The Calcium Paradoxon of Renin Release

Calcium Suppresses Renin Exocytosis by Inhibition of Calcium-Dependent Adenylate Cyclases AC5 and AC6

Christian Grünberger, Birgit Obermayer, Jürgen Klar, Armin Kurtz, Frank Schweda

Abstract—An increase in the free intracellular calcium concentration promotes exocytosis in most secretory cells. In contrast, renin release from juxtaglomerular (JG) cells is suppressed by calcium. The further downstream signaling cascades of this so called “calcium paradoxon” of renin secretion have been incompletely defined. Because cAMP is the main intracellular stimulator of renin release, we hypothesized that calcium might exert its suppressive effects on renin secretion via the inhibition of the calcium-regulated adenylate cyclases AC5 and AC6. In primary cultures of JG cells, calcium-dependent inhibitors of renin release (angiotensin II, endothelin-1, thapsigargin) suppressed renin secretion, which was paralleled by decreases in intracellular cAMP levels [cAMP]. When [cAMP] was clamped by membrane permeable cAMP derivates, renin release was not suppressed by any of the calcium liberators. Additionally, both endothelin and thapsigargin suppressed cAMP levels and renin release in isoproterenol or forskolin-pretreated As4.1 cells, a renin-producing cell line that expresses AC5 and AC6. The calcium-dependent inhibition of intracellular cAMP levels and renin release was prevented by small interfering RNA–mediated knockdown of AC5 and/or AC6 expression, underlining the functional significance of these AC isoforms in renin-producing cells. Finally, in isolated perfused mouse kidneys, angiotensin II completely inhibited the stimulation of renin secretion induced by adenylate cyclase activation (isoproterenol) but not by membrane permeable CAMP analogs, supporting the conclusion that the suppressive effect of calcium liberators on renin release is mediated by inhibition of adenylate cyclase activity. (Circ Res. 2006;99:1197-1206.)

Key Words: renin secretion ■ cAMP ■ angiotensin II ■ calcium

The renin/angiotensin system (RAS) plays a central role in the regulation of cardiovascular function under both physiological and pathophysiological conditions such as arterial hypertension and heart failure. The rate-limiting step of the RAS cascade is the proteolytic activity of renin which is released from the renin-producing juxtaglomerular (JG) cells of the kidney into the circulation by regulated exocytosis.1 Despite its major impact on cardiovascular function, the regulation of renin release at the cellular level is incompletely understood.

Presently, 3 intracellular second messenger systems are known to control renin release: the cyclic nucleotides cAMP and cGMP and the intracellular free calcium concentration [Ca2+]i.2 Although the effects of cGMP are rather complex with either stimulatory or inhibitor effects, cAMP and [Ca2+]i represent the main intracellular regulators of renin secretion.3 Thus, it had been uniformly demonstrated in a variety of in vitro and in vivo models that stimulation of CAMP generation or inhibition of cAMP degradation enhances the release of renin from JG cells (for review, see Hackenthal et al1). The direct intracellular application of cAMP in JG cells induced exocytosis as determined by patch clamp experiments on single JG cells, indicating that it is cAMP per se that controls renin release.3 The stimulatory effects of cAMP on renin release are in line with known effects of cAMP on other secretory cells, in which cAMP also promotes exocytosis. In contrast, [Ca2+]i initiates, supports, or maintains exocytosis in other secretory cells, whereas it inhibits the release of renin, a phenomenon that has been termed the “calcium paradoxon” of renin release.1,2 The concept of an inhibitory effect of [Ca2+]i on renin release is based primarily on indirect evidence. Generally, classical vasoconstrictor hormones such as angiotensin II, endothelin, or vasopressin potently inhibit renin release both in vivo and in vitro in a calcium-dependent manner.1,2 Moreover, vasoconstrictors like angiotensin II induced calcium influx into isolated JG cells and simultaneously inhibited renin release.4 Also, nonspecific measures to modulate [Ca2+]i-activated renin secretion. Thus, a reduction in the extracellular calcium concentration stimulated renin release from several models including isolated JG cells,3 whereas an activation of store-operated calcium influx by thapsigargin suppressed renin secretion rates from isolated perfused rat kidneys.6
Downstream signaling events, which mediate the inhibition of renin release by [Ca\textsuperscript{2+}], have been the subject of several studies. Thus, activation of protein kinase C and calcium/calmodulin-dependent processes and the modulation of ion channel activities in the plasma membrane of JG cells or in the renin storage vesicles have been implicated in this process.\textsuperscript{1,2} However, despite evidence supporting these pathways, no general accepted explanation detailing the calcium paradoxon has been established. In the present study, we hypothesized that calcium might inhibit renin release by a suppression of intracellular cAMP formation. As mentioned above, cAMP is the central intracellular stimulator of renin exocytosis. Therefore, suppression of intracellular cAMP by [Ca\textsuperscript{2+}], should result in inhibition of renin release.

