Primary Hyperaldosteronism

Small-Conductance Ca^{2+}-Activated Potassium Channels Negatively Regulate Aldosterone Secretion in Human Adrenocortical Cells

Tingting Yang, Hai-Liang Zhang, Qingnan Liang, Yingtang Shi, Yan-Ai Mei, Paula Q. Barrett, Changlong Hu

Abstract—Aldosterone, which plays a key role in maintaining water and electrolyte balance, is produced by zona glomerulosa cells of the adrenal cortex. Autonomous overproduction of aldosterone from zona glomerulosa cells causes primary hyperaldosteronism. Recent clinical studies have highlighted the pathological role of the KCNJ5 potassium channel in primary hyperaldosteronism. Our objective was to determine whether small-conductance Ca^{2+}-activated potassium (SK) channels may also regulate aldosterone secretion in human adrenocortical cells. We found that apamin, the prototypic inhibitor of SK channels, decreased membrane voltage, raised intracellular Ca^{2+} and dose dependently increased aldosterone secretion from human adrenocortical H295R cells. By contrast, 1-Ethyl-2-benzimidazolinone, an agonist of SK channels, antagonized apamin’s action and decreased aldosterone secretion. Commensurate with an increase in aldosterone production, apamin increased mRNA expression of steroidogenic acute regulatory protein and aldosterone synthase that control the early and late rate-limiting steps in aldosterone biosynthesis, respectively. In addition, apamin increased angiotensin II–stimulated aldosterone secretion, whereas 1-Ethyl-2-benzimidazolinone suppressed both angiotensin II– and high K^{+}–stimulated production of aldosterone in H295R cells. These findings were supported by apamin-modulation of basal and angiotensin II–stimulated aldosterone secretion from acutely prepared slices of human adrenals. We conclude that SK channel activity negatively regulates aldosterone secretion in human adrenocortical cells. Genetic association studies are necessary to determine whether mutations in SK channel subtype 2 genes may also drive aldosterone excess in primary hyperaldosteronism. (Hypertension. 2016;68:785-795. DOI: 10.1161/HYPERTENSIONAHA.116.07094.) ● Online Data Supplement

Key Words: adrenal cortex ■ aldosterone secretion ■ angiotensin II ■ apamin ■ hyperaldosteronism ■ small-conductance Ca^{2+}-activated potassium channels

Aldosterone plays a key role in the regulation of blood pressure, and it is produced by zona glomerulosa (ZG) cells of the adrenal gland. The 2 primary physiological aldosterone secretagogues, angiotensin II (Ang II) and potassium, increase aldosterone production in a Ca^{2+}-dependent manner; extracellular Ca^{2+} entry into ZG cells is necessary to maintain stimulated secretion. The contribution of plasma membrane voltage-gated Ca^{2+} channels to this obligate rise in intracellular Ca^{2+} is enabled by membrane depolarization, an action that is evoked by both Ang II and high potassium.

Primary hyperaldosteronism (PA), caused by autonomous overproduction of aldosterone, is the most common form of endocrine hypertension with an incidence of ≈10% among hypertensives. Aldosterone-producing adenoma and bilateral idiopathic PA, the 2 most common subtypes of PA, account for ≈95% of clinically diagnosed cases. Patients with PA have increased cardiovascular risks than those with equivalent essential hypertension. Notably, recent clinical studies have highlighted the pathological role of KCNJ5 potassium channels in PA. Choi et al first reported that 2 somatic mutations, G151R and L168R, in the inward rectifying potassium channel KCNJ5 were found in 8 of 22 aldosterone-producing adrenocortical cells. Genetic association studies are necessary to determine whether mutations in SK channel subtype 2 genes may also drive aldosterone excess in primary hyperaldosteronism.
channel protein, producing loss of channel selectivity for K+ with commensurate Na+ influx that results in cell membrane depolarization, elevation of intracellular Ca2+ level, and the overproduction of aldosterone.13,14,17

Small-conductance Ca2+-activated potassium (SK) channels consist of 3 subtypes: SK1, SK2, and SK3, and they are gated solely by intracellular Ca2+ elevation.19 SK channels are widely expressed in the brain, contribute to the medium depolarization, elevation of intracellular Ca2+ level, and the overproduction of aldosterone.13,14,17

SK channels may play an important role in regulating aldosterone production. In this study, we assessed the contribution of SK channels to the control of basal and stimulated aldosterone production in human adrenocortical H295R cells and in ZG cells within adrenal slices. To test the functional importance of SK channels to the regulation of aldosterone production, we manipulated channel activity pharmacologically using apamin, a prototypical inhibitor, and 1-EBIO, an agonist of SK channels, or genetically using lentiviral vector delivery of shRNA.

Methods

Cell Culture and Chemicals

Human adrenocortical cell line H295R (American Type Culture Collection) was cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1% antibiotic anti myotic solution, and 0.1% insulin, transferrin and selenious acid+ premix (BD Biosciences). Twelve hours before the experiment, cells were serum deprived in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 containing 0.1% fetal bovine serum. Reagents were purchased from TOCRIS (Apanin and 1-EBIO), Sigma Aldrich (Angiotensin II and nifedipine), and Alomone laboratories (3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]benzamide(TTA-P2)).

