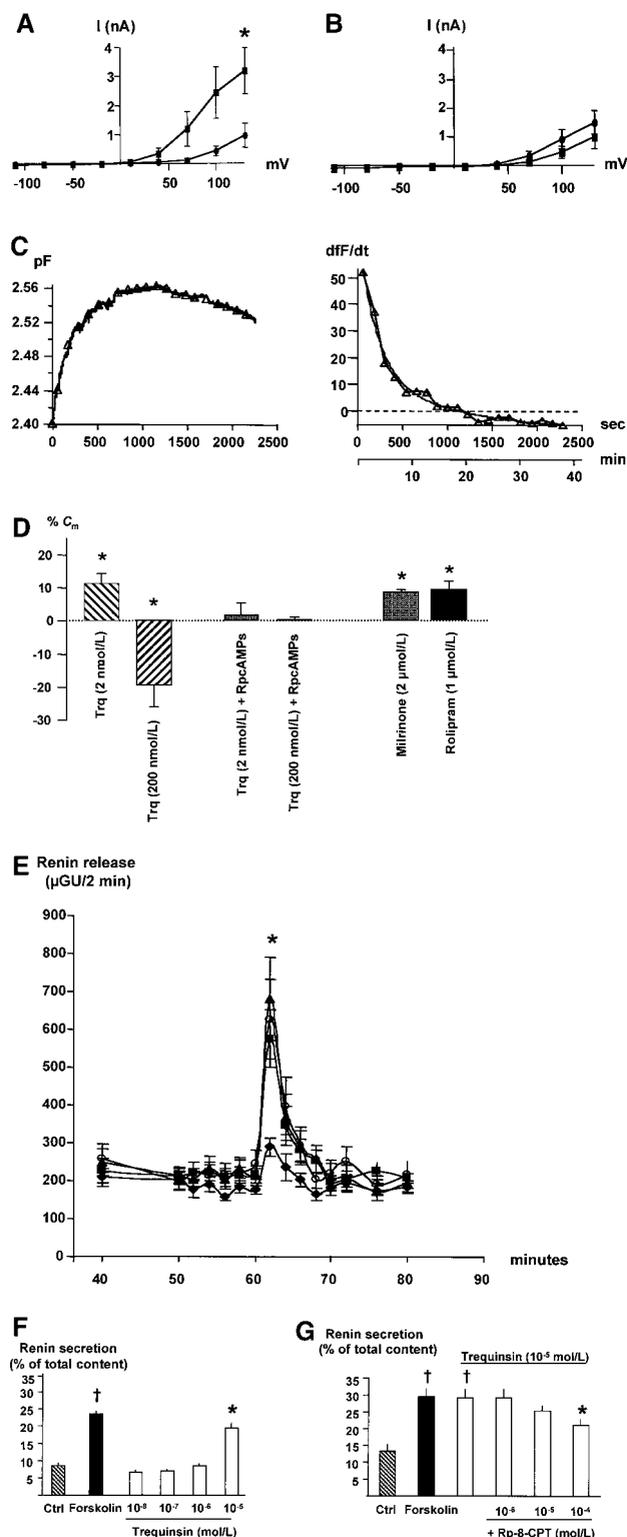
Figure 4. Effects of trequinsin on whole-cell current, C_m, and renin release. A, Steady-state average *I*-V relationships before (●) and after (■) 10 minutes of capacitance measurements. When the cells were dialyzed with trequinsin (2 nmol/L), the maximal outward current at +130 mV amounted to 1.0±0.4 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 μmol/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=3). 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 (Δ); 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 μmol/L]), milrinone (2 μmol/L), and rolipram (1 μmol/L). E, Effect of trequinsin on renin release from superfused juxtaglomerular cells. Isolated JG cells were superfused with RPMI-medium in all experiments. After 60 minutes, the superfusion medium was changed and drugs were added: trequinsin 1 μmol/L (■) (n=7); trequinsin 10 μmol/L (▲) (n=7); forskolin 10 μmol/L (○) (n=7); and control (◆) (n=7). *$P<0.05$. F, Effect of trequinsin on renin secretion from cultured JG cells. Isolated rat JG cells were incubated with trequinsin in the concentrations indicated and compared with control. 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.