In general, intracellular cAMP concentrations [cAMP], are controlled by the ratio of cAMP formation by adenylyl cyclases (ACs) and cAMP hydrolysis by phosphodiesterases (PDEs). Therefore, calcium might lower [cAMP], by either inhibiting AC activity or activating PDEs. In fact, 2 of the 9 AC isoforms are inhibited by physiological intracellular calcium concentrations.\textsuperscript{7,8} Both of these calcium-inhibited ACs, AC5 and AC6, are expressed in rat kidneys and AC6 expression has been detected in the renin-producing JG cells of human kidneys.\textsuperscript{9–11} Moreover, there is functional evidence for a role of calcium-activated PDE type 1 (PDE1) in the regulation of renin release, because pharmacological blockade of this enzyme stimulated renin secretion from isolated perfused kidneys and increased cAMP levels in renin-producing cells.\textsuperscript{12,13}

To clarify whether the calcium-dependent inhibition of renin release is mediated by [cAMP], suppression, we performed experiments using primary cultures of JG cells, the renin-producing cell line As4.1 and isolated perfused mice kidneys. Our data show that known calcium-dependent inhibitors of renin release suppress cAMP formation by inhibition of AC activity and that this pathway significantly contributes to the inhibitory effects on renin release.

Materials and Methods

Primary Culture of Mouse Renin-Producing JG Cells

JG cells of C57BL/6 mice were isolated as described.\textsuperscript{14} After 20 hours, the cultures were washed once and 100 μL of prewarmed culture medium containing the chemicals to be tested was added.

Experiments on Renin Secretion

After 4 hours of incubation, renin activity was determined in supernatants and cell lysates.\textsuperscript{15} Renin-release rates were calculated as fractional release of total active renin (ie, renin activity released/ [renin activity released+ renin activity remaining in the cells]).

Determination of cAMP Content

After 4 hours of incubation, the supernatant was removed, cells were lysed, and cAMP concentration was directly measured in the sample by an enzyme linked assay (direct cAMP Kit, Assay designs).

Determination of the Intracellular Calcium Concentration

[Ca\textsuperscript{2+}], in JG cells was assessed in fura-2 measurements. To this end, cells were seeded on glass cover slips and superfused with ringer solution throughout the measurements. For details, see the online data supplement, available at http://circres.ahajournals.org.

Determination of AC Expression in JG Cells and Aorta

Abdominal aortas were isolated from 5 C57BL/6 mice of either sex. The surrounding fat was removed, aortas were longitudinally cut, the endothelium was scraped using a scalpel blade under a stereomicroscope, and the remaining tissue was stored in liquid nitrogen. JG cells were isolated as described.\textsuperscript{14} Freshly isolated JG cells were not seeded but immediately subjected to RNA isolation. Total RNA isolation from aortas and JG cells as well as subsequent RT-PCR was performed as described.\textsuperscript{15} Semiquantification of AC mRNA expression was performed by real-time PCR.\textsuperscript{15} For details and primer sequences, see the online data supplement.

As4.1 Cell Culture

As4.1 cells were cultured as described previously.\textsuperscript{15}

Small Interfering RNA Transfection of As4.1 Cells

Cells (5000 per well) were seeded in 96-well plates 24 hours before transfection. Small interfering RNA (siRNA) specific for mouse AC5 (XM_156060) or AC6 (NM_007405) was purchased from Dharmacon as a pool of 4 independent siRNAs, respectively. A nonspecific siRNA pool (Dharmacon) served as negative control for these experiments. Transfection was performed using DharmaFECT transfection Reagent No. 2 (Dharmacon) according to the instructions of the manufacturer.

