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Somatic *ATP1A1*, *ATP2B3*, and *KCNJ5* Mutations in Aldosterone-Producing Adenomas

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Abstract

Aldosterone-producing adenomas (APAs) cause a sporadic form of primary aldosteronism and somatic mutations in the *KCNJ5* gene, which encodes the G-protein-activated inward rectifier K^+ channel 4, GIRK4, account for $\approx 40\%$ of APAs. Additional somatic APA mutations were identified recently in 2 other genes, *ATP1A1* and *ATP2B3*, encoding Na^+/K^+ -ATPase 1 and Ca^{2+} -ATPase 3, respectively, at a combined prevalence of 6.8%. We have screened 112 APAs for mutations in known hotspots for genetic alterations associated with primary aldosteronism. Somatic mutations in *ATP1A1*, *ATP2B3*, and *KCNJ5* were present in 6.3%, 0.9%, and 39.3% of APAs, respectively, and included 2 novel mutations (Na^+/K^+ -ATPase p.Gly99Arg and GIRK4 p.Trp126Arg). *CYP11B2* gene expression was higher in APAs harboring *ATP1A1* and *ATP2B3* mutations compared with those without these or *KCNJ5* mutations. Overexpression of Na^+/K^+ -ATPase p.Gly99Arg and GIRK4 p.Trp126Arg in HAC15 adrenal cells resulted in upregulation of *CYP11B2* gene expression and its transcriptional regulator *NR4A2*. Structural modeling of the Na^+/K^+ -ATPase showed that the Gly99Arg substitution most likely interferes with the gateway to the ion binding pocket. In vitro functional assays demonstrated that Gly99Arg displays severely impaired ATPase activity, a reduced apparent affinity for Na^+ activation of phosphorylation and K^+ inhibition of phosphorylation that indicate decreased Na^+ and K^+ binding, respectively. Moreover, whole cell patch-clamp studies established that overexpression of Na^+/K^+ -ATPase Gly99Arg causes membrane voltage depolarization. In conclusion, somatic mutations are common in APAs that result in an increase in *CYP11B2* gene expression and may account for the dysregulated aldosterone production in a subset of patients with sporadic primary aldosteronism.

Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension, with a prevalence of 5% to 15% among hypertensive patients and is characterized by the autonomous hypersecretion of aldosterone. Sporadic PA and 3 familial forms (familial hyperaldosteronism types I, II, and III) have been described.¹ Sporadic PA accounts for $>90\%$ of all cases and is caused by either an aldosterone-producing adenoma (APA), which can be surgically removed, or bilateral adrenal hyperplasia, which is treatable with mineralocorticoid receptor antagonists.

Somatic APA mutations in the *KCNJ5* gene, which encodes the G-protein-activated inward rectifier K^+ channel 4, GIRK4 (also called the inward rectifier K^+ channel, Kir3.4), were first identified by Choi et al.² Subsequently, Boulkroun et al.³ determined a 34% prevalence of *KCNJ5* mutations in a large European cohort of 380 APA. Intriguingly, the *KCNJ5* mutations were markedly more prevalent in women,³ and this predominance was confirmed by successive studies.^{4,5} To date, 5 different *KCNJ5* mutations causing sporadic PA have been identified, the majority of which are the substitutions p.Gly151Arg or p.Leu168Arg^{2,3} or, more rarely, p.Glu145Gln,⁵ p.Thr158Ala,⁶ and p.Ile157del.⁷ A common feature of all the mutations is that they are situated in or around the selectivity filter of the K^+ channel pore and in several cases have been shown to cause a loss of channel selectivity resulting in sodium entry and membrane depolarization and the opening of voltage-gated Ca^{2+} channels.^{2,7,8} The resultant increase in the intracellular Ca^{2+} concentration determines an increase in the transcription of the *CYP11B2* gene, which encodes aldosterone synthase, and in the production of aldosterone.^{4,8} Furthermore, APAs harboring *KCNJ5* mutations have been reported to display higher levels of *CYP11B2* expression compared with those with wild-type *KCNJ5*,^{4,9} although this was not confirmed in another study.¹⁰

To identify further genetic determinants of PA, Beuschlein et al¹¹ performed exome sequencing of APAs from male patients without somatic *KCNJ5* mutations. Novel mutations in *ATP1A1*, encoding the Na⁺/K⁺-ATPase α -subunit, and in *ATP2B3*, encoding the plasma membrane Ca²⁺-ATPase 3, were identified. In a European cohort of 308 APAs, the prevalence of these mutations in *ATP1A1* and *ATP2B3* was 5.2% and 1.6%, respectively. The *ATP1A1* mutations were primarily c.311T>G (p.Leu104Arg), less frequent was a c.995T>G mutation (p.Val332Gly), as well as 2 different in-frame DNA deletions that result in the same deletion at the amino acid level (p.Phe100_Leu104del). In *ATP2B3*, 3 different in-frame DNA deletions were identified that result in either p.Leu425_Val426del or p.Val426_Val427del. In all cases, the affected amino acids are highly conserved across species and between different members of the P-type ATPase family. In vitro functional characterization of the substitutions in Na⁺/K⁺-ATPase 1 demonstrated that they have a profound effect on K⁺ binding and ATPase activity and cause membrane depolarization in whole cell patch-clamp studies that in adrenal cells would lead to the opening of voltage-dependent Ca²⁺-channels as for the *KCNJ5* mutations.¹¹

In this study, we have analyzed 112 APAs, diagnosed and surgically removed in Italian referral hypertension centers, for mutations in *ATP1A1*, *ATP2B3*, and *KCNJ5* and searched for new mutations within known hotspots for genetic alterations in these genes.

