Mutated KCNJ5 activates the acute and chronic regulatory steps in aldosterone production

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Abstract

Somatic and germline mutations in the inward-rectifying K+ channel (KCNJ5) are a common cause of primary aldosteronism (PA) in aldosterone-producing adenoma and familial hyperaldosteronism type III, respectively. Dysregulation of adrenal cell calcium signaling represents one mechanism for mutated KCNJ5 stimulation of aldosterone synthase (CYP11B2) expression and aldosterone production. However, the mechanisms stimulating acute and chronic production of aldosterone by mutant KCNJ5 have not been fully characterized. Herein, we defined the effects of the T158A KCNJ5 mutation (KCNJ5T158A) on acute and chronic regulation of aldosterone production using an adrenal cell line with a doxycycline-inducible KCNJ5 T158A gene (HAC15-TRE-KCNJ5 T158A). Doxycycline incubation caused a time-dependent increase in KCNJ5 T158A and CYP11B2 mRNA and protein levels. Electrophysiological analyses confirm the loss of inward rectification and increased Na+ permeability in KCNJ5 T158A-expressing cells. KCNJ5 T158A expression also led to the activation of CYP11B2 transcriptional regulators, NURR1 and ATF2. Acutely, KCNJ5 T158A stimulated the expression of total and phosphorylated steriodogenic acute regulatory protein (StAR). KCNJ5 T158A expression increased the synthesis of aldosterone and the hybrid steroids 18-hydroxycortisol and 18-oxocortisol, measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). All of these stimulatory effects of KCNJ5 T158A were inhibited by the L-type Ca2+ channel blocker, verapamil. Overall, KCNJ5 T158A increases CYP11B2 expression and production of aldosterone, corticosterone and hybrid steroids by upregulating both acute and chronic regulatory events in aldosterone production, and verapamil blocks KCNJ5 T158A-mediated pathways leading to aldosterone production.

Key Words
▶ KCNJ5 mutations
▶ primary aldosteronism (PA)
▶ adrenal
▶ aldosterone
Introduction

In normal physiology, aldosterone production is mainly regulated by the renin–angiotensin–aldosterone system and plasma potassium levels. Primary aldosteronism (PA) is characterized by renin-independent aldosterone excess and represents the most common cause of secondary hypertension. The main causes of PA are aldosterone-producing adenomas (APA) and bilateral adrenal hyperplasia. In 2011, Choi and colleagues reported mutations in the selectivity filter of the inward-rectifying K+ channel KCNJ5 in 35% of APA (Choi et al. 2011). In the past few years, several other mutations localized in or near the KCNJ5 selectivity filter have been described, causing a change in permeability from only K+ ions to allow influx of Na+ ions (Choi et al. 2011, Zennaro & Jeunemaitre 2011, Akerstrom et al. 2012, 2015, Charmandari et al. 2012, Monticone et al. 2012, 2013, 2015, Mulatero et al. 2012b, Murthy et al. 2012, 2014, Scholl et al. 2012, Boulkroun et al. 2013, Scholl & Lifton 2013, Williams et al. 2014, Dutta et al. 2014, Kuppusamy et al. 2014, Hardege et al. 2015, Lenzini et al. 2015, Thiel et al. 2015). The Na+ influx triggers the activation of voltage-gated Ca2+ channels and downstream signaling pathways, which leads to elevated expression of aldosterone synthase (CYP11B2) and aldosterone production in basal and angiotensin II-treated conditions (Monticone et al. 2012, 2013, 2015, Mulatero et al. 2012b, Oki et al. 2012, Williams et al. 2014, Kuppusamy et al. 2014, Murthy et al. 2014, Hardege et al. 2015, Wang et al. 2015). The use of L-type Ca2+ channel blockers, such as nifedipine or verapamil, have been shown to block mutated KCNJ5-mediated aldosterone production in vitro (Monticone et al. 2012, 2013, Oki et al. 2012, Kuppusamy et al. 2014, Tauber et al. 2014). However, the details regarding the effects of KCNJ5T158A on signaling events downstream of intracellular Ca2+ have not been well defined.

Most in vitro studies of mutant KCNJ5 have used strategies with constitutive transgene expression in adrenocortical cells. These studies have provided useful findings regarding the effects of mutated KCNJ5 on membrane potential, CYP11B2 and NURR1 transcription and aldosterone production. Other studies have used transient methods of expression (such as electroporation), which by itself can be toxic to cells, requiring longer recovery period for cells, in addition to potentially activating stress-mediated signaling. Therefore, this strategy makes it difficult to define the acute actions of mutant KCNJ5 on adrenal cell function.