Determination of cAMP Content

After 72 hours of transfection, the medium was removed and 100 μL of prewarmed medium containing the tested compounds was added. After 4 hours of incubation, cell lysates were harvested for cAMP measurements as described for JG cells (see above).

Determination of Renin Release

As detected in pilot experiments, As4.1 cells used in this study primarily secreted renin in its inactive prorenin form (95%). Therefore, to determine renin release, intracellular and secreted prorenin was activated by trypsin basically as described previously.\textsuperscript{16} Because neither forskolin nor isoproterenol robustly stimulated renin release after 4 hours of incubation, cells were prestimulated before the measurement period. Thus, 10 μL of the drug stock solutions were added to the medium for 4 hours (experiments with forskolin treatment) or 6 hours (experiments with isoproterenol treatment). Thereafter, the medium was removed and 100 μL of prewarmed medium with the compounds to be tested was added. After another 4 hours of stimulation (76 hours after start of transfection), the medium and cells were harvested as described for JG cells and total renin activity was determined in the medium and cell lysates.

Semiquantification of AC mRNA expression was performed by real-time PCR as described previously.\textsuperscript{15} For details and primer sequences, see the online data supplement.

AC5 and AC6 Western Blot

The cell lysates from 5 wells were pooled. Total protein (5 μg per lane) was loaded, separated on a 7% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Munich, Germany). The blots were blocked overnight at 4°C and incubated for 2.5 hours at room temperature with the primary antibody (anti-AC5 1:250; PAC-501AP, FabGennix Inc) and for 45 minutes at room temperature with the secondary antibody (1:5000, horseradish peroxidase–conjugated anti-rabbit IgG; Santa Cruz Biotechnology). Detection was achieved with enhanced chemiluminescence (Amer sham), and band intensities were quantified by densitometry. For the detection of β-actin protein (mouse monoclonal antibody, 1:5000; Sigma), 2.5 μg of protein was analyzed.

Antibodies directed against AC6 (PAC-601AP FabGennix Inc; ab14781 Abcam) did not produce a specific band of the expected molecular weight in extracts of kidneys, brain, or As4.1 cells.
Isolated Perfused Mouse Kidney

Kidneys of male C57Bl/6 mice were perfused at a constant pressure of 100 mm Hg as described in detail previously.\(^{17}\)

Determination of Renin Activity

Renin activity in supernatants and cell lysates of primary cultures of JG cells and As4.1 cells as well as in the venous perfusate from isolated perfused kidneys were determined by the ability of the samples to generate angiotensin I from the plasma of bilaterally nephrectomized rats. Angiotensin I was measured by a commercially available radioimmunoassay (DiaSorin).

Statistics

Three to 5 different cell preparations were taken for each experimental protocol. In each experiment on renin release or cAMP formation, 4 individual wells were assigned per condition. Therefore, each data point represents 12 to 20 single measurements and is depicted as mean±SEM. Differences between groups were analyzed by ANOVA and Bonferroni adjustment for multiple comparisons. In isolated perfused kidney experiments, the last 2 values obtained within an experimental period were averaged and used for statistical analysis. Student paired t test was used to calculate levels of significance within individual kidneys. A value of \(P<0.05\) was considered statistically significant.

Results

Studies in Primary Cultures of JG Cells

In the first set of experiments, we determined whether angiotensin II, endothelin-1, and thapsigargin, which are known to inhibit renin release in a calcium-dependent manner in several experimental procedures, increase the intracellular calcium concentration \([Ca^{2+}]_i\) in isolated renin-producing JG cells. \([Ca^{2+}]_i\) was assessed in fura-2 measurements. As shown in Figure 1A and 1B, angiotensin II (1 \(\mu\)mol/L), endothelin-1 (10 nmol/L), and thapsigargin (1 \(\mu\)mol/L) significantly increased \([Ca^{2+}]_i\), as detected by increases in the 340/380 nm ratio. Whereas the application of angiotensin and endothelin induced a sharp peak in \([Ca^{2+}]_i\) followed by a plateau phase, thapsigargin evoked a slower increase in \([Ca^{2+}]_i\), without an initial peak.