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 μmol/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] The PKA inhibitor blocked this effect. The increase in outward current was only observed at positive holding potentials (>+20 mV), and was unlikely to be directly related to the changes in capacitance, which were recorded at a considerably lower holding potential (−30 mV). This is in accordance with previous data[28] 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

brane material. The sudden decreases in C_m (and thereby membrane surface area) observed after dialyzing JG cells with high concentrations of cAMP, cGMP, PDE inhibitors, or after stimulation of cAMP production with forskolin[17] 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

and renin release in JG-cell cultures.[4] Likewise, renin secretion is promptly stimulated in a PKA-dependent fashion by activation of the NO-cGMP pathway in the isolated perfused kidney.[16] In conclusion, our data provide evidence at the cellular level that PKA mediates stimulatory cGMP responses in JG cells.

PDE3 has been suggested to mediate cross talk between cAMP and cGMP pathways in JG cells.[8,11,16] The PDE3 enzymes are cAMP-specific PDEs that are inhibited by cGMP and comprise the 3A and 3B subtypes, which are encoded by 2 separate genes.[23,24] cGMP inhibits PDE3A activity in the micromolar range, whereas PDE3B is less sensitive.[25] PDE3B was cloned from rat adipocytes,[24] whereas PDE3A is mainly expressed in myocardium and vasculature.[23] There has been some confusion with respect 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 vessels,[20,26,27] we found more marked PDE3A expression compared with PDE3B in JG cells (and renal vascular smooth muscle cells [results not shown]). In addition, PDE4C was expressed in JG cells. In agreement with this, selective PDE3 and PDE4 inhibitors increased cAMP levels in JG cells and changed both C_m and whole-cell current in a PKA-dependent way similar to dialysis with intracellular cAMP. In keeping with this result, trequinsin stimulated renin release from superfused and incubated JG cells in a PKA-dependent way. Further evidence that cGMP affected 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.

References

1. Chen M, Schnermann J, Smart AM, Brosius FC, Killen PD, Briggs J. Cyclic AMP selectively increases renin mRNA stability in cultured juxtaglomerular granular cells. *J Biol Chem*. 1993;268:24138–24144.
2. Della Bruna R, Pinet F, Corvol P, Kurtz A. Regulation of renin secretion and renin synthesis by second messengers in isolated mouse juxtaglomerular cells. *Cell Physiol Biochem*. 1991;1:98–110.
3. Kurtz A, Della Bruna R, Pfeilschifter J, Taugner R, Bauer C. Atrial natriuretic peptide inhibits renin release from juxtaglomerular cells by a cGMP-mediated process. *Proc Natl Acad Sci U S A*. 1986;83: 4769–4773.
4. Schricker K, Kurtz A. Liberators of NO exert a dual effect on renin secretion from isolated mouse renal juxtaglomerular cells. *Am J Physiol*. 1993;265:F180–F186.
5. Scholz H, Kurtz A. Involvement of endothelium-derived relaxing factor in the pressure control of renin secretion from isolated perfused kidney. *J Clin Invest*. 1993;91:1088–1094.
6. Greenberg S, He X-R, Schnermann JB, Briggs JP. Effect of nitric oxide on renin secretion, I: studies in isolated juxtaglomerular granular cells. *Am J Physiol*. 1995;37:F948–F952.
7. Gambaryan S, Wagner C, Smolenski A, Walter U, Poller W, Haase W, Kurtz A, Lohmann SM. Endogenous or overexpressed cGMP-dependent protein kinases inhibit cAMP-dependent renin release from rat isolated perfused kidney, microdissected glomeruli and isolated juxtaglomerular cells. *Proc Natl Acad Sci U S A*. 1998;95:9003–9008.
8. Kurtz A, Götz KH, Hamann M, Wagner C. Stimulation of renin secretion by nitric oxide is mediated by phosphodiesterase 3. *Proc Natl Acad Sci U S A*. 1998;95:4743–4747.
9. Dousa TP. Cyclic-3′,5′-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int*. 1999;55:29–62.
10. Reid IA, Stockigt JR, Goldfien A, Ganong WF. Stimulation of renin secretion in dogs by theophylline. *Eur J Pharmacol*. 1972;17:325–332.
11. Chiu T, Reid IA. Role of cyclic GMP-inhibitable phosphodiesterase and nitric oxide in the β-adrenoceptor control of renin secretion. *J Pharmacol Exp Ther*. 1996;278:793–799.
12. Chiu YJ, Hu S-H, Reid IA. Inhibition of phosphodiesterase III with milrinone increases renin secretion in human subjects. *J Pharmacol Exp Ther*. 1996;290:16–19.
13. Chiu N, Park I, Reid IA. Stimulation of renin secretion by the phosphodiesterase IV inhibitor rolipram. *J Pharmacol Exp Ther*. 1996;276: 1073–1077.
14. Reid IA. Role of phosphodiesterase isoenzymes in the control of renin secretion: effects of selective enzyme inhibitors. *Curr Pharm Des*. 1999; 5:725–735.
15. Sayago CM, Beierwaltes WH. Nitric oxide synthase and cGMP-mediated stimulation of renin secretion. *Am J Physiol*. 2001;281:R1146–R1151.
16. Kurtz A, Götz KH, Hamann M, Kieninger M, Wagner C. Stimulation of renin secretion by NO donors is related to the cAMP pathway. *Am J Physiol*. 1998;274:F709–F717.
17. Friis UG, Jensen BL, Aas J, Skøtt O. Direct demonstration of exo- and endocytosis in single mouse juxtaglomerular cells. *Circ Res*. 1999;84: 929–936.
18. Poulsen K, Jørgensen J. An easy radioimmunological microassay of renin activity, concentration and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody. *J Clin Endocrinol Metab*. 1974;39:816–825.
19. Lykkegaard S, Poulsen K. Ultramicroassay for plasma renin concentration in the rat using the antibody trapping technique. *Analyt Biochem*. 1976;75:250–259.
20. Sandner P, Kornfeld M, Ruan X, Arendshorst WJ, Kurtz A. Nitric oxide/cAMP interactions in the control of rat renal vascular resistance. *Circ Res*. 1999;84:186–192.
21. Kurtz A, Penner R. Angiotensin II induces oscillations of intracellular calcium and blocks anomalous inward rectifying potassium current in

mouse renal juxtaglomerular cells. *Proc Natl Acad Sci U S A*. 1989;86: 3423–3427.

22. Lal B, Dohadwalla AN, Dadkar NK, D'Sa A, de Souza NJ. Trequinsin, a potent new antihypertensive vasodilator in the series of 2-(arylimino)-3-alkyl-9,10-dimethoxy-3,4,6,7-tetrahydro-2H-pyrimido [6,1-a]isoquinolin-4-ones. *J Med Chem*. 1984;27: 1470–1480.

23. Meacci E, Taira M, Moos M Jr, Smith CJ, Movsesian MA, Degerman E, Belfrage P, Manganiello V. Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. *Proc Natl Acad Sci U S A*. 1992;89:3721–3725.

24. Taira M, Hockman SC, Calvo JC, Taira M, Belfrage P, Manganiello VC. Molecular cloning of the rat adipocyte hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase. *J Biol Chem*. 1993;268: 18573–18579.

25. Leroy MJ, Degerman E, Taira M, Murata T, Wang LH, Movsesian MA, Meacci E, Manganiello VC. Characterization of two recombinant PDE3

26. (cGMP-inhibited cyclic nucleotide phosphodiesterase) isoforms, RcGIP1 and HcGIP2, expressed in NIH 3006 murine fibroblasts and Sf9 insect cells. *Biochemistry*. 1996;35:10194–10202.

26. Reinhardt RR, Chin E, Zhou J, Taira M, Murata T, Manganiello VC, Bondy CA. Distinctive anatomical patterns of gene expression for cGMP-inhibited cyclic nucleotide phosphodiesterases. *J Clin Invest*. 1995;95: 1528–1538.

27. Liu H, Maurice DH. Expression of cyclic GMP-inhibited phosphodiesterases 3A and 3B (PDE3A and PDE3B) in rat tissues: differential subcellular localization and regulated expression by cyclic AMP. *Br J Pharmacol*. 1998;125:1501–1510.

28. Bührle CP, Scholz H, Hackenthal E, Nobiling R, Taugner R. Epithelioid cells: membrane potential changes induced by substances influencing renin secretion. *Mol Cell Endocrinol*. 1986;45:37–47.

29. Lindau M, Neher E. Patch-clamp techniques for time-resolved capacitance measurements in single cells. *Pflügers Arch*. 1988;411:137–146.

Circulation Research

American Heart Association®

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 and Ole Skøtt

The online version of this article, along with updated information and services, is located on the
World Wide Web at:

http://circres.ahajournals.org/content/90/9/996

Data Supplement (unedited) at:

http://circres.ahajournals.org/content/suppl/2002/05/10/90.9.996.DC1