To overcome the toxic effects of transfections, we developed a doxycycline-inducible system for KCNJ5 harboring the T158A mutation (KCNJ5T158A, encoding the mutant channel Kir3.4T158A) in order to define the effect of KCNJ5T158A on acute and chronic events leading to aldosterone production.

Materials and methods

Cell culture

Human adrenocortical carcinoma (HAC15) cells were cultured as described earlier (Parmar et al. 2008, Monticone et al. 2012, 2013, Wang et al. 2012). The pLenti-CMV-rtTA3-hygro vector was used to produce a lentivirus to generate the HAC15-CMV-rtTA3 cell line expressing the reverse tetracycline-controlled transactivator 3 (rtTA3) (Campeau et al. 2009). These cells were further transduced with a lentivirus-expressing KCNJ5 cDNA harboring the T158A mutation (KCNJ5T158A) under the CMV promoter containing a Tet operator element (TO), to generate the HAC15-TRE-KCNJ5T158A cells. Incubation with doxycycline prevents rtTA3 from physically binding to the TO, thus permitting the expression of KCNJ5T158A. The HAC15-CMV-rtTA3-hygro and HAC15-TRE-KCNJ5T158A cells were grown in Dulbecco’s Modified Eagle’s/Ham’s F-12 medium (DMEM/F-12) containing 5% Cosmic calf serum (CCS, Hyclone, Logan UT), 1% ITS (Gibco) and antibiotics (penicillin–streptomycin and gentamicin). HAC15-TRE-KCNJ5T158A cells were further selected with 10μg/mL puromycin. For experiments, cells were plated at a density of 50,000 cells/well in a 48-well dish (for studies involving gene expression analyses) or at 1 million cells/well in a 6-well dish (for nuclear protein isolation) for 48h. After incubation in low-serum medium (DMEM/F-12 containing 0.1% CCS and antibiotics) for 24h, KCNJ5T158A induction was initiated with 1μg/mL doxycycline for the indicated times. For LC-MS/MS measurement of steroids, cells were incubated in doxycycline for 60h. Inhibitor studies involved treatment of cells with 10μM verapamil for 30min before and during the course of doxycycline treatment.

For electrophysiological studies, HAC15-TRE-KCNJ5T158A cells treated with doxycycline for 60h were plated on circular coverslips in 12-well plates at a density of 200,000 cells/well for 8–10h. During patching, the coverslips were rinsed in normal Tyrode’s solution before patching. Due to previous observations that endogenous Kir3.4 is non-functional in HAC15 cells (Kienitz et al. 2015), cells were transduced with lentiviruses for constitutive expression of wild-type KCNJ5 (KCNJ5WT, encoding channel Kir3.4WT) and selected with for antibiotic resistance for
blasticidin. These cells over-expressing KCNJ5WT were used for the purpose of measuring control currents.

**Viral transduction**

Cells were plated for 24 h and treated with appropriate amounts of virus in growth medium devoid of any antibiotics and containing 8 μg/mL polybrene. Cells were centrifuged at 290g for 2 h and incubated overnight to attain maximal transduction efficiency (Wu et al. 2009). Recovery was performed with the addition of 2x growth medium devoid of any antibiotics/polybrene. After an additional 48 h, cells were sub-cultured and maintained in normal growth medium with 10μg/mL puromycin.

**RNA isolation and real-time quantitative PCR (RT-qPCR)**

Gene expression analyses were performed using Taqman primer-probes (to make a total of 900 nM of each primer and 400 nM probe per reaction; Life Technologies) and Kapa Probe fast qPCR kit master mix ABI Prism (Kapa Biosystems, Boston, MA, USA). Negative controls consisted of nuclease-free water in place of cDNA. The PCR program, consisting of 40 amplification cycles, was performed following the manufacturer's recommendations. Normalization of gene expression within each sample was performed using the respective expression levels of PPIA (cyclophilin A) to calculate the ΔCt. Relative increase in KCNJ5 T158A protein levels was calculated using the 2−ΔΔCt method.

**LC-MS/MS measurements of steroids**

A mixture of 60μL aliquot of medium, internal standards and 3% isopropyl alcohol was loaded on supported liquid extraction plates (Novum, Phenomenex, Torrance, CA, USA) and subsequently eluted with 2mL of methyl-t-butyl ether. The organic phase was concentrated under nitrogen. The dried extract was reconstituted with 60μL of methanol/deionized water (1:1) and transferred to a 0.25-mL vial insert. Samples (10μL) were injected via autosampler and resolved with an Agilent 1290 binary pump HPLC on a Kinetex 50 × 2.1 mm, 3μm particle-size biphenyl column (Phenomenex) using gradient elution with 0.25-mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and 0.25 mmol/L ammonium fluoride and methanol.

**Electrophysiology**

Ion currents were recorded in whole-cell patch-clamp configuration using a MultiClamp 700B amplifier and Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes had resistances of 6–10 MΩ when filled with intracellular pipette solution and placed in extracellular solution. The external solution consisted of a simple HEPES-based Tyrode's solution (140mM NaCl, 5mM KCl, 1.8 mM CaCl₂, and 1.8 mM MgCl₂). The internal pipette solution contained 95mM K-glucuronate, 15 mM KCl, 15 mM NaCl, 0.726 mM CaCl₂, 3 mM EGTA, 2.38 mM MgCl₂, 3 mM K₂-ATP, 0.025 mM K₂-GTP, and 0.5 mM GTP γ-S (pH 7.2 adjusted with KOH). In order to confirm Na⁺ permeability of mutated KCNJ5 channels, NaCl in the external bath solution was replaced by choline chloride. Barium chloride (1mM) was used to inhibit K⁺ conductance. Data acquisition and analysis were performed using pCLAMP software (ver.10.3; Molecular Devices, Sunnyvale, CA, USA). Current amplitudes were divided by cell capacitance (Cm) and expressed as current densities (pA/pF) to normalize for variable cell sizes.

**Results**

**Doxycycline causes time-dependent induction of KCNJ5T158A**

The HAC15-TRE-KCNJ5T158A cell model was generated with doxycycline-regulated KCNJ5T158A transgene expression using lentiviral transduction. HAC15-TRE-KCNJ5T158A cells were treated with 1μg/mL doxycycline for the indicated times to induce KCNJ5T158A expression. KCNJ5T158A exhibited a time-dependent increase in mRNA (Fig. 1, Panel A) and protein (Fig. 1, Panel B). Transcript expression increased after 6h of doxycycline incubation, peaked at 36h (increasing by ~40-fold) and plateaued thereafter. Western analysis indicated an increase in KCNJ5T158A protein levels. Semi-quantitative densitometric analysis indicated that KCNJ5T158A protein increased approximately 5-fold to 10-fold between...
24 and 60 h. Electrophysiological analyses indicated that the expression of Kir3.4T158A channel by doxycycline incubation also revealed increased conductance to Na\(^+\) in addition to K\(^+\), as indicated by sensitivity to both barium and choline. By contrast, in cells constitutively expressing the Kir3.4WT channel, channel conductance was sensitive to barium but not choline (Fig. 2, Panels A–C). The Kir3.4T158A channel also showed a loss of the inward rectification property compared with the Kir3.4WT channel (Fig. 2, Panels B and C).

**KCNJ5T158A increased CYP11B2 expression and aldosterone production, which were reversed by blockade of calcium channels**

The temporal effects of doxycycline (1 μg/mL) on CYP11B2 expression and aldosterone production in HAC15-TRE-KCNJ5T158A cells were investigated. The data show that, along with increased KCNJ5T158A expression, there was a time-dependent stimulation in CYP11B2 mRNA and protein levels (Fig. 3, Panels A and B). Specifically, transcript expression peaked at 36 h and plateaued thereafter (Fig. 3, Panel A). CYP11B2 protein levels doubled every 12 h after 24 h doxycycline incubation, attaining a 5-fold increase over basal at 48 h and almost 10-fold over basal at 60 h. Treatment with the agonist angiotensin II (Ang II) served as a positive control and led to an increase in CYP11B2 mRNA (by ~60-fold over basal) and protein (by 8-fold) (Fig. 3, Panels A and B). Aldosterone levels also exhibited a time-dependent increase in response to Ang II and KCNJ5T158A expression (Fig. 3, Panel C). The stimulatory effects of Ang II were observed as early as 24 h, while the increase in KCNJ5T158A-induced aldosterone levels was detected at 48 h. At 60 h Ang II or doxycycline treatment, aldosterone levels were elevated (normalized to protein) by over 4-fold and 10-fold (compared with basal), respectively (Fig. 2, Panel D). Finally, treatment with the L-type Ca\(^{2+}\) channel blocker verapamil (1, 3, 10 and 30 μM) blocked the stimulatory effects of Kir3.4T158A- and Ang II-induced aldosterone production in a concentration-dependent manner (Fig. 3, Panel D). Although the highest inhibitory effect on aldosterone was observed using 30 μM verapamil, this dose also exhibited some cytotoxic effects. Therefore, the most efficient dose for verapamil was 10 μM, which inhibited Kir3.4T158A- and Ang II-stimulated aldosterone production by approximately 65% and 70%, respectively. Treatment with 10 μM verapamil also abrogated Kir3.4T158A- and Ang II-induced CYP11B2 mRNA and protein expression (by approximately 85% and 70%, respectively) (Fig. 3, Panels E and F).