To determine whether increases in \([Ca^{2+}]_i\), modulate cAMP levels in renin-producing cells, primary cultures of JG cells were incubated with the calcium liberators for 4 hours and the cAMP content of the cells was determined thereafter. Stimulation of cAMP generation by the \(\beta\)-adrenoreceptor agonist isoproterenol at its maximum effective concentration of 100 nmol/L increased the cAMP concentration \([cAMP]_i\) from 41±12 to 368±60 fmol/well and simultaneously stimulated renin release from 9.9±1.2% to 18.0±0.7% (Figure 2A). The concomitant application of angiotensin II, endothelin-1, or thapsigargin prevented the stimulation of renin release by isoproterenol and completely abolished increases in cAMP by the activation of \(\beta\)-adrenoreceptors (Figure 2A). Also, the direct activation of AC by forskolin (5 \(\mu\)mol/L) significantly elevated intracellular cAMP concentrations (from 56±11 to 539±36 fmol/well) and renin release (from 12.0±1.1% to 22.5±1.7%) (Figure 2B). The concomitant application of angiotensin II, endothelin, or thapsigargin not only inhibited renin release by approximately 40% but also, in parallel, suppressed [cAMP], by 40%. The inhibitory effects of angiotensin II on renin release and cAMP content were mediated by angiotensin II type 1 (AT1) receptors because they were completely blocked by the concomitant application of losar-
The application of the PDE inhibitor IBMX (100 μmol/L) (Figure 2C) resulted in cAMP accumulation (42±14 versus 413±53 fmol/well) and stimulation of renin release (from 11.7±1.2% to 24.6±2.8%). Because under the pharmacological blockade of PDE activity the calcium liberators significantly reduced [cAMP], these experiments indicate that the suppression of cAMP was not related to activation of cAMP degradation but to inhibition of cAMP generation. Notably, both renin release and [cAMP], were again decreased to
addition of calcium-liberating agents (Figure 3, "normal calcium values; dashed line, regression line for high-calcium values.

Figure 3. Relationship between cAMP levels and renin release. cAMP levels and renin release were determined under control conditions and after stimulation by either isoproterenol (100 μmol/L), forskolin (5 μmol/L), IBMX (100 μmol/L), in the absence (normal calcium, white circles) or the presence (high calcium, black circles) of angiotensin II, endothelin-1, or thapsigargin. Solid line indicates regression line for normal calcium values; dashed line, regression line for high-calcium values.

By guest on November 12, 2017 Downloaded from http://circres.ahajournals.org/ calcium-inhibited AC isoforms, AC5 and AC6, and moreover AC1, AC4, and AC9 (Figure 4). Because JG cells are closely related to vascular smooth muscle cells, AC expression in denuded aortas was determined for comparison. Interestingly, besides the AC isoforms detected in JG cells, AC3 and AC8 were expressed in the aorta.

Studies in the Renin-Producing Cell Line As4.1

To test the functional relevance of AC5 and AC6 in the regulation of intracellular cAMP and renin release, we used the siRNA technique for specific knockdown of these adenylyl cyclase isoforms in the renin-producing JG mouse cell line As4.1. Because As4.1 cells have a high basal PDE activity and to exclude the modulation of [cAMP], by changes in PDE activity, all experiments were performed in the presence of IBMX (100 μmol/L).

After transfection of the cells with siRNA directed against either AC5 or AC6 for 72 hours, the respective mRNA expressions were markedly reduced compared with cells transfected with control siRNA. Double transfection with a combination of AC5 siRNA and AC6 siRNA downregulated AC gene expression to values <20% of control transfected cells (Figure 5A). Downregulation of AC5 mRNA expression was paralleled by a reduction in AC5 protein expression as determined by Western blot (Figure 5B). As mentioned in the methods, AC6 protein expression could not be determined because of the lack of a specific antibody selectively detecting AC6.

