Stimulation of Renin Secretion by Catecholamines Is Dependent on Adenylyl Cyclases 5 and 6

Fadi Aldehni, Tong Tang, Kirsten Madsen, Michael Plattner, Andrea Schreiber, Ulla G. Friis, H. Kirk Hammond, Pyung Lim Han, Frank Schweda

Abstract—The sympathetic nervous system stimulates renin release from juxtaglomerular cells via the β-adrenoreceptor-cAMP pathway. Recent in vitro studies have suggested that the calcium-inhibited adenylyl cyclases (ACs) 5 and 6 possess key roles in the control of renin exocytosis. To investigate the relative contribution of AC5 and AC6 to the regulation of renin release in vivo we performed experiments using AC5 and AC6 knockout mice. Male AC5−/− mice exhibited normal plasma renin concentrations, renal renin synthesis (mRNA and renin content), urinary volume, and systolic blood pressure. In male AC6−/− mice, plasma renin concentration (AC6−/−: 732±119; AC6+/+; 436±78 ng of angiotensin I per hour*mL−1; P<0.05), and renin synthesis were stimulated associated with an increased excretion of dilute urine (1.55-fold; P<0.05) and reduced blood pressure (−10.6 mm Hg; P<0.001). Stimulation of plasma renin concentration by a single injection of the β-adrenoreceptor agonist isoproterenol (10 mg/kg IP) was significantly attenuated in AC5−/− (male: −20%; female: −33%) compared with wild-type mice in vivo. The mitigation of the plasma renin concentration response to isoproterenol was even more pronounced in AC6−/− (male: −63%; female: −50% versus AC6+/+). Similarly, the effects of isoproterenol, prostaglandin E2, and pituitary adenylyl cyclase-activating polypeptide on renin release from isolated perfused kidneys were attenuated to a higher extent in AC6−/− (−51% to −98% versus AC6+/+) than in AC5−/− (−31% to 46% versus AC5+/+). In conclusion, both AC5 and AC6 are involved in the stimulation of renin secretion in vivo, and AC6 is the dominant isoform in this process. (Hypertension. 2011;57:460-468.) Online Data Supplement

Key Words: ADCY5 ▪ ADCY6 ▪ renin ▪ renal function ▪ blood pressure

The renin-angiotensin-aldosterone system (RAAS) is centrally involved in the control of salt and water homeostasis and blood pressure regulation. Moreover, the RAAS is of major importance in the pathophysiology of heart and renal diseases.

Because the activity of the circulating RAAS is controlled by the enzymatic activity of renin, renin release from juxtaglomerular (JG) cells into the circulation is a critical step in the regulation of RAAS. Renin release is controlled by a complex interplay of several local and systemic factors, such as salt intake, arterial blood pressure, and the sympathetic nervous system. The fundamental role of catecholamines and renal nerve activity in the regulation of renin synthesis and secretion has been shown in numerous studies using pharmacological approaches or renal denervation.1,2 Moreover, basal plasma renin concentration (PRC), renal renin gene expression, and the increase in PRC in response to different stimuli are markedly reduced in β1/β2-adrenoreceptor double knockout mice.3

The complex picture of systemic and local factors that regulate renin release becomes more concise at the intracellular level, where renin exocytosis is regulated by cAMP, cGMP, and the cytosolic calcium concentration (Ca2+). Although cGMP can either stimulate or inhibit renin release, cAMP is considered the main stimulator and Ca2+ the main suppressor of renin secretion.4 Several important renin-stimulating hormones, such as catecholamines, prostaglandin E2, and others, signal via G protein–coupled receptors, thereby activating adenylyl cyclase activity and cAMP generation.1,4,5 Accordingly, conditional deletion of the stimulatory G protein Gsα in renin-producing cells results in a drastic reduction of PRC under control conditions and in response to several physiological stimuli of renin release,6 which underlines the central role of the activation of adenylyl cyclases and cAMP generation in the regulation of renin release in vivo. However, thus far, it has not been determined which adenylyl cyclase isoform is activated by Gsα in JG cells or mediates the stimulation of renin release in vivo.
Recent studies in renin-producing cells indicate that the stimulatory cAMP pathway and the inhibitory Ca\(^{2+}\) pathway of renin secretion are linked in a way that an increase in intracellular Ca\(^{2+}\) suppresses intracellular cAMP levels and renin secretion,\(^7\) whereas a reduction of the free cytosolic Ca\(^{2+}\) by calcium chelation has the opposite effect.\(^6\) Of the 9 membrane-bound adenyl cyclase isofoms, adenyl cyclases 5 and 6 (AC5 and AC6) are inhibited by physiological increases in the cytosolic calcium concentration.\(^9,10\) In fact, using small interfering RNA–mediated gene knockdown or pharmacological approaches in cultured renin-producing cells, it has been shown that these calcium-inhibited ACs connect the Ca\(^{2+}\) and cAMP pathways and control renin secretion.\(^7,8,11\) Therefore, although other mechanisms contribute to the Ca\(^{2+}\)-dependent inhibition of renin release,\(^1,12,13\) AC5 and AC6 appear to have a central role in the cellular control of renin secretion because of their involvement in the stimulatory and the inhibitory signaling pathways of renin secretion.