**Effect of KCNJ5T158A on adrenal steroidogenesis**

To better define the effects of KCNJ5T158A expression on adrenal steroidogenesis, cells were incubated in doxycycline for 60 h, and LC-MS/MS was performed to analyze the steroids in the pathway leading to aldosterone and cortisol production, as well as the hybrid steroids 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF). Figure 4, Panel A provides a schematic representation of the steroidogenic pathways for the synthesis of glucocorticoids and mineralocorticoids. KCNJ5T158A expression led to
an increase in the production of aldosterone, as well as aldosterone precursors 11-deoxycorticosterone (DOC) and corticosterone, and cortisol (Fig. 4, Panel B). No changes in progesterone, 17-hydroxyprogesterone and 11-deoxycortisol were observed. In addition, KCNJ5 T158A increased the production of the hybrid steroids 18OHF and 18oxoF by over 6-fold.

Effect of KCNJ5 T158A on transcriptional events that regulate CYP11B2 expression

The transcription factors NURR1 and ATF2 are the main regulators of CYP11B2 promoter activation and are activated by intracellular calcium levels (Bassett et al. 2000, 2004a, Nogueira et al. 2009, Hattangady et al. 2012). We, therefore, investigated the effect of KCNJ5 T158A on the expression of NURR1 and the phosphorylation of ATF2. Analyses for NURR1 transcript levels indicate a time-dependent increase upon KCNJ5 T158A induction, with NURR1 mRNA peaking at 8-fold over basal at 36 h and plateauing thereafter (Fig. 5, Panel A). An increase in nuclear-localized NURR1 was also observed at 48 h of KCNJ5 T158A activation by doxycycline treatment (Fig. 5, Panel B). Stimulation of NURR1 mRNA and protein was inhibited by pretreatment of cells with verapamil (10 μM) by approximately 70% (Fig. 5, Panel B). Treatment with Ang II as a positive control similarly elevated NURR1 transcript and protein by 5-fold at 60 h of incubation. Verapamil pretreatment inhibited the Ang II-mediated effects by approximately 50%. To define the effect of KCNJ5 T158A on the activation of ATF2 (a member of the cAMP-response element-binding protein (CREB) family), nuclear protein from cells incubated with doxycycline or Ang II in the presence or absence of verapamil was assessed for phosphorylation. Both Ang II and doxycycline treatment increased the phosphorylation of ATF2 (normalized to total ATF2 levels) by ~5-fold and 4-fold, respectively (Fig. 5, Panel C). These effects were reversible by calcium channel blockade with verapamil, which inhibited doxycycline and Ang II effects by 50% and 30%, respectively. Overall, these data indicate that KCNJ5 T158A-mediated calcium dysregulation leads to the activation of transcriptional events needed for CYP11B2 transcription.

Effect of KCNJ5 T158A on acute regulatory events in aldosterone production

To further characterize the role of acute events in KCNJ5 T158A-mediated events, we investigated the effects on steroiogenic acute regulatory protein (StAR), an established acute regulator of aldosterone synthesis. The regulation of STAR transcription, as well as the post-translational modification of StAR, was determined. Figure 6, Panel A shows a representative western blot from three experiments examining phosphorylated StAR, total
StAR and GAPDH (as a protein loading control). After 24h of KCNJ5<sup>T158A</sup> transgene expression by doxycycline incubation, phospho-StAR was elevated by approximately four-fold (P<0.05) after normalization to GAPDH (Fig. 6, Panel B). A three-fold (P<0.05) increase in total STAR was observed following KCNJ5<sup>T158A</sup> expression (Fig. 6, Panel C). Pretreatment with verapamil (10 μM) inhibited KCNJ5<sup>T158A</sup>-mediated stimulatory effects on total and phospho-StAR. Initial time-response curves for total and phospho-StAR (data not shown) indicated that KCNJ5<sup>T158A</sup> increased phospho-StAR at 24 h. Increases in total STAR were observed starting 6 h post doxycycline treatment.