Despite the marked downregulation of AC expression, baseline [cAMP], or renin release were not altered by any of the transfection protocols (Figures 6 and 7). Forskolin or isoproterenol stimulated cAMP levels 5.8-fold and 4.6-fold compared with vehicle in control cells, whereas the stimulation was significantly reduced in cells with suppressed AC5 expression (forskolin, 2.4-fold stimulation; isoproterenol 2.4-fold stimulation) or AC6 expression (2.3- and 2.1-fold stimulation, respectively) (Figure 6). Thapsigargin and endothelin-1 markedly lowered [cAMP], (Figure 6) and renin release (Figure 7) in cells transfected with control siRNA, although they did not significantly reduce [cAMP], or renin release in cells transfected with siRNA directed against either
AC5 or AC6. Downregulation of both AC5 and AC6 not only further reduced increases in cAMP in response to forskolin (1.4-fold, \( P < 0.085 \)) and isoproterenol (1.4-fold, \( P = 0.074 \)) but also completely prevented the stimulation of renin release (Figures 6 and 7).

Notably, angiotensin II did not produce any effect on cAMP levels and renin release in As4.1 cells. This somewhat unexpected finding is well explained by the fact that the As4.1 cells used in this study did not express AT1 receptors as tested by RT-PCR and Western blot (data not shown).

**Studies in Isolated Perfused Mouse Kidneys**

To expand the results of the cell culture experiments to the whole-organ level, additional experiments using isolated perfused kidneys of mice were performed (Figure 8). Activation of ACs by isoproterenol (30 nmol/L) stimulated renin release to levels similar to that of the addition of the membrane permeable cAMP analog 8br-cAMP (250 \( \mu \)mol/L). Angiotensin II (3 nmol/L) completely reversed (Figure 8, top) or prevented (Figure 8, bottom) the stimulation of renin release by isoproterenol, whereas it only moderately attenuated renin secretion rates stimulated by 8br-cAMP.

**Discussion**

The calcium paradoxon of renin secretion describes the phenomenon in which hormones or substances known to elevate the intracellular free calcium concentration \([\text{Ca}^{2+}]\), inhibit renin release. Because downstream signaling pathways mediating this calcium-dependent inhibition of renin release have not been satisfactorily identified, the present study attempted to examine whether the inhibition of renin release by \([\text{Ca}^{2+}]\) is mediated by suppression of the intracellular cAMP pathway, which is known to be the central stimulator of the renin system. Indeed, our data clearly show that in native JG cells and in the renin-producing cell line As4.1, an increase in \([\text{Ca}^{2+}]\) accompanies a reduction in intracellular cAMP levels. Because net cAMP levels are determined by cAMP formation and cAMP degradation, and JG cells express the calcium-activated PDE1, it appeared possible that cAMP concentrations might be lowered by enhanced hydrolysis. However, unselective pharmacological blockade of PDEs with IBMX, a maneuver that both enhanced cAMP levels and renin release, did not prevent the suppression of cAMP and renin levels by either of the applied calcium liberators. Moreover, cAMP levels raised by cAMP-AM, a cAMP derivate that is easily metabolized in cells by PDEs,\(^{18}\) were not affected by \([\text{Ca}^{2+}]\) as one would expect if PDE activity was stimulated by these agents. Therefore, we focused on the role of the calcium-inhibited AC isoforms AC5 and AC6 on the regulation of cAMP in renin-producing cells, which are indeed expressed in freshly isolated JG cells (Figure 4) and in As4.1 cells (Figure 5). Knockdown of either AC isoform by specific siRNAs markedly attenuated the maximum stimulation of cAMP formation in response to forskolin and isoproterenol and prevented the suppression of cAMP levels by both of the applied calcium liberators. Moreover, cAMP levels raised by cAMP-AM, a cAMP derivate that is easily metabolized in cells by PDEs,\(^{18}\) were not affected by \([\text{Ca}^{2+}]\), as one would expect if PDE activity was stimulated by these agents. Therefore, we focused on the role of the calcium-inhibited AC isoforms AC5 and AC6 on the regulation of cAMP in renin-producing cells, which are indeed expressed in freshly isolated JG cells (Figure 4) and in As4.1 cells (Figure 5). Knockdown of either AC isoform by specific siRNAs markedly attenuated the maximum stimulation of cAMP formation in response to forskolin and isoproterenol and prevented the suppression of cAMP levels by endothelin-1 and thapsigargin. Moreover, double-transfection with siRNA directed against both AC5 and AC6 further suppressed the stimulation of cAMP levels without completely inhibiting cAMP. The weak residual stimulation of cAMP might either be explained by the remaining activity of AC5 and AC6 that had not been completely downregulated by the siRNA approach or by the activity of other AC isoforms expressed in As4.1 cells.