AC5 and AC6 are the major AC isofoms in the heart, and their roles in cardiac physiology and pathophysiology have been the subjects of several studies.\(^14–18\) Very recently, the role of AC6 in renal function has been investigated in different AC6 knockout mouse lines.\(^19,20\) AC6\(^{-/-}\) mice display normal glomerular filtration rates but an impaired ability to concentrate urine, a phenotype that is compatible with the widespread expression of AC6 in the nephron and especially in the collecting duct.\(^20,22\) Despite their important role in cardiovascular and renal function, the functional roles of AC5 and AC6 in the regulation of renin release in vivo have not yet been investigated. Because, moreover, previous in vitro studies suggested central roles for AC5 and AC6 in the cellular control of renin exocytosis, the present study was set out to investigate whether and to what extent AC5 and AC6 contribute to the regulation of renin release in vivo.

### Materials and Methods

#### Animals

AC5-deficient and AC6-deficient mice were offspring of heterozygous breeder pairs of the respective knockout strains generated by Lee et al\(^{21}\) (AC5) and Tang et al\(^{16}\) (AC6). All of the animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the institutional committee and the local government.

#### In Vivo Experiments

Blood samples (25 \(\mu\)L) were obtained from age-matched, conscious mice of either sex by submandibular venipuncture. Blood was collected into heparinized tubes containing EDTA to prevent clotting. Plasma was separated by centrifugation and frozen at \(-20^\circ\)C until further processing. Three weeks after the first blood withdrawal, the mice received a single injection of isoproterenol (10 mg/kg body weight IP\(^{21}\) in isotonic NaCl), and a blood sample was collected 50 minutes later. Thereafter, the mice were deeply anesthetized with sevoflurane, euthanized by cervical dislocation, and kidneys were removed and frozen in liquid nitrogen.

### Aldehni et al AC5- and AC6-Dependent Stimulation of Renin Release

#### Isolated Perfused Kidney

Kidneys of male AC5 and AC6 knockout mice were perfused ex situ at a constant perfusion pressure (100 mm Hg) as described in detail previously.\(^25\) Samples of the venous perfusate were collected every 2 minutes for the determination of renin activity. Three samples were taken during each experimental period. Renin secretion rates were calculated as the product of the renin activity and the venous flow rate (milliliter/minute*gram of kidney weight\(^{\text{-1}}\)). For details please see the online Data Supplement at http://hyper.ahajournals.org.

#### Determination of PRC in Plasma and Plasma Renin Activity in Perfusate Samples

PRC in plasma samples and renin activity in perfusate samples of isolated perfused kidneys were measured on the basis of the generation of angiotensin I after the addition of plasma from bilaterally nephrectomized male rats as excess renin substrate. The generated angiotensin I (in nanograms/milliliter/hour\(^{-1}\)) was determined by radioimmunoassay (DiaSorin).

#### Determination of mRNA Expression by Real-Time PCR

Total RNA was isolated from the frozen kidneys or freshly isolated JG cells using TRIzol reagent (Life Technologies). After reverse transcription (Mo1oney murine leukemia virus reverse transcriptase, Superscript, Invitrogen), real-time RT-PCR was performed to assess renin, AC, and \(\beta\)-actin expression using a LightCycler Instrument (Roche Diagnostics Corp).\(^7\) JG cells of mouse kidneys were isolated as described in detail previously.\(^7\) In brief, kidney cortices were minced and digested with a trypsin/collagenase mixture. The cell suspension was filtered (22.4-\(\mu\)m nylon mesh) and separated by centrifugation in a Percoll density gradient. The cellular layer with the highest specific renin activity was resuspended in TRIzol reagent. For primer sequences please see the online Data Supplement.

#### Determination of Renal Renin Content

The renal renin content was determined by measuring the capacity of homogenized kidneys to generate angiotensin I in the presence of excess renin substrate as described previously.\(^26\)

#### Immunofluorescence for Renin, AC5, and AC6

For immunofluorescence of renin, kidneys of AC5\(^{-/-}\), AC6\(^{-/-}\), and their wild-type littermates were perfused fixed with 4% paraformaldehyde. Immunolabeling was performed on 5-\(\mu\)m paraffin sections using a chicken antimouse antibody (generated by Davids Biotechnology) overnight at 4\(^\circ\)C, followed by incubation with a fluorescent secondary antibody.