Discussion

Herein we defined the acute and chronic events involved in KCNJ5<sup>T158A</sup>-induced aldosterone production. We developed a cell line with a doxycycline-inducible construct allowing for conditional expression of KCNJ5<sup>T158A</sup>, a mutation that occurs both as a germline mutation in familial hyperaldosteronism type III and as a somatic mutation in APA (Oki et al. 2012, Mulatero et al. 2012b). Transient or stable constitutive expression of mutated KCNJ5 causes increases in aldosterone production and expression of CYP11B2, through cell depolarization and resultant activation of calcium-dependent kinases (Monticone et al. 2012, 2013, Oki et al. 2012, Williams et al. 2014). The specific advantage of the inducible expression of KCNJ5<sup>T158A</sup> is the ability to investigate the temporal events of molecular responses. Characterization of the cell line demonstrated time-dependent increases in KCNJ5<sup>T158A</sup> transgene mRNA and protein expression leading to increased CYP11B2 transcript and protein levels, as well as aldosterone production, while these effects were absent in control-treated wild-type HAC15 cells (Supplementary Fig. 1).
In summary, the cell line represents a model to study molecular pathophysiological changes following the initial event of KCNJ5 gene mutation.

The KCNJ5T158A mutation showed effects on the traditional steroids from both the mineralocorticoid and glucocorticoid pathways, as well as on the hybrid steroids, 18OHF and 18oxoF, which are frequently elevated in PA (Oki et al. 2012, Mulatero et al. 2012). The largest stimulatory effects were observed in 18OHF and 18oxoF, most likely mirroring increased CYP11B2 activity. Increases in aldosterone and its precursors, DOC and corticosterone, were also observed. KCNJ5T158A expression resulted in a modest increase in cortisol production, in agreement with previous studies that demonstrate a modest increase in constitutive KCNJ5T158A-mediated CYP11B1 transcript levels (Oki et al. 2012). In addition, due to increased CYP11B2 expression, cortisol was further converted into hybrid steroids, 18OHF and 18oxoF, by CYP11B2. Verapamil inhibited the KCNJ5T158A effects on aldosterone, 18OHF and 18oxoF, but not on corticosterone and DOC. Thus, stimulatory effects of KCNJ5T158A as well as the inhibitory effects of verapamil appear to be specific to CYP11B2.

From a molecular perspective, the physiological regulation of aldosterone production in response to Ang II or elevated serum potassium involves two key regulatory steps. The acute rate-limiting step includes the activation of StAR (occurring within seconds to minutes of agonist treatment). StAR regulates the transport of cholesterol from the outer to the inner mitochondrial membrane. The chronic limiting step involves CYP11B2-mediated conversion of DOC to aldosterone, which is mainly controlled on the transcriptional level. Ang II and K+ lead to the expression of NURR1, as well as the activation by phosphorylation of members of the CREB family, including ATF2 (Bassett 2004a,b, Nogueira et al. 2009, Xing et al. 2011, Hattangady et al. 2012). Both of these steps are regulated by elevated intracellular calcium and the activation of calcium–calmodulin kinases (CaMKs) (Pezzi et al. 1996, 1997). These kinases activate the CREB family and NURR1 expression, which then translocate to the nucleus and bind the CYP11B2 promoter to initiate its transcription. In vitro studies have shown that mutated KCNJ5-mediated aldosterone excess in PA involves the elevation of intracellular calcium. Additionally, our studies are the first to show increased nuclear protein levels of phosphorylated ATF2 after induction of KCNJ5T158A. These results are in agreement with recent findings in APA, which support the activation of calcium–calmodulin kinases I (CaMK I) and elevated nuclear localization of active, phosphorylated CREB (Sackmann et al. 2011).
KCNJ5 T158A increases the activation of transcription factors required for CYP11B2 transcription, including NURR1 (A and B) and ATF2 (C). Doxycycline incubation caused a time-dependent increase in NURR1 mRNA (Panel A). Pre-incubation with verapamil (10 μM) abrogates KCNJ5 T158A-mediated and Ang II-stimulated NURR1 mRNA and nuclear protein expression (Panel B). Expression of KCNJ5 T158A and Ang II treatment stimulates the phosphorylation of ATF2 (normalized to total ATF2) (Panel C). Ca2+ channel blocker, verapamil (10 μM), inhibited the stimulatory effect of KCNJ5 T158A. The western analyses for nuclear-localized NURR1 and ATF2 (Panels B and C) are representative of three independent experiments. Black and gray bars represent the absence and presence of verapamil (10 μM), respectively (Panels B and C). Results represent the mean±s.e.m. of at least five independent experiments for mRNA (Panels A and B) and three independent westerns (Panels B and C). Statistical analyses were performed using one-way ANOVA (*P<0.05 vs basal, $P<0.05$ vs corresponding treatment in the absence of verapamil).