Similar to native JG cells (Figure 4), As4.1 cells do not exclusively express AC5 and AC6 (RT-PCR, data not shown). These additional AC isoforms are supposedly stimulated by forskolin, resulting in increased cAMP levels, independent of the downregulation of AC5 and AC6. Interestingly, the knockdown of 1 of the 2 AC isoforms was sufficient to abolish the calcium-dependent suppression of cAMP formation, an observation that points to a close interdependence of the 2 calcium-inhibited AC isoforms.
Besides the demonstration that calcium-dependent inhibitors of renin release suppress cAMP formation in renin-producing cells, our study provides 3 lines of evidence that this process is centrally involved in their inhibitory effects on renin secretion. First, we found that the stimulation of renin release under cAMP clamp conditions (cAMP-AM) was not inhibited by angiotensin II, endothelin-1, or thapsigargin in native JG cells. Moreover, the calcium effectors did not shift the correlation between cAMP and renin release as expected considering the fact that calcium induced an additional effect on renin release besides the effects mediated via suppression of cAMP formation. Finally, knock down of AC5 or AC6 in As4.1 cells completely prevented the inhibition of renin release by calcium-dependent inhibitors of renin release, clearly indicating a central role of the calcium inhibited ACs in the regulation of renin release.

In contrast to JG cells, in which angiotensin II inhibits cAMP formation, it has been shown that angiotensin II stimulates cAMP formation in cardiac fibroblasts and in vascular smooth muscle cells of renal-resistance vessels and the aorta.\(^{19-21}\) This response to angiotensin II is accompanied by differential AC isoform expression patterns. Thus, expression of calcium-inhibited adenylyl cyclase type 6 (AC6) is lower in aortic cells compared with JG cells (Figure 4). On the other hand, AC3 and AC8, which are activated by calcium,\(^{8,21}\) are not expressed in JG cells, but in aortic cells, and might therefore mediate angiotensin II–mediated cAMP stimulation (Figure 4 and online data supplement). The cellular expression of several AC isoforms is not uncommon\(^{21,22}\) but raises the question how differently regulated AC isoforms can respond selectively within a single cell. The localization of the respective cAMP signaling cascades occurs within restricted plasmalemmal microdomains in a highly organized manner. For instance, in cardiac fibroblasts AC3 and AC5/6 are localized in caveolin-rich membrane fractions, whereas AC2, -4, and -7 are excluded.\(^{21}\) Moreover, in cardiac myocytes, AC6 colocalizes with \(\beta_1\)-adrenoreceptors in caveolin-rich membranes, whereas prostaglandin E2 (EP2) receptors are not located in these membrane areas, resulting in a missing functional coupling of EP2 receptor activation and AC6-dependent cAMP formation.\(^{23}\) A close spatial and functional coupling of \(\beta_1\)-adrenoreceptors to AC5 and AC6 could explain why the increase in \([\text{Ca}^{2+}]_i\), completely prevented the stimulation of cAMP generation by isoproterenol in JG cells. Based on the close coupling phenomena, isoproterenol would preferentially activate AC5 and AC6, and this stimulation would be blocked by an increase in \([\text{Ca}^{2+}]_i\). In contrast, forskolin stimulates all expressed AC isoforms. Because only the activities of AC5 and AC6 are inhibited by \([\text{Ca}^{2+}]_i\), residual cAMP generation by the other calcium-independent AC isoforms prevents complete blockade of forskolin-mediated [cAMP] stimulation, which was the case in our experiments (Figure 2).

The functional relevance of our concept of calcium-dependent inhibition of renin release was further underlined by experiments using isolated perfused mouse kidneys. Thus, the suppressive effects of angiotensin II were markedly reduced when renin release was stimulated by the direct application of cAMP compared with an indirect elevation of cAMP levels by isoproterenol. The finding that the effect of