Methods

An expanded Methods section is available in the online-only Data Supplement.

Patient Selection

Patients were selected as described previously.¹² PA was diagnosed in accordance with Endocrine Society guidelines.¹³ Further details are available in the online-only Data Supplement.

The protocol was approved by our local ethics committee, and all participants gave their written informed consent.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction

Adrenal tissues were homogenized in 1 mL TRI reagent for RNA extraction, and first-strand cDNA was synthesized from total RNA (5 μ g) as described.¹⁴

DNA Sequencing of *KCNJ5*, *ATP1A1*, and *ATP2B3*

Polymerase chain reaction (PCR) primers used to amplify cDNA fragments for direct sequencing of *ATP1A1* and *ATP2B3* are shown in Table S1 in the online-only Data Supplement. The validity of novel mutations was confirmed by sequencing both strands of an independently amplified PCR fragment. The present cohort constitutes 112 APAs from different centers and included 32 samples from the Beuschlein study.¹¹ A full description of these samples is included in the online-only Data Supplement. No mutations were identified in paired peripheral blood DNA samples (n=43), and mutations were absent from paired peritumoral tissue (n=23 of which 7 carried *KCNJ5* mutations in the corresponding APA).

KCNJ5 and *ATP1A1* Expression Vectors

The rat *Atp1a1* cDNA was used for this study so that endogenous human *ATP1A1* in the HAC15 cell line could be inactivated by RNA interference using the strategy described.¹¹ For clarity, throughout this article, when referring to either rat or human *ATP1A1*, the numbering used to describe mutations follows the human cDNA or amino acid numbering.

Modeling of Protein Structures

Structural models of Na⁺/K⁺-ATPase 1 (PDB code 2ZXE) were prepared using PyMOL software (www.pymol.org).

Cell Transfection and Gene Silencing

HAC15 cells were grown in culture⁴ and transfected with plasmids and siRNAs by Amaxa nucleofection using program X005.⁹ Equivalent transfection rates of rat cDNAs and silencing of HAC15 *ATP1A1* were confirmed by real-time TaqMan PCR.

TaqMan Gene Expression Assays

Gene expression levels in transfected cells and in adrenal tissues were determined by real-time PCR using TaqMan gene expression assays using *GAPDH* as the endogenous reference gene.¹⁴ Gene expression levels in APA were compared with those in corresponding surrounding tissue (peri-APA) for 7 of 8 APA with *ATP1A1* or *ATP2B3* mutations and for 11 APA without mutations in *KCNJ5*, *ATP1A1*, or *ATP2B3*.

Na⁺/K⁺-ATPase Functional Assays

COS cells were transiently transfected with rat wild-type or mutated *Atp1a1* encoding Na⁺/K⁺-ATPase p.Gly99Arg together with a siRNA to interfere specifically with the expression of the endogenous COS cell *Atp1a1*.¹¹ ATPase activity assays, Na⁺ dependence of phosphorylation by MgATP and K⁺ inhibition of phosphorylation by MgATP, were as described.¹¹

Electrophysiological Characterization of Cells Expressing Na⁺/K⁺-ATPase Gly99Arg

The full-length cDNA encoding wild-type rat *Atp1a1* and the mutated *Atp1a1* c.295G>A (p.Gly99Arg) were subcloned into the bicistronic pIRES-CD8 expression vector.¹⁵ Human embryonic kidney cells were transfected transiently with Lipofectamine, anti-CD8–labeled Dynabeads were used to identify transfected cells, and patch-clamp whole cell recordings were performed using an EPC-10 amplifier without leak subtraction.¹¹

Statistical Analyses

All data are expressed as mean±SEM for normally distributed variables and as median (25th–75th percentile) for non-normally distributed variables. The Student *t* test or the Kruskal–Wallis test was used for quantitative variables, and the χ^2 test or the Fisher exact test was applied for qualitative variables. Confidence intervals were calculated for frequency data using Wilson's method.¹⁶