RNA Extraction and Quantitative Real-Time Polymerase chain Reaction

Total RNA was extracted (RNeasy mini kit, QIAGEN) and reverse transcribed (2 μg RNA). Real-time polymerase chain reaction was performed in 20 μL reactions containing: 2 μL of template, 0.4 μmol/L of each paired primer, and SYBR Green Polymerase Chain Reaction master mix. The thermocycling conditions were 94°C, 10 minutes; 38 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; and 72°C, 8 minutes. Results were normalized by β-actin mRNA or in lentiviral infection, hypoxanthine phosphoribosyltransferase mRNAs. Data were calculated by 2−ΔΔCt method and reported as fold change over control. The primers used for real-time polymerase chain reaction are listed in Table S1 in the online-only Data Supplement.

Electrophysiology

H295R whole-cell currents were recorded using an Axopatch 200B or 700B amplifier (Axon Instruments). The bath solution contained (in mmol/L): 116 NaCl, 2 KCl, 10 HEPES, 2 MgCl2, 2 CaCl2, 10 glucose, 20 TEACl, and 5 4-aminopyridine, pH 7.4 (adjusted with NaOH). The internal solution contained (in mmol/L): 155 K gluconate, 10 KCl, 10 HEPES, 10 EGTA, 1 CaCl2, 1 MgCl2, and 2 Mg-ATP (pH adjusted to 7.3 using KOH). In some studies; Figure S1, we used solutions that augmented the recorded SK currents (online-only Data Supplement). For calcium current recording, the bath solution contained (in mmol/L): 143 TEACl, 10 CaCl2, 2 MgCl2, 10 HEPES, and 10 gua cose, pH 7.4 (adjusted with TEAOH). The internal solution contained (in mmol/L): 125 CsCl, 10 HEPES, 10 EGTA, 1 MgCl2, 1 CaCl2, 5 Mg-ATP, and 0.3 Tris-GTP (pH adjusted to 7.2 using CsOH). For current clamp recording, the bath solution contained (in mmol/L):140 NaCl, 3 KCl, 10 HEPES, 2 MgCl2, 2 CaCl2, and 10 glucose (pH 7.3). The internal solution contained (in mmol/L):29 KMeSO4, 10 NaCl, 10 HEPES, 1 MgCl2, 0.5 EGTA, 3 Mg-ATP, and 0.3 Tris-GTP (pH 7.2). Recording pipettes (capillary tubing, BRAND) had resistances of 3 to 7 MΩ under solution conditions. All recordings were performed at room temperature. Currents were sampled at 10 kHz and filtered at 2 kHz. Modulators were applied by gravity perfusion.

Steroid Assay

Cell culture supernatants collected after treatment were stored at −80°C. Medium aldosterone concentrations were analyzed using an Aldosterone ELISA Kit (ENZO Life Science) following the manufacturer’s recommendations. Results were normalized by total protein (BCA Protein Kit, GeneRay).

Measurement of Intracellular Ca2+ Levels

Intracellular Ca2+ was detected using fura-2 AM as previously reported.20 Briefly, after serum starvation for 12 hours, cells were incubated in fresh 0.1% serum Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 without amino acids, washed twice with balanced salt solution. (Balanced salt solution) buffer (in mmol/L): 145 NaCl, 2.5 KCl, 10 HEPES, 1 MgCl2, 10 glucose, and 2 CaCl2. Subsequently, cells were incubated in balanced salt solution containing 10 μmol/L fura-2 AM (37°C, 45 minutes), washed 3x with balanced salt solution, and fluorescence captured at 505 nm after excitation at 340 and 380 nm. Basal Ca2+ signal was acquired for 30 s before the application of Ang II (10 nmol/L, with 0.05% BSA) or high K+ (22 mmol/L) by pressure delivery (ALA Scientific Instruments). Fluorescence intensity was quantified using Metafluor software (Universal Imaging Corporation).

Plasmids and Lentiviral Production

The shRNA hairpin sequences were inserted into MluI-ClaI sites of pLVTHM targeting vector as previously reported.18 Oligonucleotides specifying the shRNA are 5′-GAA GCT AGA ACT TAC CAA A-3′ and scramble, 5′-AAG GAT ACA CCG ATA ACA T-3′. Lentivirus was produced in the 293T cells using a packaging vector psPAX2 and an envelope plasmid pMD2.G. The viral supernatant was harvested after 48 to 72 hours and centrifuged at low speed to remove cellular debris. The virus was collected by ultracentrifugation at 4°C and resuspended with PBS containing 0.1% BSA for storage at −80°C. Lentivirus infection was performed 24 hours after seeding H295R cells with 5 μg/μL polynucle (added during infection) to complete medium. Seventy-two hours after infection, cells were serum deprived for 12 hours using low serum (0.1%) Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12, and then the medium replaced with or without apamin. After incubation (24 hours), supernatants were collected for measurement of aldosterone and cells for measurement of mRNA.

Immunofluorescence and Aldosterone Secretion of Human Adrenal Gland

Adrenal gland samples were obtained from 11 patients (5 men and 6 women, age 31–67 years) undergoing radical nephrectomy to remove kidney cancer and adjacent ipsilateral adrenal. The protocol for obtaining and using human adrenal tissues in this work was reviewed and approved by the Ethics Committee of Fudan University Shanghai Cancer Center, Fudan University. Written informed consent from all patients was obtained.

Human adrenal samples were transported in cold PBS. Fat tissue was removed, and adrenals were kept in ice-cold bicarbonate-buffered...
saline containing (in mmol/L): 140 NaCl, 2 KCl, 0.1 CaCl₂, 5 MgCl₂, 26 NaHCO₃, and 10 glucose, bubbled with 95% O₂ and 5% CO₂. Tissue was embedded in low-melting temperature agar (2.5% in bicarbonate-buffered saline, 10×10 mm) and sectioned (200 μm, DSK microslicer, DOSAKA). The slices were placed on Millicell-CM membranes (pore size 0.4 μm; Millipore) and incubated in MEM Hanks (GIBCO) supplemented with (in mmol/L) 1 l-Glutamine, 1 CaCl₂, 2 MgCl₂, 30 HEPES, 12.8 NaHCO₃, 12-mg/L bovine insulin, 0.12% ascorbic acid, and 20% horse serum (GIBCO) at 37°C with 5% CO₂ for 2 hours before 6 hours of treatment with the indicated agents.

For immunofluorescence, adrenal glands were fixed in 4% paraformaldehyde (12–16 hours at 4°C) and transferred sequentially to 10%, 20%, and 30% sucrose/PBS at 4°C. After optimal cutting temperature compound (Tissue-Tek, SAKURA) embedding, tissues were sectioned (20 μm, Leica CM1950 cryostat) and dried at room temperature. Slices were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed (cold PBS), and incubated in 0.5% Tween-20 in PBS for 10 minutes. Slices were blocked (10% horse serum, 0.3% Triton X-100 in PBS, 2 hours, room temperature) and then incubated with primary antibody: rabbit anti-KCNN2 (1:200, Alomone Laboratories), 1% horse serum, 0.3% Triton X-100 in PBS, 1 day, 4°C. After washing with PBS, slices were incubated in the secondary antibody (Cy3-labeled goat anti-rabbit IgG, 1:500, Beyotime, China) overnight, 4°C, washed with PBS, and 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride treated before coverslipping. Confocal images were obtained using a Leica SP2 confocal microscope. For blocking studies, the KCNN2 peptide antigen was added (1:1) to the primary antibody solution. All protocols were in accordance with institutional guidelines.

**Statistics**

Data analysis was performed with Clampfit 10.2 (Axon Instruments) and Origin 8.0 software (OriginLab). Statistical analysis consisted of unpaired or paired Student t tests. Data are given as means±SEM, n indicates the number of tested cells or independent tests. P<0.05 was considered statistically significant. Multiple comparisons were analyzed using a 1-way ANOVA followed by post hoc Tukey testing.

**Results**

**H295R Cells Express SK Channels**

A previous study suggested that the human adrenal cortex may express SK channels. Therefore, we tested for SK channel mRNA expression in the human adrenocortical cell line H295R. As shown in Figure 1F, mRNA for SK1, SK2, and SK3 was detected in H295R cells. All bands corresponded to the predicted product sizes generated from genes KCNN1-3, and their SK channel identity was confirmed by sequencing.

To determine whether functional SK channels are expressed in H295R cells, we recorded K⁺ currents (Figure 1A through 1E) and used pharmacological manipulation with apamin, the prototypical SK channel inhibitor, or 1-EBIO, an SK channel agonist, to identify SK channel currents. K⁺ currents elicited by 10-mV depolarizing steps from a holding potential of ~95 mV elicited outward currents of small magnitude (Figure 1A). Although small, the elicited whole-cell currents were amplified robustly by 1-EBIO (100 μmol/L), a modulation that could be antagonized by apamin (100 nmol/L). A determination of the apamin-sensitive component of 1-EBIO–induced currents (AS-1EBIO) pharmacologically isolated the SK component of current. We note that because our standard recording solutions contained high TEA in the bath and EGTA in the internal, the measured SK component of current was modestly diminished in agreement with the 17% increase observed (Figure S1) when TEA and EGTA were removed.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** H295R cells express functional small-conductance Ca²⁺-activated potassium (SK) channels. **A** to **C**, Representative K⁺ current traces in the absence and presence of SK channel activator 1-EBIO or inhibitor apamin. **D**, Apamin-sensitive SK channel currents obtained by subtracting C from B. **E**, Current–voltage relationship (I–V curves) generated from peak current density at each test voltage. Data are presented as means±SEM from 10 cells. **F**, SK1, SK2, and SK3 channel mRNA expression were detected in H295R cells by real-time polymerase chain reaction.
Apamin Increases Aldosterone Secretion and Depends on Ca2+ Entry Through T-Type Ca2+ Channels

To test for a role of SK channels in the regulation of basal aldosterone secretion, we used apamin to block SK currents. As shown in Figure 2A, apamin increased aldosterone secretion from H295R cells in a dose-dependent manner (n=3–15, P<0.05 compared with control by ANOVA). Production was stimulated to a maximal level of 50% with an EC50 of ≈0.08 nmol/L. Because aldosterone production critically depends on Ca2+, we tested whether block of SK channels by apamin was sufficient to increase intracellular Ca2+ levels in H295R cells. As shown in Figure 2B, the basal level of intracellular Ca2+ was significantly and consistently increased after pretreatment with apamin (1 nmol/L, 24 hours).

Native ZG cells isolated from human, rodent, or bovine adrenal glands express both low-voltage activated, T-type, and high-voltage activated, L-type, Ca2+ channels in variable proportions.6 To assess the relative contribution of T-type

Figure 2. Apamin increases aldosterone secretion mediating Ca2+ entry through T-type Ca2+ channels in H295R cells. A. Apamin increased aldosterone secretion dose dependently. Twelve-hour serum-starved H295R cells were incubated in 0.1% fetal bovine serum Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 for 24 h with/without apamin at various concentrations. Data were expressed as fold increase over control. P<0.05 compared with control using a 1-way ANOVA test. (n=3–12). B. Twenty-four hours of treatment with 1 nmol/L apamin increased basal intracellular calcium concentration. Results are presented as mean±SEM of fluorescence excitation ratios. *P<0.05 compared with control using Student t test. C and D, Ca2+ currents were evoked by 80-ms steps (-60 to +35 mV in 5-mV increments) applied every 6 s from a Vh of -90 or -50 mV. Shown are representative currents evoked by steps to -40, -10, or +10 mV from a Vh of -90, or Vh of -50 mV to reduce Cav3.3 channel availability. Current–voltage relationship was constructed from peak currents (n=7). E, Effects of L-type Ca2+ channel inhibitor nifedipine (3 μmol/L) and T-type Ca2+ channel inhibitor 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2) (2 μmol/L) on Ca2+ currents. F, TTA-P2 but not nifedipine precluded apamin-induced aldosterone production (n=4, *P<0.05 compared with control by ANOVA).
versus L-type Ca\(^{2+}\) currents in H295R cells used in these studies, we used patch clamp electrophysiology to characterize Ca\(^{2+}\) currents. We elicited test depolarizations in 5-mV increments from −60 to +35 mV from a holding potential of −90 mV, to measure the combined activities of T-type and L-type Ca\(^{2+}\) channels or from a holding potential of −50 mV (6 s) to inactivate T-type Ca\(^{2+}\) channels and determine the activity of L-type Ca\(^{2+}\) channels in relative isolation. Notably, holding at −50 mV, reduced maximal peak current by 49% and induced a rightward shift in the potential at which maximal peak current is recorded (from −10 mV to +15 mV) in agreement with the removal of a major component of low-voltage activated (T type) channel current from the recorded current (Figure 2C and 2D). We used nifedipine, a dihydropyridine L-type Ca\(^{2+}\) channel blocker,\(^{22}\) or TTA-P2, a selective T-type Ca\(^{2+}\) channel blocker,\(^{23}\) to assess the relative importance of each channel subtype to the apamin-induced stimulation of basal aldosterone production. From a holding potential of −90 mV, 50 ms step depolarizations to −20 mV elicited a peak Ca\(^{2+}\) current that was inhibited maximally by 45.7%±4.0% when nifedpine (3 \(\mu\)mol/L) was applied alone. The addition of TTA-P2 (2 \(\mu\)mol/L) increased current inhibition to 94%±2.0%, indicating nearly equal contributions of T and L current to the total elicited current (Figure 2E).

At these functionally effective concentrations, neither nifedipine nor TTA-P2 inhibited basal aldosterone output. By contrast, TTA-P2, but not nifedipine, prevented the apamin-induced increase in aldosterone production in agreement with the privileged role previously reported for T-type Ca\(^{2+}\) channel currents in the control of aldosterone output from isolated ZG cells\(^{24}\) (Figure 2F).