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**Figure 6.** Effects of forskolin 5 μmol/L and isoproterenol 100 nmol/L on cAMP levels of renin-producing As4.1 cells with or without endothelin 10 nmol/L or thapsigargin 1 μmol/L. Cells were transfected with control siRNA, siRNA directed against AC5 or AC6, or a combination of AC5 and AC6 siRNA for 72 hours before stimulation. *P<0.05 vs vehicle, #P<0.01 vs vehicle; n=6.
angiotensin II on renin secretion was strongly attenuated but not completely prevented by the cAMP clamp, as we observed in the cell culture experiments, is not unexpected because at the whole-organ level angiotensin II not only directly affects the renin-producing cells but also regulates intrarenal hemodynamics, endothelial function, and tubular salt transport, which all indirectly modulate renin release. Therefore, changes in renin secretion rates from isolated kidneys reflect the sum of direct and indirect effects on the renin producing cells. Thus, although these experiments are not suited to definitively prove our cellular concept detailing the calcium paradoxon of renin release, they indicate that

Figure 7. Effects of forskolin (5 μmol/L) and isoproterenol (100 nmol/L) on renin release (percentage of total renin content) from As4.1 cells with or without endothelin (10 nmol/L) or thapsigargin (1 μmol/L). Cells were transfected with control siRNA, siRNA directed against AC5 or AC6, or a combination of AC5 and AC6 siRNA for 72 hours before stimulation. *P<0.05 vs vehicle, #P<0.01 vs vehicle; n=5.
modulation of cAMP generation by angiotensin II is a central element of the inhibitory effects of angiotensin II on renin release, even at the whole-organ level.

The molecular mechanisms by which [Ca\textsuperscript{2+}] inhibits adenylyl cyclase activity have been the subject of several previous studies. All AC activities are inhibited by high supraphysiological concentrations of calcium, whereas only AC5 and AC6 are inhibited by physiological concentrations of [Ca\textsuperscript{2+}]. This physiological inhibition of enzyme activity does not depend on an interaction of calcium with calmodulin but might be related to a direct binding of Ca\textsuperscript{2+} to the enzyme. Based on these data and our result that inhibition of AC activity directly in JG cells. However, this assumption seems to be somewhat at odds with previous studies on isolated JG cells from our laboratory demonstrating that calmodulin antagonists markedly stimulate renin release arguing for a suppressive role of the calcium/calmodulin complex on renin secretion. However, this stimulation is not necessarily mediated by a stimulation of AC activity because calmodulin activates cAMP degradation via stimulation of PDE1 or facilitates calcium influx into the cytosol, both potentially interfering with renin release. Therefore, further studies are needed to clarify whether the inhibition of AC activity by [Ca\textsuperscript{2+}] leading to a suppression of renin release at the cellular level is dependent on calmodulin or whether it is mediated directly by Ca\textsuperscript{2+}.

In future studies, we must also examine the exact point at which calcium-dependent inhibition of cAMP generation interferes with renin release from JG cells. Thus, our data do not allow us to clearly discern whether the observed inhibition of cAMP generation only suppresses the exocytotic events of renin secretion or also attenuates the upstream processes of vesicle transport or intracellular prorenin processing, which all could result in decreased regulated renin release from JG cells.

Taken together, our data illustrate that the suppression of renin release by [Ca\textsuperscript{2+}] is mediated by an inhibition of AC5 and AC6, resulting in a reduction of intracellular cAMP levels and providing a functional basis for the so-called calcium paradoxon of renin release.

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Disclosures

None.

References

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Expanded Methods

Measurement of the intracellular calcium concentration:
JG cells were isolated as described in\textsuperscript{1} and seeded in 500µL aliquots on glass cover slips placed in 24 well cell culture plates. After 24 hours cells were incubated with 5µmol/L fura-2AM (Invitrogen) with 0.02% pluronic (Molecular Probes) in RPMI medium for 60 minutes at room temperature. Cover slips were transferred to a superfusion chamber and cells were superfused with ringer solution (mmol/L: 145 NaCl, 0.4 KH2PO4, 1.6 K2HPO4, 5 glucose, 1 MgCl2, 1.3 Ca\textsuperscript{2+} gluconat) at 37°C. Cell fluorescence was measured continuously using an inverted microscope (Olympus) and a high speed polychromator system. Fura-2 was excited at 340/380nm and emission was recorded between 470 and 550 nm using a CCD camera (CoolSnap HQ, Visitron Systems). Experiments were analyzed using the Meta-Fluor software package (Universal Imaging).