For description of the immunohistochemistry procedures used to detect AC5 and AC6, please see the online Data Supplement.

#### Blood Pressure and Heart Rate Measurements

Systolic blood pressure and heart rate in AC5 and AC6 mice were assessed noninvasively by the tail-cuff method in conscious male mice (TSE). In an additional set of 4 male AC6\(^{-/-}\) and 4 AC6\(^{-/-}\), blood pressure was determined by radiotelemetry for 5 days. For detailed descriptions please see the online Data Supplement.

#### Urine Collection and Determination of Osmolality and Electrolyte Concentrations

After a 2-day habituation period, 24-hour urine collection was performed in metabolic cages during the 3 following days. Urine osmolality was determined using the freezing point depression
method (Osmomat 030, Gonotec), and sodium concentration was determined by flame photometry (Jenway Ltd).

Single Cell RT-PCR of Renin-Producing JG Cells
JG cells were isolated from the renal cortex of wild-type mice and sampled using a patch pipette.27 The subsequent RT-PCR of single JG cells has been described in detail previously.27

In Situ Hybridization
The mRNA expression of AC5, AC6, and renin was explored by in situ hybridization using digoxigenin-labeled riboprobes on cryosections of mouse kidneys. The sense and antisense probes were generated by in vitro transcription of the respective cDNAs (DIG RNA Labeling Mix, Roche). For details please see the online Data Supplement.

Statistical Analysis
Values are presented as the mean±SEM. Differences between groups were analyzed by ANOVA, followed by Bonferroni adjustment for multiple comparisons. In the 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 the levels of significance within individual kidneys. P values <0.05 were considered statistically significant.

Results
Expression of AC5 and AC6 in Renin-Producing JG Cells
In situ hybridization with specific antisense mRNA probes for AC5 and AC6 produced a clear and reproducible staining in the JG cell area (Figure 1A). A renin probe was used as control and showed staining at the typical localization of JG cells (Figure 1A). AC5 and AC6 mRNA expressions were detected in 15% and renin mRNA in 35% of glomeruli. Sense mRNA probes did not produce any signal. Moreover, mRNA expression for both AC5 and AC6 was detected in single JG cells that had been sampled by a patch pipette (Figure 1B).

Deletion of AC5 or AC6 did not result in a compensatory upregulation of other AC isoforms in freshly isolated JG cells (Figure 1C) or in whole kidneys (not shown). As shown previously, freshly isolated JG cells not only express AC5 and AC6 but, moreover, AC1, AC4, and AC9 (Figure 1C and Reference7).

To determine the expression pattern of the AC5 and AC6 proteins in JG cells, we performed immunofluorescence staining of kidney sections. However, despite the use of several different fixation protocols and antibodies, we were unable to detect any specific or reproducible staining for AC5 or AC6 in the kidney (for details, please see the online Data Supplement).

Regulation of PRC in AC5 and AC6 Knockout Mice
Renal renin mRNA expression, renin content, and PRC did not differ between AC5−/− and AC5+/+ mice under control conditions (Table). Moreover, no differences in systolic blood pressure, heart rate, and urine excretion were detected.

Figure 1. A, In situ hybridization using antisense mRNA probes specific for AC5 (left), AC6 (middle), and renin (right). Hybridization with sense mRNA probes did not produce staining. B, mRNA expression of AC5 (left) and AC6 (right) in single mouse JG cells. C, mRNA expression of AC isoforms in isolated JG cells of AC5 (left) and AC6 (right) knockout mice compared with their wild-types (n=4 each genotype).
However, the stimulation was significantly attenuated in AC6−/− mice, because the increase in PRC was reduced by 50% in male mice. PRC indicates plasma renin concentration.

Baseline Functional Characteristics of AC5−/− and Their Wild-Type Littermates (AC5+/+)

Table.

<table>
<thead>
<tr>
<th>Basal Parameters</th>
<th>AC5+/+</th>
<th>AC5−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC female, ng of Ang I/h*ml</td>
<td>281±37 n=14</td>
<td>256±33 n=14</td>
</tr>
<tr>
<td>PRC male, ng of Ang I/h*ml</td>
<td>257±67 n=12</td>
<td>306±48 n=12</td>
</tr>
<tr>
<td>Renal renin mRNA renin/β-actin mRNA</td>
<td>0.72±0.17 n=8</td>
<td>0.58±0.03 n=8</td>
</tr>
<tr>
<td>Renal renin content, μg of Ang I/g of kidney weight</td>
<td>8.6±2.6 n=8</td>
<td>10.3±2.3 n=8</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>120.8±2.6 n=8</td>
<td>116.8±2.1 n=8</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>624.5±8.7 n=8</td>
<td>632.6±7.1 n=8</td>
</tr>
<tr>
<td>Urine excretion, μL/24 h*g of body weight</td>
<td>74.8±6.2 n=4</td>
<td>66.5±2.9 n=4</td>
</tr>
<tr>
<td>Urine osmolality, μmolosmol/g</td>
<td>2587±70 n=4</td>
<td>2886±96 n=4</td>
</tr>
<tr>
<td>Sodium excretion, μmol/24 h*g of body weight</td>
<td>11.39±0.43 n=4</td>
<td>10.59±0.31 n=4</td>
</tr>
</tbody>
</table>

Except for plasma renin concentration (PRC female), all of the data were obtained in male mice. Ang indicates angiotensin.

between male AC5−/− and AC5+/+ mice (Table). In AC6−/− mice, renal renin mRNA expression, renal renin content and PRC were significantly enhanced compared with AC6+/+ (Figure 2). Accordingly, the kidneys of AC6−/− mice displayed a robust increase in renin immunofluorescence and a recruitment of renin-producing cells in the afferent arteriole (Figure 3). Systolic blood pressure, determined by tail-cuff measurements, was reduced in AC6−/− by 10.6 mm Hg (P < 0.001 versus AC6+/+; n=12 each genotype), whereas heart rate was unaltered (Figure 2). The reduced blood pressure of AC6−/− was confirmed by radiotelemetry (systolic: AC6+/+ 122.3±1.3 mm Hg, AC6−/− 109.8±2.1 mm Hg, P < 0.05; diastolic: AC6+/+ 85.2±2.6 mm Hg, AC6−/− 78.1±0.9 mm Hg, P < 0.05; heart rate: AC6+/+ 572±8, AC6−/− 570±5 bpm, P value not significant; n=4 each genotype). As has been demonstrated previously, AC6−/− mice excrete a high volume of dilute urine18,20 and have enhanced urinary sodium excretion (Figure 2).

Because the sympathetic nervous system stimulates renin release via activation of β-adrenoreceptors, we used a single injection of the β-adrenoreceptor agonist isoproterenol (10 mg/kg of body weight IP) to mimic sympathetic activation.24 Isoproterenol stimulated PRC in AC5−/−, AC6−/−, and their respective wild-types (Figure 4). However, the stimulation was significantly attenuated in the knockout mice: the increase in PRC in response to isoproterenol was reduced in female (−33%) and male (−20%) AC5−/− compared with their wild-type littermates (Figure 4). This attenuation was pronounced in AC6−/− mice, because the increase in PRC was reduced by 50% in female and by 63% in male AC6−/− mice compared with AC6+/+ (Figure 4).

Regulation of Renin Release From Isolated Perfused Kidneys of AC5 and AC6 Knockout Mice

Because the stimulability of renin secretion in vivo might be dampened by the systemic phenotype of AC6−/− mice, that is, the increased diuresis and reduced blood pressure, we performed additional experiments using the isolated perfused kidney model. This model allows for studying the acute regulation of renin secretion without interference by confounding systemic phenotypes or adverse effects of the experimental drug.

Renin secretion rates did not differ between AC5−/− or AC6−/− and their respective wild-type littermates at control conditions (Figure 5A and 5B). Isoproterenol stimulated renin

![Figure 2. Baseline physiological characteristics of AC6 knockout (AC6 KO, □) and wild-type mice (AC6 WT, □). *P<0.05 vs AC6 WT; **P<0.01 vs AC6 WT.](http://hyper.ahajournals.org/supplemental/doi/10.1161/HYPO.117.115472/figure-2)
secretion concentration dependently in all of the genotypes (Figure 6). In AC5−/− kidneys, the stimulation of renin release was attenuated at high concentrations of isoproterenol only (−43% at 30 nmol/L; −46% at 100 nmol/L), whereas in AC6−/− kidneys the effects of isoproterenol were reduced over the entire concentration range (1 to 100 nmol/L). Of note, the stimulation of renin secretion by isoproterenol 1 and 3 nmol/L was completely abrogated in AC6−/− kidneys (Figure 6). Therefore, similar to the in vivo experiments, the impairment of the isoproterenol effects was pronounced in AC6−/− compared with AC5−/−.

In addition to the attenuation of catecholamine-induced renin release, the stimulation of renin release in response to 10 nmol/L of prostaglandin E2 (PGE2) was reduced by 31% in AC5−/− and by 51% in AC6−/− mouse kidneys compared with their respective wild-type littermates (Figure 5A and 5B). The pituitary adenyl cyclase-activating polypeptide (PACAP; 10 nmol/L), which is known to stimulate renin release via activation of G protein–coupled receptors and an increase in cAMP, enhanced renin secretion rates in all of the genotypes. Again, the effects of PACAP were impaired in AC6−/− kidneys to a greater extent than in AC5−/− kidneys (Figure 5A and 5B).

Renin release was not generally affected in AC-deficient kidneys, because the membrane-permeable cAMP analog 8-bromo-cAMP (200 μmol/L) enhanced renin secretion rates to similar extents in the kidneys of AC5−/− and AC6−/− mice compared with their wild-type controls (Figure 5A and 5B). Moreover, angiotensin II suppressed renin secretion rates in a concentration-dependent manner to similar levels in all of the genotypes (Figure 5C and 5D).

**Discussion**

Catecholamines and other renin-stimulating hormones stimulate renin release from renal JG cells via G protein–coupled receptors, Gsα-mediated activation of adenylyl cyclases, and an increase in intracellular cAMP. Previous cell culture experiments suggested that the calcium-inhibited AC isoforms AC5 and AC6 play a central role in the cellular control of renin release.7,8,11 Accordingly, the present study was set out to test whether and to what extent AC5 and AC6 contribute to the stimulation of renin release by sympathetic activation, because the increase in PRC or in renin secretion rates in response to the β-adrenoreceptor agonist isoproterenol was attenuated in AC5−/− and AC6−/− mice both in vivo and in the isolated perfused kidney model. Similarly, the stimulation of renin release by PGE2 and PACAP, which both potently stimulate renin release, was significantly reduced in both AC5−/− and AC6−/− mice. Importantly, the stimulations of PRC or renin secretion rates were reduced by 51% to 98% in AC6−/−, depending on the experimental model and the concentration of isoproterenol used, suggesting that AC6 mediates half of the effect at least. In contrast, the stimulations of PRC or renin secretion rates were diminished by only 20% to 46% in AC5−/− compared
with wild-types, demonstrating that the activation of AC5 is accountable for less than half of the stimulation of renin release. Moreover, catecholamine-induced stimulation of renin release rates was attenuated only at very high concentrations of isoproterenol in isolated perfused kidneys of AC5/H11002/H11002, whereas in AC6/H11002/H11002 it was abrogated at low concentrations and markedly reduced at higher concentrations of isoproterenol. These results allow 3 conclusions: (1) both AC5 and AC6 are involved in the regulation of renin release by catecholamines, PGE2 and PACAP; (2) AC6 contributes to the stimulation of renin secretion to a greater extent than AC5; and (3) neither AC5 nor AC6 exclusively mediates the stimulation of renin secretion in response to catecholamines, PGE2 and PACAP.

In contrast to β1/β2 double knockout mice and Gsα-deficient mice, PRC was not reduced in the AC knockout strains under control conditions. Moreover, basal renin secretion rates from isolated perfused AC5/H11002/H11002 and AC6/H11002/H11002 kidneys were not different compared with their wild-type kidneys. These findings indicate that the lack of either AC5 or AC6 can be fully compensated under baseline conditions. Whereas AC5 compensates for the lack of AC6 in AC6/−/− and vice versa or if other AC isoforms functionally contribute to the compensation at control conditions cannot be clearly concluded from our data. We have shown in a previous and confirmed in the present study that freshly isolated JG cells not only express AC5 and AC6 but also AC1, AC4, and AC9, which might, in general, contribute to the regulation of renin release. However, our functional data argue against a major role of these other isoforms in the stimulation of renin release. Thus, the reduction of the PRC response observed in AC5/H11002/H11002 plus the reduction observed in AC6/H11002/H11002 adds up to a ≥82% attenuation of the response to isoproterenol, PGE2, and PACAP, indicating that AC5 and AC6 mediate most of the stimulation of renin release.

Somewhat unexpectedly, PRC, renin mRNA, and renin content were significantly elevated in AC6/−/− compared with wild-type mice under control conditions. This activation of the renin system is most likely the consequence of an enhanced renal volume and salt loss (Figure 2). This renal phenotype of AC6 knockout mice has been reported recently and is mediated by a defect in postreceptor signaling of the
vasopressin V2 receptor, which signals via activation of AC6 and AC3 in collecting duct cells.\textsuperscript{19,20,22} Moreover, because an inverse relation between blood pressure and PRC exists, the reduced blood pressure of AC6\textsuperscript{−/−} (Figure 2) might contribute to the stimulation of PRC in AC6\textsuperscript{−/−} under control conditions. Whether the lower blood pressure of AC6\textsuperscript{−/−} is the result of the increased renal fluid and salt excretion in combination with the inability to adequately increase PRC or whether other mechanisms are responsible for this phenotype remains to be determined in future studies. Together, these data further underline the conclusion that the regulation of the renin system by physiological stimulators, such as blood pressure and water and salt content, is not completely abrogated in AC6\textsuperscript{−/−} mice.

The main results of the in vivo study are corroborated by experiments that used the isolated perfused kidney model. This model allows the investigation of the regulation of renin release without confounding systemic factors and phenotypes. In fact, baseline renin release rates from AC6\textsuperscript{−/−} and wild-type kidneys were equivalent, suggesting that the enhanced PRC observed in AC6\textsuperscript{−/−} mice in vivo is the indirect result of a systemic phenotype. Similar to the in vivo experiments, the stimulation of renin release in response to isoproterenol was significantly attenuated in AC5\textsuperscript{−/−} and AC6\textsuperscript{−/−} kidneys compared with their wild-type kidneys. Importantly, kidneys from AC6\textsuperscript{−/−} mice demonstrated a more pronounced phenotype compared with those from AC5\textsuperscript{−/−} mice, which is similar to our observations in vivo. In contrast, the membrane-permeable cAMP analog 8-br-cAMP, which acts independent of adenyl cyclase activation, stimulated renin release rates to similar extents in all of the genotypes. Likewise, angiotensin II consistently suppressed renin secretion in AC5\textsuperscript{−/−} and AC6\textsuperscript{−/−} kidneys, which indicated that the stimulatory and the inhibitory pathways of renin release were not generally affected in the knockout mice.

Although AC5 mRNA expression is restricted to the glomerulus and initial portions of the collecting duct, AC6 is widely expressed throughout the entire nephron.\textsuperscript{21,29} The expression pattern of AC in renin-producing cells is less clear. The expression of AC6, but not of AC5, mRNA in JG cells has been demonstrated in sections of human kidneys by in situ hybridization.\textsuperscript{30} Colocalization of AC5 and renin protein was observed in cultured mouse JG cells by immunofluorescence, whereas immunostaining for AC6 was negative in these cultured cells.\textsuperscript{11} To determine the distribution of AC5 and AC6 protein in the kidney we performed immunostaining of kidney sections from wild-type mice, as well as AC5\textsuperscript{−/−} and AC6\textsuperscript{−/−}. Despite the use of different fixation methods and antibodies, we were unable to achieve reproducible and specific staining of either isoform in the kidney, which is most likely the consequence of low protein expression levels and the inability of some of the antibodies to discriminate between AC5 and AC6 (see the online Data Supplement). However, we detected the expression of both AC5 and AC6 mRNA in single JG cells that had been sampled by a patch pipette and in JG cells in kidney sections by in situ hybridization. Therefore, we conclude that both AC5 and AC6 are expressed in JG cells of mice, at least at the mRNA level.

The possible contribution of both AC5 and AC6 in the regulation of renin release initially appears to be at odds with a recent study that suggested a central role for only AC5 in JG cells.\textsuperscript{31} Thus, NKY-80, a “selective” AC5 blocker, prevented the stimulation of renin release from isolated JG cells in response to a reduction of intracellular calcium. However, it should be taken into account that the reduction of intracellular calcium does not necessarily activate the same intracellular pathways elicited by catecholamines resulting in stimulation of renin secretion. Moreover, NKY-80 has been shown to have greater selectivity for AC5 compared with AC2 and AC3, whereas its affinity for AC6 has not been investigated.\textsuperscript{31}
Accordingly, it is well possible that NKY-80 not only blocks AC5 but also AC6 with similar affinity.

Taken together, our data clearly indicate that both AC5 and AC6 are involved in the stimulation of renin release in response to catecholamines PGE2 and PACAP, with AC6 being the dominant isoform. Neither AC5 nor AC6 mediates the stimulation of renin release exclusively, but the expression of both isoforms is necessary to evoke a full response.

Perspectives
The release of renin from JG cells is a critical step in regulating the activity of the renin-angiotensin cascade and, therefore, of angiotensin II formation. Because angiotensin II exerts a negative feedback on renin secretion, pharmacological tools that block the activity (renin antagonists and ACE inhibitors) or the signaling (angiotensin II receptor blockers) of the RAAS result in an undesirable stimulation of renin release into the circulation. Accordingly, the inhibition of renin release into the circulation might be an alternative approach to block the RAAS. Our data indicate that AC5 and AC6 are potentially suitable targets for this purpose, because the stimulation of renin release in response to sympathetic activation is significantly reduced in AC5 and AC6 knockout mice. However, under control conditions, PRC is not reduced in the knockout mice compared with wild-type mice, which suggests that blockade of a single AC isoform is not sufficient to attenuate baseline renin release. Finally, AC6−/− mice have an increased diuresis. This phenotype might be favorable in congestive heart failure, which is also characterized by an activation of sympathetic activation is significantly reduced in AC5 and AC6 knockout mice. Whether the deletion of AC5 and/or AC6 in fact results in a reduced activation of the RAAS or in a symptomatic benefit in this pathological condition remains to be clarified in future studies.

Acknowledgments
The expert technical assistance of Katharina Ehnh, Marlies Hamann, and Regine Volkmann is gratefully acknowledged. We thank Dr Charlotte Wagner (University of Regensburg) for supplying renin cDNA.

Sources of Funding
This study was financially supported by the Deutsche Forschungsgemeinschaft (SFB699 to F.S.); Beginning Grant-In-Aid from the American Heart Association Western States Affiliate (to T.T.); National Institutes of Health grants P01HL066941, HL081741, and HL088426 (to H.K.H.); and Veterans Affairs Merit Review Award (to H.K.H.).

Disclosures
None.

References


Stimulation of Renin Secretion by Catecholamines Is Dependent on Adenylyl Cyclases 5 and 6
Fadi Aldehni, Tong Tang, Kirsten Madsen, Michael Plattner, Andrea Schreiber, Ulla G. Friis, H. Kirk Hammond, Pyung Lim Han and Frank Schweda

Hypertension. 2011;57:460-468; originally published online January 31, 2011; doi: 10.1161/HYPERTENSIONAHA.110.167130

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/57/3/460

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2011/01/28/HYPERTENSIONAHA.110.167130.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Online Supplement

STIMULATION OF RENIN SECRETION BY CATECHOLAMINES IS DEPENDENT ON
ADENYLYL CYCLASES AC5 AND AC6

Fadi Aldehni, ²Tong Tang, ¹Kirsten Madsen, Michael Plattner, Andrea Schreiber, ¹Ulla G. Friis, ²H.
Kirk Hammond, ³Pyung Lim Han, Frank Schweda

Institute of Physiology, University of Regensburg, 93053 Regensburg, Germany

¹ Department of Physiology and Pharmacology, University of Southern Denmark, 5000 Odense C,
Denmark

² VA San Diego Healthcare System, Department of Medicine, University of California San Diego, San
Diego, California 92161, USA

³ Division of Nano Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea
In situ hybridization

The mRNA expression of AC5, AC6 and renin in mouse kidney sections was explored by in situ hybridization using digoxigenin-labelled riboprobes. The sense and antisense probes were generated by in vitro transcription of the respective cDNAs (DIG RNA Labeling Mix, Roche). AC5 and AC6 probes were isolated from a mouse brain cDNA library using the following primer pairs:

AC5:  5'-AGAGCTCCTGGAGGAGCGTCGAGGAAA-3' and 5'-ATGGTACCGGTCCTGGGAGTTGGTGTGC-3',

AC6:  5'-AGAGCTCATGAACCAGAGCAGCCTCAC-3' and 5'-ATGGTACCCATCTCCATGGCAACGTGC-3'.

After digestion (SacI and KpnI), the cDNA fragment was cloned into pBluscript SK-(Stratagene). After linearization with BspHI or ACC65I, Digoxigenin-11-UTP-labeled RNA probes (sense or antisense, 630bp) were synthesized by \emph{in vitro} transcription (DIG RNA Labeling Mix, Roche) by T7 or T3 polymerase, respectively.

A pSP73 vector containing a 194bp fragment of renin cDNA was provided by Dr. C. Wagner (University of Regensburg, Germany). Linearization with EcoRI or BamHI and \emph{in vitro} transcription with T7 or Sp6 polymerase yielded a 248bp probe.

Cryosections (12-µm) of mouse kidneys were fixed (4% paraformaldehyde, 0.1% glutaraldehyde) and treated with proteinase K (10µg/ml) followed by acetylation (0.1M triethanolamine and 0.25 % acetic anhydrate; 10 minutes at room temperature). Sections were prehybridized for 3 h (60°C; 50% formamide, 0.75M \textit{NaCl}, 25mM \textit{Pipes} pH 6.8, 1mM \textit{EDTA}, 1% SDS, 2.5X Denhardt’s solution, 250 µg/ml tRNA, 250 µg/ml salmon sperm DNA). Hybridization with 10 ng/µl of specific RNA probe (sense or antisense) was performed overnight at 60°C in prehybridization solution and 10 % dextran sulphate. Sections were rinsed twice (2 x SSC), nonhybridized probe was digested by RNase A (10 µg/ml, 2 minutes at room temperature) followed by washing of the sections (1 X SSC, 0.5 X SSC, 0.2 X SSC; 30 minutes at 60°C each step). Finally, digoxigenin staining was performed according to the manufacturers protocol (Roche, Germany). Endogenous activity of alkaline phosphatase in the tissue was blocked by adding 5 mM levamisole (Roche) to the substrate solution.

Immunofluorescence staining for AC5 and AC6

Antibodies used for immunostaining of either AC5 or AC6:

Anti-AC5: PAC-501AP  (FabGennix International, USA), sc-74300 (Santa Cruz Biotechnology Inc., USA).

Anti-AC6: PAC-601AP  (FabGennix International, USA), sc-68137 (Santa Cruz Biotechnology Inc., USA).

The following fixations and embedding protocols were applied to kidneys of AC5-/-, AC6-/- mice and their wildtype littermates:

1. Kidneys were perfusion-fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned into 5-µm-thick samples.
2. Kidneys were perfusion-fixed in 4% paraformaldehyde, frozen in TissueTek O.C.T. embedding medium (Sakura Finetek) and sectioned into 5-µm-thick samples using a cryostat.
3. Kidneys were frozen unfixed in TissueTek O.C.T. embedding medium (Sakura Finetek) and were sectioned into 5-µm-thick samples using a cryostat and fixed in methanol at −20°C for 20 min.
4. In additional experiments kidney sections were prepared according protocols 1-3 and boiled in either 10mM sodium citrate buffer (pH 6) or TRIS-EDTA (pH 8.5) for antigen unmasking.

Sections were prepared according protocols 1-4 and incubated at 4°C overnight with the primary antibodies (dilutions 1:50 – 1:200). Thereafter they were incubated for 90 min at room temperature with a appropriate secondary antibodies. Sections were mounted with Dakocytomation Glycergel mounting medium (Dako) and viewed with an Axiovert microscope (Zeiss; Jena, Germany).

Despite of the different fixation and embedding protocols either no staining at all or no specific staining (in terms of no staining in the respective knockout kidney) could be observed using the antibodies indicated above.

**Blood pressure and heart rate measurements**

**Tail cuff method:** Mice were conditioned by placing them in the holding devices on seven consecutive days before the first measurement was performed. Subsequently, blood pressure was determined on 5 consecutive days, and the values obtained at the five days were averaged.

**BP Telemetry:** The Data Sciences International telemetry system (St. Paul, MN) was used for experiments. Transmitters (model TA11PA-C10) were magnetically activated >24 hours before implantation. Under inhalation anesthesia (sevoflurane) the telemeter catheter was inserted into the left carotid artery and advanced into the aortic arch, with the telemeter body positioned in a subcutaneous pocket on the right flank. After a 1-week recovery, recordings were begun on the morning of the eighth day, with 1-minute samplings every 3 minutes for 5 days for each animal. Radio signals were processed using a model RPC-1 receiver, a 20-channel data exchange matrix, APR-1 ambient pressure monitor, and a Data Quest ART Silver 2.3 acquisition system. The recording room was maintained at 21 to 22°C with a 12-hour light/12-hour dark cycle. All blood pressure and heart rate values obtained were averaged.

**Determination of mRNA expression by real-time PCR**

The following primers were used:

**renin:****

5´-atg aag ggg gtc gtc gga ggg gca cct-3´ (sense)
5´atg cgg gga ggg tgg gca cct-3´ (antisense)

**AC1**

5´-ggctcaacagctctctc-3´ (sense)
5´-acgggatgtctctcagt-3´ (antisense)

**AC4**

5´-ggtctctacctcctcactc-3´ (sense)
5´-aatctctctgactctcagc-3´ (antisense)

**AC5**

5´- gcg tgc tct cgg ctt agg ca -3´ (sense)
5´- gcg ccc gac gca gag atg tc-3´ (antisense)

**AC6**

5´- gca tgc gca agc tgg cca tga cc-3´ (sense)
5´- gac gcc aag cag tag atc ata g-3´ (antisense)

**AC9**

5´-ccaggagcacca tcag-3´ (sense)
5’-ggtgcctctgtatggagga-3’ (antisense)

β-actin:  
5´- cgg gat ccc cgc cct agg cac cag ggt g-3´ (sense)  
5´ - gga att agg ctt ggt tgt tga agg tct caa a-3´(antisense)

**Isolated perfused kidney**

The isolated kidney model using male AC5 and AC6 knockout mice was performed as described previously in detail ². Briefly, the animals were anaesthetized with an intraperitoneal injection of 12 mg/kg xylazine and 80 mg/kg ketamine-HCl, the abdominal aorta was cannulated and the right kidney was excised and placed in a thermostated moistening chamber (37°C). Single-pass perfusion was performed at constant pressure (100 mmHg) using a modified Krebs-Henseleit solution supplemented with 6 g/100 ml bovine serum albumin and with freshly washed human red blood cells (10 % hematocrit). Finally, the renal vein was cannulated, and samples of the venous perfusate were collected every 2 minutes for the determination of the renin activity. Three samples were taken during each experimental period. Renin secretion rates were calculated as the product of the renin activity and the venous flow rate [ml/min*g kidney weight].

**References**