**Effect of Apamin on mRNA Expression of Steroid Biosynthetic Enzymes in H295R Cells**

To further investigate the mechanism by which apamin stimulates basal aldosterone production in H295R cells, we examined levels of mRNA for key steroidogenic enzymes and proteins that participate in aldosterone synthesis. After 24 hours of treatment with 1 nmol/L apamin, mRNA expression of 3β-hydroxysteroid dehydrogenase-2 (HSD3B2), cytochrome P450, family 11, subfamily B, polypeptide 1 (CYP11B1), and aldosterone synthase (CYP11B2) were significantly increased by 1.5-, 2.2- and 2.2-fold compared with untreated cells (Figure 3A), whereas cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2) gene expression were decreased by 0.7-fold (Figure 3A). By contrast, apamin did not alter the mRNA for cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1), and steroidogenic acute regulatory protein (STAR) after 24 hours of treatment (Figure 3B). Nevertheless, apamin did stimulate STAR mRNA expression in a time-dependent manner, with a maximal transient rise attained after 6 hours of incubation (Figure 3C). A transient rise in STAR expression has been observed in H295R cells stimulated with very-low-density lipoprotein or Ang II.\(^{25}\)

**Effect of SK2 Channel Suppression on Aldosterone Production in H295R Cells**

Among SK channels, SK2 channels are the most sensitive to inhibition by apamin (EC\(_{50}\): SK1 0.7–12.2 nmol/L, SK2 27–140 pmol/L, and SK3 0.6–4 nmol/L).\(^{26–31}\) Because stimulation of aldosterone production by apamin was near maximal

![Figure 3](http://hyper.ahajournals.org/) Effect of apamin on mRNA expression of the key proteins that control steroid biosynthesis: STAR, CYP11A1, HSD3B2, CYP21A2, CYP17A1, CYP11B1, and CYP11B2. A and B, Apamin increased HSD3B2, CYP11B1, and CYP11B2 but decreased CYP21A2 mRNA expression. Twelve-hour serum starved H295R cells were incubated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 with 0.1% fetal bovine serum for 24 h with/without 1 nmol/L apamin. Data are expressed as fold change over control. \(P<0.05\) compared with control (\(n=4–7\)). C, Apamin upregulated the expression of STAR at early time points. \(P<0.05\) compared with control (\(n=3\)).
at 0.1 nmol/L (Figure 1A), we predicted that regulation by apamin was the result of inhibition of SK2 channel activity. Therefore, to independently evaluate the contribution of SK2 channels to the regulation of aldosterone production, we used lentivirus vectors to deliver shRNA targeted to affect post-translational silencing of the SK2 gene in H295R cells. Seventy-two hours after infection, H295R cells were serum deprived (0.1% serum, 12 hours) and fresh medium with or without apamin substituted (24 hours). As shown in Figure 4, shRNA-targeting SK2 channels, but not the scrambled control, reduced SK2 channel expression by 70% (Figure 4A). This reduction was sufficient to increase basal aldosterone production by 35% (Figure 4B) and importantly abrogated the apamin-induced increase in basal aldosterone output. The knockdown of SK2 channels also mimicked apamin-induced changes in mRNA expression, increasing that of CYP11B1, CYP11B2, and HSD3B2 (fold increase: CYP11B1, 1.3±0.1, n=5; CYP11B2, 2.3±0.3, n=4; and HSD3B2, 1.24±0.07, n=4; P<0.05 compared with scramble control), decreasing that of CYP21A2 while sparing that of CYP17A1, CYP11A1, or STAR (Figure 4C). Taken together, these data indicate that SK2 channel activity underlies the regulation of aldosterone production by apamin.

**Effect of Apamin/1-EBIO on Ang II– and K+-Induced Aldosterone Secretion**

To assess the overall role of SK channels in aldosterone production, we tested for the apamin or 1-EBIO evoked modulation of Ang II– and K+-induced aldosterone secretion. We used 1-EBIO, an activator of SK channels, to hyperpolarize and depolarize H295R cells and verified these predicted changes measuring membrane voltage in current clamp (voltage change: 1-EBIO, −9±2 mV, n=8 and apamin, +6±1mV, n = 12). As observed for basal aldosterone output, 1 nmol/L apamin increased aldosterone production stimulated by low and high concentrations of Ang II (1 and 10 nmol/L; 1.6- and 1.34-fold, respectively; Figure 5A), whereas 100 μmol/L 1-EBIO suppressed Ang II–induced aldosterone secretion, in agreement with their opposing effects on membrane voltage. Commensurate with an increase in output, 24 hours of treatment with apamin augmented the Ang II–induced rise in intracellular Ca2+ (Figure 5B) consistent with depolarization and the opening of voltage-dependent Ca2+ channels.

In vivo, aldosterone production is also modulated by extracellular K+ within the physiological range (2–5 mmol/L). Although H295R cells are responsive to extracellular K+, supraphysiological concentrations are required to stimulate production. Raising extracellular K+ to 22 mmol/L increased aldosterone production by 5.3-fold (Figure 6A). However, unlike the more modest but maximal stimulation of production elicited by 10 nmol/L Ang II, aldosterone production evoked by 22 mmol/L K+ was not increased further by apamin but was reduced by 100 μmol/L 1-EBIO (fold increase: 22 mmol/L K+, 5.3±0.3 and K+1-EBIO, 3.4±0.2, P<0.05 compared with 22 mmol/L K+ alone, n=6; Figure 6A). Consistent with a lack of effect of apamin on K+-induced aldosterone production, 1 nmol/L apamin failed to increase intracellular Ca2+ evoked by 22 mmol/L K+ (Figure 6B). Notably, the failure to raise Ca2+ and stimulate aldosterone production in cells stimulated by 22 mmol/L K+ could be explained by the inability of apamin...
to further reduce membrane voltage in H295R cells that were already ≈20 mV depolarized by 22 mmol/L K+(Figure 7).

**Effect of Apamin on Aldosterone Production in ZG Cells Retained Within a Human Adrenal Slice**

Finally, to assess the role of SK2 channels in the regulation of aldosterone production in native human glands, we tested first for their expression in human adrenal slices. As shown in Figure 8A, SK2 channels are highly expressed in the ZG layer of the human adrenal. Target antigen fluorescence was strong in the ZG layer, weak in the zona fasciculata and completely competed with antigenic peptide. Importantly, expressed SK2 channels were also functional as 1 nmol/L apamin significantly and consistently increased aldosterone secretion from adrenal tissue slices prepared from 11 individual patients (n=11, P<0.05 compared with control Figure 8B). Similar to modulation evoked by 1 nmol/L apamin in H295R cells, apamin further increased aldosterone production from human adrenal slices stimulated by 10 nmol/L Ang II (Ang II and Ang II+apamin and 1.7- and 2.3-fold, respectively; P<0.05; Figure 8D) but failed to augment aldosterone production evoked by 15 mmol/L K+ (P>0.05, Figure 8E).

**Discussion**

To date, SK channels are the only known target for apamin; therefore, the inhibition of SK channel activity should account for the apamin-induced effects reported here. Accordingly, in this study we found that apamin increased aldosterone secretion from H295R cells and that the genetic knockdown of SK2 channel expression by shRNA evoked a similar degree of steroid stimulation that was resistant to apamin modulation. Moreover, both apamin block and silencing SK2 channel gene expression evoked equivalent changes in the expression of steroidogenic enzymes and regulatory proteins, notably, increasing the mRNA expression of STAR and CYP11B2, which control the early and late rate-limiting step of aldosterone biosynthesis, respectively. Together, these data argue that in human H295R cells, SK channels have a significant conductance in the basal state that negatively regulates or restrains the production of aldosterone.

SK channels are activated by submicromolar concentrations of internal Ca²⁺, and among SK channel subtypes, SK2 channels have the highest Ca²⁺ sensitivity. Such high Ca²⁺ sensitivity can allow for their activation by distal Ca²⁺ sources, such as intracellular Ca²⁺ stores. However, our data indicate that Ca²⁺ entry through voltage-gated plasma membrane CaV3.2 channels was the source of Ca²⁺ that activated SK channels because apamin-evoked aldosterone production was blocked selectively by TTA-P2, the selective CaV3.2 Ca²⁺ channel blocker. Therefore, it is formally possible that SK channels indeed form Ca²⁺ nanodomains with CaV3.2 channels in ZG cells. Whether SK channels indeed form Ca²⁺ nanodomains with CaV3.2 channels in ZG cells remains to be determined.

Because isolated ZG cells remain electrically quiescent, operating within a narrow voltage range of −85 to −40 mV, Cav3.2 channels have been found to be the primary Ca²⁺ entry
pathway, mediating Ang II/K⁺-stimulated aldosterone secretion in vitro.34–38 However, as we have previously reported, ZG cells retained within an acutely prepared adrenal slice are electrically excitable, generating autonomous Vm oscillations. By providing the opportunity for recurrent channel activation, Vm oscillations allow an otherwise transiently activating Ca²⁺ channel (T type) to transduce a substantial steady-state Ca²⁺ current that can support increased steroidogenesis for minutes to hours. 39 However, electric excitability also provides the opportunity for high-voltage activated conductances to participate in the regulation of aldosterone production. Previous studies have shown that genetic deletion of either the pore-forming, KCNQ1 or the regulatory, KCNE1 subunit of the large conductance, Ca²⁺- and voltage-activated potassium (BK) channel produces hyperaldosteronism in mice. 40,41 BK channel mRNA expression is detected in both human adrenal samples and NCI-H295 cell line,42 and the expression of SK channels in human adrenal cortex was detected by the Human Gene 1.0 ST Array. 13 The relative importance of SK versus BK channel types in the regulation of aldosterone production from the human adrenal cortex in vivo is as yet unexplored, but precedence for coordinated regulation of catecholamine secretion by these channel types is found in electrically excitable cells of adrenal medulla.43

Ang II and K⁺ are the 2 most important physiological aldosterone secretagogues, and intracellular Ca²⁺ elevation and membrane depolarization are key features of their mechanisms of action. In this study, we found that SK channel activity regulated Ang II– but not K⁺-stimulated aldosterone secretion in H295R cells, as apamin augmented Ang II–stimulated aldosterone production but failed to modulate aldosterone production evoked by high K⁺. Apamin also failed to augment the rise in intracellular Ca²⁺ evoked by high K⁺, suggesting a loss of capacity to alter membrane voltage during high K⁺ stimulation. Indeed, here we show that apamin functionally depolarized Ang II–stimulated H295R cells but was not able to alter membrane voltage in cells whose potential

Figure 6. Effects of apamin and 1-EBIO on high K⁺-stimulated aldosterone secretion in H295R cells. A, Apamin did not alter K⁺-stimulated aldosterone secretion, whereas 1-EBIO suppressed it. *P<0.05 compared with control. #P<0.05 compared with high K⁺ alone. B, Twenty-four hours of incubation with apamin did not alter high K⁺-induced elevation of intracellular calcium (left). Baseline Ca²⁺ fluorescent signal was recorded for 30 s, and the high K⁺–induced signal was recorded for 120 s. Statistical analysis of high K⁺–induced intracellular Ca²⁺ level in control or apamin-treated cells (right). Data expressed as fold increase over control cells. P>0.05 compared with control cells (n=4).

Figure 7. Changes in membrane voltage (Vm) of H295R cells elicited by Ang II or K⁺ with/without Apamin. Membrane voltage was determined in current clamp. Depolarization by apamin was additive to that of 10 mmol/L Ang II but was subsumed by the voltage change induced by 22 mmol/L K⁺ (n=5–7). *P<0.05 compared with Ang II alone.
was clamped by 22 mM K⁺. Nevertheless, these results do not rule out the possibility that SK channels contribute to the regulation of aldosterone production in vivo by physiological levels of K⁺ (2–5 mM) that modestly shift the equilibrium potential for K⁺ and thus could allow the activity of SK2 channels to change ZG cell membrane voltage.

Previous studies suggested that the human adrenal cortex may express SK2 channels. Here, we showed that SK2 channels are predominantly expressed in the ZG layer of the human adrenal cortex compared with that of the zona fasciculata, an expression pattern that is similar to that observed in rat adrenal. Although apamin did not significantly increase K⁺-evoked aldosterone secretion from human adrenal slices stimulated by supraphysiological levels of K⁺ (15 mM), SK channels may still contribute to the regulation of production evoked by physiological levels of K⁺ in vivo. In this regard, it is noteworthy that the K⁺ concentration of the MEM Hanks used in our human adrenal steroidogenesis studies is ≈5.7 mM. Therefore, the stimulation of basal rates of aldosterone production by apamin reported here predicts and supports the modulation of K⁺-elicited production of aldosterone by SK2 channels in vivo.

**Perspectives**

Here, we show for the first time that SK channels negatively regulate aldosterone secretion in H295R cells and ZG cells within human adrenal slices. Blockage of SK channels increases mRNA expression of STAR and CYP11B2 that control the early and late rate-limiting steps for aldosterone biosynthesis. By regulating membrane voltage and evoked Ca²⁺ currents carried by T-type Ca²⁺ channels, SK channels modulate both basal and Ang II–stimulated aldosterone secretion in...
human adrenal glomerulosa cells. Together, our data support the possibility that SK channels contribute to the regulation of aldosterone secretion from native human adrenals. Whether genetic variation in the KCNN2 gene, like that of the KCNJ5 gene, also drives hyperaldosteronism in PA warrants further investigation.

Sources of Funding
This work was supported by the National Natural Science Foundation of China (NSFC 31271240); the National Key Research and Development Program of China (2016YFA0100080); the Specialized Research Fund for the Doctoral Program of Higher Education of China (20100071120022) and the Shanghai Leading Academic Discipline Project [B111] awarded to C. Hu, and the National Institutes of Health grants HL089717 and HL036977 awarded to P.Q. Barrett.

Disclosures
None.

References
Yang et al  SK Channels Regulate Aldosterone Secretion  795


**Novelty and Significance**

**What Is New?**
- This study demonstrates that small-conductance Ca2+-activated potassium (SK) channel activity negatively regulates both basal and angiotensin II–evoked aldosterone secretion from human adrenal cortical cells by alterations in membrane voltage and intracellular Ca2+ concentration.
- This regulation is orchestrated predominantly by SK2 channels activated selectively by Ca2+ entry through Ca3.3.x channels.

**What Is Relevant?**
- Autonomous overproduction of aldosterone from zona glomerulosa cells causes primary hyperaldosteronism.
- Recent clinical studies have highlighted the pathological role of KCNJ5 potassium channel in primary hyperaldosteronism.