KCNJ5 T158A activates steroidogenic acute regulatory protein (StAR) expression and post-translational modification. Cells treated with doxycycline (1 μg/mL) for 24 h, in the absence or presence of the calcium channel blocker, verapamil (10 μM), were analyzed for StAR induction by western analyses. A representative of three independent sets of protein analyses for total StAR, phosphorylated StAR and GAPDH (protein loading control) is shown in Panel A. Panels B and C represent semi-quantitative analyses by densitometry of KCNJ5 T158A effects on total StAR levels and phosphorylation of StAR, respectively. Black and gray bars represent the absence and presence of verapamil (10 μM), respectively. Statistical analyses were performed using one-way ANOVA (*P<0.05 vs basal, $P<0.05$ vs doxycycline treatment in the absence of verapamil).
StAR regulates the movement of cholesterol from the outer to the inner mitochondrial membrane. StAR activity is regulated at the level of transcription and translation, as well as by post-translational modification. The transcriptional and translational regulation of StAR controls the level of expression of the 37-kDa StAR protein, while phosphorylation of StAR is crucial for its activity (Krueger & Orme-Johnson 1983, Alberta et al. 1989, Clark et al. 1994, Estabrook & Rainey 1996, Clark & Stocco 1997). Increased intracellular calcium levels, following Ang II treatment, cause increased StAR activation in the H295R cell line (Clark et al. 1995, Olala et al. 2014, Nanba et al. 2015) as well as primary cultures of bovine adrenocortical cells (Betancourt-Calle et al. 2001, Yamazaki et al. 2006). In agreement with these studies, KCNJ5 T158A expression increased the expression and phosphorylation of StAR. Finally, maximum KCNJ5 T158A-induced activation of StAR was observed at 24h (earliest time point for StAR phosphorylation, data not shown), mirroring the fact that transcription of the transgene and translation of StAR protein following doxycycline treatment are necessary. Ang II treatment led to StAR activation as early as 30min (data not shown).

We also evaluated the effects of constitutively expressed KCNJ5 T158A in primary cultures of human adrenal fasciculata-reticularis (ZF/ZR) (Supplementary Fig. 2). While a dose-dependent increase in KCNJ5 T158A mRNA was observed, no increase in CYP11B2 mRNA or aldosterone production was observed. A positive control consisting of ZF/ZR cells treated with ACTH did yield increased CYP11B2 mRNA expression and aldosterone production. Importantly, the ZF/ZR cells were also unresponsive to Ang II or K+. These results, along with reports from other laboratories describing a ZF-like phenotype in APA harboring KCNJ5 mutations (Azizan et al. 2012), may be the cause that mutations in KCNJ5 alone in ZF cells do not sufficiently lead to cause autonomous aldosterone production. The inherent challenge herein is the ability to obtain Ang II-responsive ZG cells, as normal adrenal glands generally lack a distinct CYP11B2-expressing ZG, but rather harbor a few, scattered CYP11B2-expressing cell clusters (Nishimoto et al. 2010).

In summary, this study has defined the effects of KCNJ5 T158A on (1) acute events (including StAR phosphorylation) and (2) chronic transcriptional events (including induction and activation of ATF2 and NURR1), (3) adrenal steroidogenesis in HAC15 cells and (4) confirmed the ability of verapamil to block KCNJ5 T158A activation of aldosterone production.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0324.

Declaration of interest
We hereby declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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