Results

Prevalence of Somatic APA Mutations in ATPases

Sequencing analysis of 112 APA DNA samples collected from 5 different Italian centers demonstrated a prevalence of *ATP1A1* mutations of 6.3% (Table 1). A novel *ATP1A1* c.295G>A mutation (p.Gly99Arg) was identified in a single sample, and the *ATP1A1* c.311T>G mutation (p.Leu104Arg)¹¹ was present in 6 samples (Figure 1A and B; Table 1). The c.995T>G mutation (p.Val332Gly) and the deletion mutations (p.Phe100_Leu104del) described previously¹¹ were not detected. However, 1 APA with an *ATP2B3* mutation (c.1272-1277delGCTGGT corresponding to p.Leu425-Val426del) was identified (Figure 1C; Table 1). Together, the mutations in these 2 ATPases were present in 7.2% of APAs and were absent from the corresponding peripheral blood DNA, thereby demonstrating their somatic nature. This prevalence is in

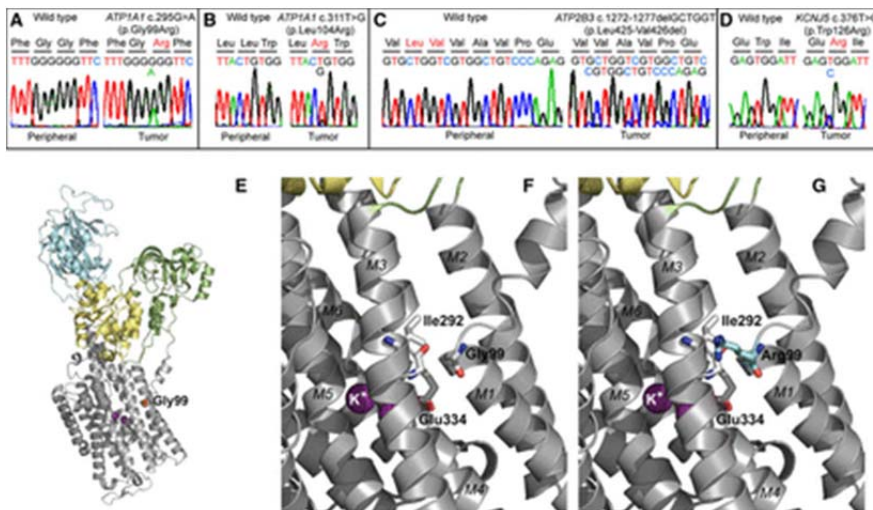
close agreement with the 6.9% frequency reported by Beuschlein et al,¹¹ but there was no correlation with increased preoperative aldosterone concentrations or with reduced serum K⁺ levels (Table S2).

Table 1. Prevalence of Somatic APA Mutations in *ATP1A1* and *ATP2B3* in Different Italian Centers

Center	No. of Samples	<i>ATP1A1</i> c.311T>G (p.Leu104Arg)	<i>ATP1A1</i> c.295G>A (p.Gly99Arg)	Total No. of <i>ATP1A1</i> Mutations (%; CI)	<i>ATP2B3</i> c.1272-1277delGCTGGT (p.Leu425-Val426del; %; CI)
Torino 1	54	4	1	5 (9.3%, 4.0–19.9)	1 (1.9%, 0.3–9.8)
Torino 2	19	0	0	0	0
Ancona	21	2	0	2 (9.5%, 2.7–28.9)	0
Padova	10	0	0	0	0
Pisa	8	0	0	0	0
Total	112	6	1	7 (6.3%, 3.1–12.3)	1 (0.9%, 0.2–4.9)

APA indicates aldosterone-producing adenoma; and CI, confidence interval.

Figure 1.



Somatic aldosterone-producing adenoma (APA) mutations in *ATP1A1*, *ATP2B3*, and *KCNJ5*. A novel *ATP1A1* c.295G>A mutation (p.Gly99Arg) was identified in the APA of 1 patient but not in the peripheral blood DNA (A); the somatic *ATP1A1* c.311T>G mutation (p.Leu104Arg)¹¹ was identified in 6 APAs (B); a single APA sample carried an *ATP2B3* c.1272-1277delGCTGGT (p.Leu425-Val426del) mutation that was absent from peripheral DNA (C); a novel *KCNJ5* c.376T>C mutation (p.Trp126Arg) was identified in the APA of 1 patient but not in the patient's peripheral DNA (D); structural overview of Na⁺/K⁺-ATPase (PDB code 2ZXE). The backbone carbons of Gly99 in transmembrane helix M1 are highlighted in orange. The A-domain, N-domain, P-domain, and transmembrane regions are shown in green, cyan, yellow, and grey, respectively. Violet spheres indicate K⁺ ions (E); close up of the relevant region in E shows residue Gly99 in close proximity to Ile292 in M3 and Glu334 in M4. The Gly99-Ile292 pair functions as a pivot for the movement of M1 leading to opening of the gate at the entrance to the cation binding pocket, whereas Glu334 is part of the gate¹⁶ (F); introduction of a large, positively charged arginine residue in the position of Gly99 is likely to cause a steric clash with an impact on the surrounding residues, including Glu334 that binds K⁺ (G).

Novel Somatic APA Mutation in *ATP1A1*

The novel APA mutation in *ATP1A1* was identified in the Torino 1 group of samples, *ATP1A1* c.295G>A (p.Gly99Arg). The patient harboring the p.Gly99Arg mutation displayed a particularly severe form of PA with very low serum potassium (1.4 mEq/L), and the phenotype of this patient is described in the online-only Data Supplement.

Novel Somatic APA Mutation in *KCNJ5*

Sequence analysis of *KCNJ5* demonstrated the presence of 4 different somatic APA mutations in this sample set: the frequently reported c.451G>A/C (p.Gly151Arg) and c.503T>G (p.Leu168Arg) substitutions,^{2,3} c.472A>G (p.Thr158Ala)⁶ and a novel c.376T>C (p.Trp126Arg) mutation that affects a highly conserved residue (Figure 1D; Table 2; and Figure S1). In agreement with other reports,^{3,5} the p.Gly151Arg substitution was identified more frequently than p.Leu168Arg (31.5% versus 9.0%; $P<0.02$). A wide variation in prevalence was observed between different centers (20.0%–47.6%), but the overall prevalence was 39.3%. There was no correlation with adenoma size, age at diagnosis, or preoperative aldosterone and K^+ levels (Table S2). The phenotype of the patient harboring the p.Trp126Arg mutation is described in the online-only Data Supplement.

Table 2. Prevalence of Somatic APA Mutations in *KCNJ5* in Different Italian Centers

Center	No. of Samples	c.451G>A/C (p.Gly151Arg)	c.503T>G (p.Leu168Arg)	c.472A>G (p.Thr158Ala)	c.376T>C (p.Trp126Arg)	No. of Mutations (%, CI)
Torino 1	54	16	5	1	0	22 (40.7%, 28.7–54.0)
Torino 2	19	3	3	0	1	7 (36.8%, 19.2–59.0)
Ancona	21	9	1	0	0	10 (47.6%, 28.3–67.6)
Padova	10	2	0	0	0	2 (20%, 5.7–51.0)
Pisa	8	2	1	0	0	3 (37.5%, 13.7–59.4)
Total	112	35	10	1	1	44 (39.3%, 30.7–48.5)

APA indicates aldosterone-producing adenoma; and CI, confidence interval.

Prevalence of APA Mutations According to Sex

In this study, the ATPase mutations were distributed equally between men and women ($P=0.5$), rather than predominantly in men.¹¹ In accordance with previous reports,^{3–5} the *KCNJ5* mutations were more prevalent in women than in men (50.0% APA women carried *KCNJ5* mutations versus 29.3% men; $P=0.02$; Table 3).

Table 3. Prevalence of APA Mutations According to Sex

Gene	Mutations in Women/Total Women (%, CI)	Mutations in Men/Total Men (%, CI)
<i>KCNJ5</i>	27/54 (50.0%, 37.1–62.9)	17/58 (29.3%, 19.2–42.0)
<i>ATP1A1+ATP2B3</i>	4/54 (7.4%, 2.9–17.6)	4/58 (6.9%, 2.7–16.4)

APA indicates aldosterone-producing adenoma; and CI, confidence interval.

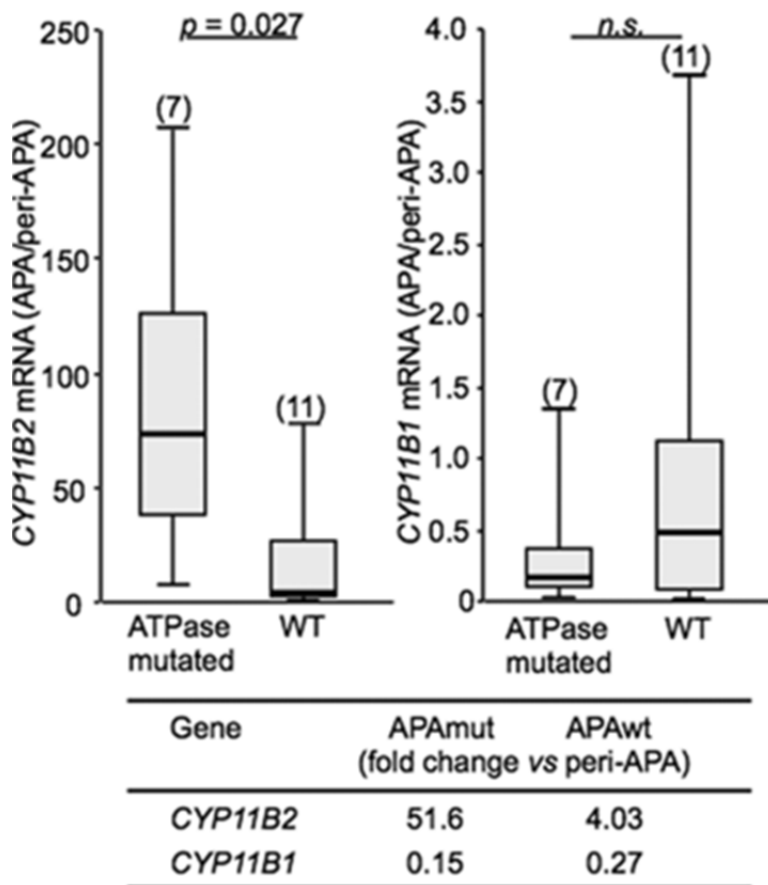
Structural Effects of the Na^+/K^+ -ATPase Gly99Arg Mutation

Gly99 resides in the transmembrane domain M1 (Figure 1E and 1F) in close proximity to residues Ile292 and Glu334 in domains M3 and M4, respectively (Figure 1F). The Gly99-Ile292 pair functions as a pivot for the movement of M1 leading to the opening of the gate at the entrance to the cation binding pocket, whereas Glu334 is part of the gate¹⁷ (Figure 1F). Introduction of the large positively charged arginine side-chain is likely to cause a severe structural alteration affecting surrounding residues including Glu334 that binds K^+ (Figure 1G).

APAs Carrying *ATP1A1* and *ATP2B3* Mutations Display Increased *CYP11B2* Gene Expression

Analysis of gene expression levels by TaqMan real-time PCR demonstrated that the expression of *CYP11B2* in APAs with ATPase mutations (in either *ATP1A1* or *ATP2B3*) compared with corresponding peri-APA tissue was significantly higher than that in APAs without mutations in either *KCNJ5*, *ATP1A1*, or *ATP2B3* (51.6- versus 4.03-fold; $P < 0.027$). In contrast, there was no significant difference in *CYP11B1* gene expression (Figure 2).

Figure 2.



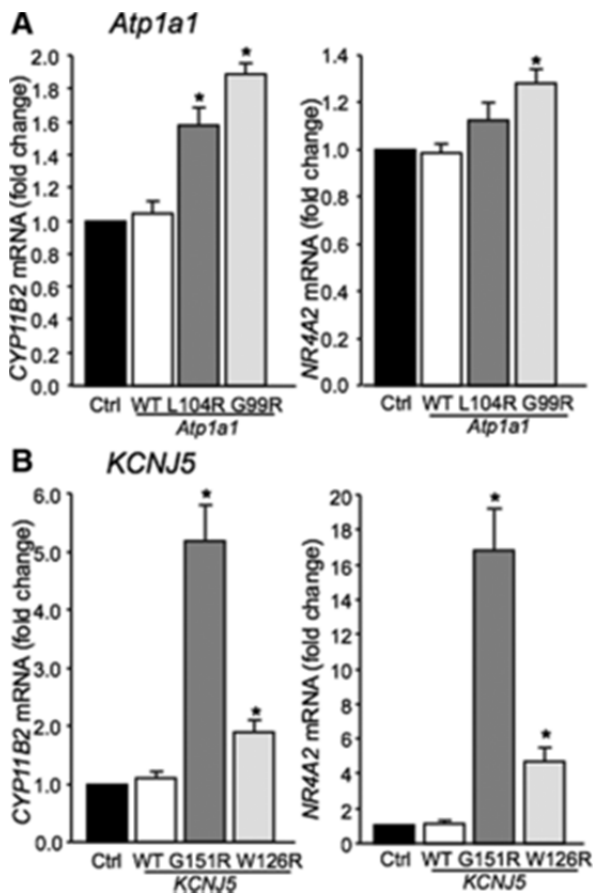
CYP11B2 and *CYP11B1* gene expression in aldosterone-producing adenomas (APAs) harboring *ATP1A1* and *ATP2B3* mutations. TaqMan assays were used in real-time polymerase chain reaction to determine fold changes ($2^{-\Delta\Delta Ct}$ quantification method) in *CYP11B2* (left) and *CYP11B1* (right) gene expression in APAs compared with corresponding surrounding tissue (peri-APA) in APAs with *ATP1A1* or *ATP2B3* APA mutations (ATPase mutated) and in APA samples without mutations in *ATP1A1*, *ATP2B3*, or *KCNJ5* (WT, wild-type). *GAPDH* was used as the endogenous reference gene. In the box plots, the horizontal line represents the median, and the box and bar indicate the 25th to 75th and 5th to 95th percentiles, respectively. The numbers in parentheses above the boxes indicate the number of paired APA-peri-APA samples. Statistical significance between groups was calculated for non-normally distributed data using the Mann-Whitney *U* test. The overall differences in gene expression (fold changes) in APAs compared with the corresponding paired peri-APA tissue are shown in the table below the box plots where APAmut, APAs with *ATP1A1* or *ATP2B3* mutations; APAwT, APAs without *ATP1A1*, *ATP2B3*, or *KCNJ5* mutations; and n.s., not significant.

p.Gly99Arg and p.Leu104Arg Mutations in Na^+/K^+ -ATPase 1 Increase *CYP11B2* Expression In Vitro

Expression of rat *Atp1a1*.c.311T>G and c.295G>A (p.Leu104Arg and p.Gly99Arg, respectively) in HAC15 adrenal cells, in which endogenous *ATP1A1* had been silenced, resulted in 1.58 ± 0.10 -fold and 1.89 ± 0.07 -fold increases in *CYP11B2* gene expression, respectively, compared with control cells transfected with empty vector and a control siRNA ($P < 0.01$; Figure 3A). Expression of the p.Gly99Arg mutation also resulted

in a 1.28 ± 0.06 -fold increase ($P < 0.01$) in the expression of *NR4A2*, a transcriptional regulator of *CYP11B2*¹⁸; in contrast, p.Leu104Arg expression had no statistically significant effect on *NR4A2* (Figure 3A).

Figure 3.



CYP11B2 and *NR4A2* gene expression in HAC15 cells expressing *ATP1A1* or *KCNJ5* mutations. Cells were transfected by Amaxa nucleofection, and total RNA was extracted 48 hours post-transfection after an overnight incubation in serum-free medium. After reverse transcription, gene expression levels were determined by TaqMan real-time polymerase chain reaction using *GAPDH* as the endogenous reference gene. In *Atp1a1* experiments, cells were cotransfected with the empty expression vector and a control (Ctrl) siRNA or with rat wild-type (WT) *Atp1a1* or rat *Atp1a1* cDNA encoding either the p.Leu104Arg (L104R) or the novel p.Gly99Arg (G99R) mutation together with a siRNA to specifically interfere with the expression of the endogenous human *ATP1A1*,¹¹ and the expression of *CYP11B2* (left) and *NR4A2* (right) was determined (A); in *KCNJ5* experiments, cells were transfected with the empty expression vector (Ctrl) or with human wild-type *KCNJ5* (WT) or *KCNJ5* cDNA encoding either the p.Gly151Arg (G151R) or the novel p.Trp126Arg (W126R) mutation, and the expression of *CYP11B2* (left) and *NR4A2* (right) was determined (B). Values are the mean \pm SEM of 6 independent experiments. * $P < 0.01$ vs both Ctrl and WT.

GIRK4 p.Trp126Arg Mutation Increases *CYP11B2* and *NR4A2* Expression In Vitro

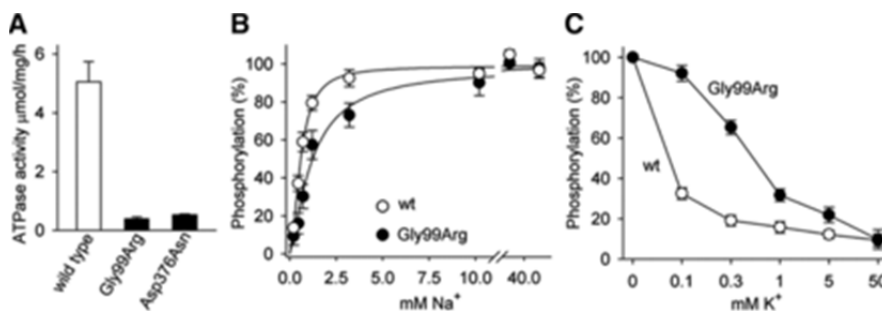
Expression of GIRK4 p.Trp126Arg (*KCNJ5* c.376T>C) in HAC15 cells resulted in a 1.90 ± 0.18 -fold increase in the expression of *CYP11B2* compared with control cells and a 4.70 ± 0.73 -fold increase in the expression of *NR4A2* ($P < 0.01$; Figure 3B). Expression of the most frequent GIRK4 mutation in this study, Gly151Arg, resulted in 5.19 ± 0.61 - and 16.78 ± 2.39 -fold increases ($P < 0.01$) in *CYP11B2* and *NR4A2* gene expression, respectively (Figure 3B).

Na⁺/K⁺-ATPase Gly99Arg Mutation Displays Altered Functional Activity

The maximal Na⁺/K⁺-ATPase activity of Gly99Arg was compared with both the wild-type and a nonphosphorylatable inactive mutant (Asp376Asn). The ATPase activity of Gly99Arg was impaired severely and displayed levels of activity comparable with those of the inactive mutant, which represent background

detection (Figure 4A). In addition, the Gly99Arg mutant displayed a reduced affinity for Na^+ activation of phosphorylation by MgATP with a half maximal activation at 1.19 mmol/L Na^+ compared with 0.58 mmol/L for the wild-type Na^+/K^+ -ATPase (Figure 4B). In the Na^+/K^+ exchange mechanism, the ATPase is dephosphorylated after K^+ binding. Therefore, ATPase phosphorylation is inhibited by K^+ , which in vitro can be used for measuring K^+ affinity. Accordingly, the K^+ inhibition of Gly99Arg phosphorylation by MgATP was compared with that of wild-type Na^+/K^+ -ATPase. Gly99Arg was markedly less sensitive to inhibition of phosphorylation by K^+ with half maximal inhibition occurring at 0.62 mmol/L K^+ compared with 0.074 mmol/L K^+ of the wild-type ATPase (Figure 4C).

Figure 4.

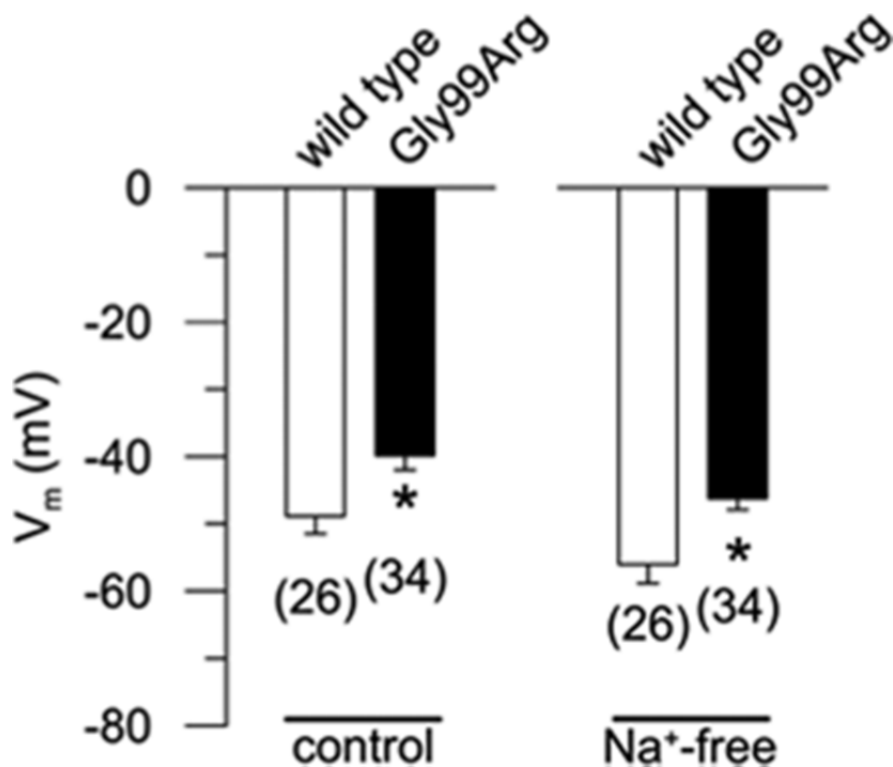


Functional characterization of Na^+/K^+ -ATPase Gly99Arg. Maximal Na^+/K^+ -ATPase activity of the wild-type (WT) and the mutants Gly99Arg and Asp376Asn. Mutation of Asn376 prevents phosphorylation and indicates background activity. Columns represent the mean of 3 to 7 independent experiments. Error bars indicate SEM (A). Na^+ dependence of phosphorylation by MgATP. Each value corresponds to the mean of 4 to 7 independent experiments. Error bars indicate SEM (B); K^+ inhibition of phosphorylation by MgATP. Each value corresponds to the mean of 5 to 12 independent experiments. Error bars indicate SEM (C).

Na^+/K^+ -ATPase Gly99Arg Mutation Causes Membrane Voltage Depolarization

The effect of the Na^+/K^+ -ATPase Gly99Arg mutation on the membrane voltage of transfected human embryonic kidney cells was measured by whole cell patch-clamp. The membrane voltage was depolarized in cells expressing the Gly99Arg mutation compared with those expressing the wild-type ATPase (Figure 5). The difference in membrane voltage was also observed when Na^+ was removed from the bath indicating that the depolarization was not caused by an increased Na^+ conductance in transfected cells, but rather by a disturbed ionic gradient and/or loss of net charge transport in cells expressing p.Gly99Arg.

Figure 5.



Electrophysiological characterization of human embryonic kidney (HEK) cells expressing Na⁺/K⁺-ATPase Gly99Arg. Membrane voltages of HEK cells expressing the Na⁺/K⁺-ATPase Gly99Arg mutant were depolarized compared with cells expressing the WT Na⁺/K⁺-ATPase under control conditions (left). After removal of bath Na⁺, the difference of the membrane voltage was preserved suggesting that the depolarization was caused by disturbed intracellular ion composition and/or loss of net charge transport by the mutant pump (right). Numbers of cells are shown in parentheses, * $P < 0.05$ vs WT.

Discussion

Mutations in the *KCNJ5* gene, which encodes the GIRK4 K⁺ channel, have been identified that cause both sporadic and familial forms of PA.¹⁻³ The mutations are located around the selectivity filter of the K⁺ channel and cause a loss of ion selectivity and cell depolarization that in adrenal glomerulosa cells result in the opening of voltage-gated Ca²⁺ channels.^{2,6,8} The Ca²⁺ influx stimulates both *CYP11B2* and *NR4A2* (a transcriptional regulator of *CYP11B2*) gene expression, and subsequently aldosterone secretion is increased.^{4,8} In this study, we report a novel somatic mutation in *KCNJ5* (GIRK4 p.Trp126Arg), and, therefore, a total of 6 different somatic *KCNJ5* mutations have been identified to date with the most prevalent being p.Gly151Arg and p.Leu168Arg. The p.Trp126Arg mutation affects a tryptophan residue that is conserved across species and in close proximity to residues Pro128 and Cys129 that form hydrogen bonds with Thr158,² a residue mutated to an Ala in both familial hyperaldosteronism type III² and in sporadic APA.⁶ We show that overexpression of GIRK4 p.Trp126Arg in adrenal cells results in an increase in *CYP11B2* and *NR4A2* gene expression as described for other GIRK4 mutations.^{4,8}

Beuschlein et al¹¹ recently reported several newly described somatic APA mutations in 2 different genes, *ATP1A1* and *ATP2B3*, that encode Na⁺/K⁺-ATPase 1 and Ca²⁺-ATPase 3, members of the P-type family of ATPases. In their sample set of 308 APAs, a combined prevalence of 6.8% *ATP1A1* and *ATP2B3* mutations and 38.3% *KCNJ5* mutations was reported. The data from the present study are in close agreement with these observations: in our 112 tumor samples, which included 32 samples from the Beuschlein study,¹¹ we describe a prevalence of 7.2% *ATP1A1* and *ATP2B3* mutations and 39.3% *KCNJ5* mutations. In both studies,

concomitant mutations within the same tumor were not observed. However, we found that the ATPase mutations were distributed equally between men and women, although our sample size of 8 APAs with ATPase mutations is smaller than that of the previous study,¹¹ which included 21 ATPase mutations.

Furthermore, we did not find a correlation of *KCNJ5* or *ATP1A1/ATP2B3* mutational status with clinical characteristics related to adenoma size or disease severity (higher preoperative aldosterone levels and lower serum potassium levels) that has been observed in some studies^{11,19} but not in others.^{2,3,5,20} Differences in clinical and biochemical characteristics of patients with or without somatic APA mutations, as well as the prevalence of mutations in different cohorts, could be determined by ethnicity and by patient selection and diagnostic strategies.

For each ATP hydrolyzed, the Na⁺/K⁺-ATPase couples the extrusion and uptake of 3 cytoplasmic Na⁺ for 2 extracellular K⁺ ions across the plasma membranes of most higher eukaryotes.^{21,22} This ion transport pathway comprises a channel on each side of the lipid bilayer that access the ion binding pocket.²² The Na⁺/K⁺-ATPase Leu104 residue is located in transmembrane domain M1 and positions Glu334 that functions in the binding and occlusion of potassium ions.²³ The p.Leu104Arg substitution identified in APAs causes a severe disturbance of K⁺ binding and ATPase activity resulting in depolarization of the cell membrane.¹¹ In glomerulosa cells, the inactivation of the Na⁺/K⁺-ATPase α -subunit by the inhibitor ouabain has been reported to cause a nifedipine-sensitive elevation in the cytoplasmic Ca²⁺ concentration and a stimulation of aldosterone production.^{24,25} The novel Na⁺/K⁺-ATPase p.Gly99Arg mutation identified in this study is located in transmembrane α -helix M1, intriguingly in the same region as the p.Leu104Arg mutation, in close proximity to residues Ile292 and Glu334, which function in gating entry to the cation binding pocket. The p.Gly99Arg mutation is likely to cause a steric clash with an impact on surrounding residues, including Glu334 that binds K⁺. Consistently, we show that the p.Gly99Arg mutant displayed minimal ATPase activity, reduced Na⁺ affinity, and markedly reduced K⁺ affinity when compared with the wild-type ATPase as observed in phosphorylation studies. These results are in agreement with a disturbed gating mechanism, in particular, affecting K⁺ binding to the mutated pump. Adrenal zona glomerulosa cells display a distinctive high resting K⁺ conductance that maintains the high negative membrane potential (-80 mV) of these cells.²⁶ We demonstrate by whole cell patch-clamp studies that the p.Gly99Arg mutation results in the depolarization of the membrane voltage. In adrenal glomerulosa cells, this depolarization results in the opening of voltage-gated Ca²⁺ channels.^{2,8} In accordance with an increase in Ca²⁺ influx, we show that the overexpression of p.Gly99Arg or p.Leu104Arg in adrenal cells results in an increase in *CYP11B2* gene expression and also, for the p.Gly99Arg mutation, *NR4A2*. Finally, the expression of *CYP11B2* in APAs harboring ATPase mutations was significantly higher compared with those without these or *GIRK4* mutations; an effect of heterozygous ATPase mutations on glomerulosa cells is consistent with the unique sensitivity of these cells to small changes in intracellular K⁺ and membrane potential.^{26,27} Taken together, these observations are in agreement with a pathophysiological link between the presence of these mutations and the dysregulated aldosterone secretion in patients with APA.

Perspectives

Genetic alterations that lead to cell depolarization and increased transcription of the *CYP11B2* gene in adrenal cells account for a large proportion of APAs (47%). The majority of these mutations affect the *GIRK4* K⁺ channel, whereas a far smaller number alter Na⁺/K⁺-ATPase 1 and Ca²⁺-ATPase 3. In general, the mutations cluster around hotspot regions and in this study we report a rare Na⁺/K⁺-ATPase p.Gly99Arg mutation that affects the same protein domain as the more frequent p.Leu104Arg mutation involved in gating of the ion transport pathway. The understanding of new molecular pathways responsible for the dysregulation of aldosterone production may lead to the development of novel therapeutic targets aimed at treating the most common form of secondary hypertension.

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