**Summary**

Blockade of SK channels by apamin or knockdown of SK channel subtype 2 gene expression by shRNA increased basal aldosterone production from human adrenal cortical cells. By regulating membrane voltage and evoked Ca2+ currents carried by T-type Ca2+ channels, SK channels regulate both basal and angiotensin II–stimulated secretion of aldosterone.
Small-Conductance Ca\textsuperscript{2+}-Activated Potassium Channels Negatively Regulate Aldosterone Secretion in Human Adrenocortical Cells

Tingting Yang, Hai-Liang Zhang, Qingnan Liang, Yingtang Shi, Yan-Ai Mei, Paula Q. Barrett and Changlong Hu

*Hypertension*. 2016;68:785-795; originally published online July 18, 2016; doi: 10.1161/HYPERTENSIONAHA.116.07094

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2016 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/68/3/785

Data Supplement (unedited) at:

http://hyper.ahajournals.org/content/suppl/2016/07/18/HYPERTENSIONAHA.116.07094.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 Supplements

SK CHANNELS NEGATIVELY REGULATE ALDOSTERONE SECRETION IN HUMAN ADRENOCORTICAL CELLS

Tingting Yang¹, Hai-Liang Zhang², Qingnan Liang¹, Yingtang Shi³, Yan-Ai Mei¹, Paula Q. Barrett³, Changlong Hu¹#

¹Department of Physiology and Biophysics, School of Life Sciences, Institutes of Brain Science, Fudan University, Shanghai, China
²Department of Urology, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan university, Shanghai, China
³Department of Pharmacology, University of Virginia, Charlottesville, Virginia, USA.

Short title: SK channels regulate aldosterone secretion.

Total number of tables and figures: 2 Supplemental Figures and 1 Supplemental table.

#, corresponding author and person to whom reprint requests should be addressed:
Changlong Hu, Ph.D
Department of Physiology and Biophysics, School of Life Sciences, Institutes of Brain Science, Fudan University, 2005 Songhu Road, Shanghai 200438, China.
Tel: (86)-21-51630652.
Fax: (86)-21-51630521.
Email: clhu@fudan.edu.cn
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP11B1</td>
<td>CCAGGTGGAGACACCTAAC</td>
<td>GTTGATGGCTCTGAAGGT</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>TTGGTACAGGTTTTTCC</td>
<td>TTGGTACAGGTTTTTCC</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>AACAACCGTAAGGTTATCGC</td>
<td>TTGGTTACCGCCACGAAG</td>
</tr>
<tr>
<td>CYP21A2</td>
<td>ATCAAGGACGACAACCTTAATGC</td>
<td>GGAAGAACCTGAGAAAGGGAAT</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>CCTGCTGGAAGTAGTGAG</td>
<td>CGCTGACCTCTTCAACAGGA</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>TGTTGACAAATGGCTGGCTAA</td>
<td>TGCCGACTTCTTCAACAGGA</td>
</tr>
<tr>
<td>STAR</td>
<td>GTGGGCAAGGTGTTCCG</td>
<td>CGCACGCTCACAAGTCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>GTGGACATCCCGCAAAGAC</td>
<td>AAAGGGTGTAACGCAACTAA</td>
</tr>
<tr>
<td>HPRT</td>
<td>CCTGGCGTCTGATTAGTG</td>
<td>TGATTAATAAACCCCTTTCCA</td>
</tr>
<tr>
<td>SK1</td>
<td>ACGATGACGGAGGATGA</td>
<td>TTGAGTGCAGTGAAGTA</td>
</tr>
<tr>
<td>SK2</td>
<td>CTGAATGACCAAGCAA</td>
<td>CTGAATGACCAAGCAA</td>
</tr>
<tr>
<td>SK3</td>
<td>AACACTCTGGTGACCTT</td>
<td>CGCCAATCTGCTTCTCC</td>
</tr>
</tbody>
</table>
Figure S1. H295R cells express functional SK channels. (A-C) representative K+ current traces in the absence and presence of the SK channel activator 1-EBIO, or inhibitor apamin. (D) Apamin-sensitive SK channel currents obtained by subtracting C from B. (E) Current-voltage relationship (I–V curves) generated from peak current density at each test voltage. The bath solution contained (in mmol/L): 140 NaCl, 2 KCl, 10 HEPES, 2 MgCl$_2$, 2 CaCl$_2$, 10 Glucose, pH 7.4 (adjusted with NaOH). The internal solution contained (in mmol/L) 140 K Gluconate, 10 KCl, 10 HEPES, 0.5 EGTA, 1 MgCl$_2$, 2 Mg-ATP, pH adjusted to 7.3 using KOH. Data are presented as means ± S.E.M. from 6 cells. Currents were recorded without TEA and 4-AP in bath solution, and with 0.5mmol/L EGTA in internal solution.
Figure S2. Schematic model of ionic regulation of aldosterone secretion in health and in PA. Calcium the critical second messenger driving the production of aldosterone is increased by the activities of voltage-dependent Ca^{2+} channels (T-type/Cav3.2 and L-type/Cav1.3). By contrast, aldosterone production is restrained by hyperpolarizing K^+ conductances, such as KCNJ5 channels, and as reported here, apamin-sensitive SK2 channels. Somatic mutations in the coding regions of KCNJ5 and Cav1.3 channels are prevalent in aldosterone producing adenomas of patients with primary hyperaldosteronism (PA). Based on our findings, we hypothesize that loss-of-function mutations in SK2 channels could drive aldosterone overproduction and thus, many be present in a cohort of patients with PA.