Determination of AC mRNA expression in aortas and isolated JG cells
Juxtaglomerular cells of 6-8 week old C57BL/6 mice were isolated as described in detail previously \textsuperscript{1}. In brief, 1 mouse was killed by cervical dislocation under deep anesthesia (sevoflurane), the kidneys were removed quickly, minced with a scalpel blade and digested for 60 minutes at 37°C with a trypsin/collagenase mixture. The resulting cell suspension was filtered through a 22.4-µm nylon filter, washed two times, and centrifuged at 27 000g for 30 minutes at 4°C in a 30% isosmotic Percoll density gradient (Pharmacia). Four cell layers with different specific renin activities were obtained. The cellular layer (density, 1.07 g/mL) with the highest specific renin activity was recovered. The cells were washed in physiological saline and immediately subjected to RNA isolation. Total time from kidney removal to the
beginning of RNA isolation was 120 minutes. JG cells of 5 independent preparations (5 mice) were used for AC mRNA expression analysis.

After removal of the kidneys (see above) abdominal aortas were isolated from the same 5 C57BL/6 mice of either sex. After the surrounding fat was removed under a stereomicroscope, aortas were cut longitudinally and the endothelium was scraped using a scalpel blade. All procedures were carried out in ice cold physiological saline. Finally, the remaining tissue was stored in liquid nitrogen until isolation of total RNA. Total time from removal of the aorta to the storage in liquid nitrogen was 15 minutes.

Total RNA was extracted according to the acid-guanidinium-phenol-chloroform protocol of Chomczynski and Sacchi². RT-PCR was performed according to standard protocols for 32 cycles as described in³. As negative controls samples in which reverse transcription was performed without the addition of reverse transcriptase (RT-) were used.

Semiquantification of AC mRNA expression was performed by Real-Time PCR using the Light Cycler System and the Light Cycler DNA Master SYBR Green I kit provided by Roche Molecular Biochemicals (Mannheim, Germany). Each reaction (20 µl) consisted of 2 µl cDNA, 3.0 mM MgCl₂, 1 pmol of each primer, and 2 µl of Fast Starter Mix (containing buffer, dNTPs, SYBR Green, and hotstart Taq polymerase). The amplification program consisted of 1 cycle at 95°C for 10 min, followed by 40 cycles with a denaturing phase at 95°C for 15 s, an annealing phase of 5 s at 60°C, and an elongation phase at 72°C for 15 s. A melting curve analysis was done after amplification to verify the accuracy of the amplicon.
Primers used for RT-PCR and Real time PCR:

To test for the specificity of the PCR primers used, RT-PCR was performed using cDNA of mice brain and kidneys and PCR products were analyzed on an ethidium bromide-stained 2% agarose gel. All primer pairs produced PCR products of the expected length. PCR bands were excised, purified using a standard PCR cleaning kit (Wizard SV Gel and PCR Clean-Up System, Promega) and finally the PCR products were sequenced (sequiserve, Germany).

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Length of PCR product (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>sense 5'-ggcctaaccagctctcctc-3' antisense 5'-acgggatttctctcagctc-3'</td>
<td>169</td>
</tr>
<tr>
<td>AC2</td>
<td>sense 5'-ggagatctgaaccatggaga-3' antisense 5'-atggagccaacagcatagca-3'</td>
<td>150</td>
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<tr>
<td>AC3</td>
<td>sense 5'-tgaggagacatcaacaagc-3' antisense 5'-tgggtgactctgagctg-3'</td>
<td>157</td>
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<tr>
<td>AC4</td>
<td>sense 5'-gtgcccctctcactcact-3' antisense 5'-aagtctctcgtctcgttca-3'</td>
<td>170</td>
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<tr>
<td>AC5</td>
<td>sense 5'-ctgctctcgctggttggc-3' antisense 5'-gccggtgctctggttggc-3'</td>
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<tr>
<td>AC7</td>
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<tr>
<td>AC9</td>
<td>sense 5'-ccaggacacctctcaag-3' antisense 5'-ggctgtcctctctgaggg-3'</td>
<td>220</td>
</tr>
</tbody>
</table>
**Results**

**Online Figure 1**: Expression of adenylyl cyclase mRNA in juxtaglomerular cells (JG cells, panel A) and aorta (panel B) determined by RT-PCR.

Negative controls (RT-) did not produce any specific bands (not shown). Small white arrows indicate length of expected bands of AC2 and AC7.
References:

