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Regulation of Aldosterone Biosynthesis by the Kir3.4 (KCNJ5) Potassium Channel

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Summary

1. The G-protein-activated inwardly rectifying potassium channel Kir3.4 is expressed in the zona glomerulosa cell membrane and transports potassium out of the cell.
2. Angiotensin II stimulation of aldosterone secretion is mediated in part by suppression of the transcription of *KCNJ5*, the gene coding for Kir3.4, and blocking channel activity. This results in membrane depolarization, mobilization of intracellular calcium, activation of the calcium-calmodulin pathway, and increasing gene transcription of steroidogenic enzymes required for aldosterone secretion.
3. In 40–60% of aldosterone-producing adenomas there is a somatic mutation in the region of the *KCNJ5* gene that codes for the selectivity filter that decreases potassium selectivity, allowing sodium to leak into the cells, thus depolarizing the membrane and initiating events that result in increased aldosterone synthesis.
4. The mechanism by which mutated KCNJ5 induces cell proliferation and adenoma formation remains unclear.

Introduction

Primary aldosteronism, the most common form of secondary hypertension, is the cause of high blood pressure in approximately 5–10% of unselected hypertensive patients.[1] About half of these have an aldosterone-producing adenoma (APA); the others suffer adrenal zona glomerulosa hyperplasia and hyperfunction of unknown origin, also known as idiopathic hyperaldosteronism. Recent significant advances in exome sequencing has added to our understanding of somatic gene mutations in APA and have uncovered several mutations in the selectivity filter of the G protein-activated inward rectifying potassium channel Kir3.4 (also called KCNJ5 or GIRK4) coded by the *KCNJ5* gene. Together, these mutations have been found in 40–60% of APA.[2–7] The selectivity filter is the region of the channel pore that allows the specific transport of potassium and exclusion of other cations. Mutations within the filter that allow sodium to enter the zona glomerulosa cell depolarize the membrane, resulting in calcium mobilization and activation of the calcium signal cascade, ultimately increasing aldosterone synthesis and cell proliferation.[2] The predominant KCNJ5 mutations

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are G151R and R168L. Germinal mutations of KCNJ5 have also been found in rare families with familial hyperaldosteronism type 3.[2, 8–10] More recently, other less common somatic mutations of the sodium/potassium ATPase gene *ATP1A1* and the calcium ATPase gene *ATP2B3* have been discovered in APA with normal KCNJ5 genes.[11]

Potassium Channels in the adrenal

Intracellular recordings from adrenocortical tissues from multiple species have shown that adrenal cells maintain negative resting potentials determined primarily by plasma membrane permeability to K^+.[12–14] Adrenal zona glomerulosa (ZG) cells are normally hyperpolarized by a predominant potassium conductance mediated by the 'leak' K^+ channels of the 2-pore domain /4 transmembrane family, TASK1 (*KCNK3*), TASK3 (*KCNK9*) and TREK1 (*KCNK2*), the expression of which varies depending on the animal species.[14–20] In rodents and bovines 'leak' channels TASK1, TASK3 and TREK1 appear to set the resting potential;[14, 16, 21, 22] in humans it appears to be the TASK1.[15] In ZG cells, membrane polarity is also controlled by Kir (K^+ inwardly rectifying) channels some of which are members of the G protein-activated inwardly rectifying potassium channel family. Four Kir3 channel subunits coded by the *KCNJ* genes have been identified in mammals. Kir proteins have two putative membrane-spanning domains (Fig 1)[23] and form a tetrameric complex linked by an extracellular pore-forming region and cytoplasmic amino and carboxy terminal domains.[24] Kir3.4, coded by *KCNJ5*, can form homo-tetramers or more commonly, hetero-tetramers with Kir3.1 (*KCNJ3*), Kir3.2 (*KCNJ6*) or Kir3.3 (*KCNJ9*)[24] and the combination of Kir3 subunits in each channel varies among tissues and cell types.[25] Kir 3.4 is expressed in the zona glomerulosa[2] and Kir3.1 is expressed throughout the adrenal including the zona glomerulosa (unpublished). Kir channels hyperpolarize the membrane of excitable cells such as cardiac myocytes and neurons [24]. Stimulation of thyrotrophic cells by thyrotropin-releasing hormone causes the vesicles that contain the potassium channel subunit Kir3.4 co-localized with Kir3.1subunit to fuse with the plasma membrane, resulting in an increase in the expression of these subunits in the plasma membrane, thus enhancing potassium currents when the cells are stimulated by dopamine or somatostatin [26] and suppressing TSH release. Dopamine inhibits prolactin release in the pituitary lactotropes by increasing the activity of G-protein activated K^+ channels containing Kir3.4 and Kir3.1 by hyperpolarizing the membrane.[27] Dopamine also inhibits aldosterone release,[28] suggesting that activation of Kir3.4/Kir3.1 heterotetramer in the adrenal zona glomerulosa may inhibit aldosterone production by contributing to the hyperpolarization of the membrane. KCNJ potassium channel selectivity for potassium is conferred by a GYG motif at the narrowest part of the pore[29] and mutations of this region decrease the selectivity of the channel for potassium and have been associated with APA.[2–5, 7, 8, 30]

Role of KCNJ5 in adrenal zona glomerulosa function

KCNJ5 is expressed in the zona glomerulosa of the adrenal.[2] The role of KCNJ5 in normal physiology of aldosterone regulation has been studied using the adrenal carcinoma cell line HAC15.[31] Angiotensin II stimulation of the human adrenal carcinoma cell line 15 (HAC15) results in down-regulation of KCNJ5 expression at the mRNA (−41.2%) and protein level (−52.7%) and a decrease in membrane potential, leading to the increase in the transcription of enzymes required for adrenal steroid synthesis. The calcium ionophore A23187 produced a similar effect on KCNJ5 expression, suggesting that the effect of angiotensin II on KCNJ5 expression is mediated by intracellular calcium mobilization. Naringin activates the Kir3.1–3.4 heterotetrameric channel by binding the tyrosines 148 and 150 of the M1–M2 loop[32] and partially abrogates the effect of angiotensin II on membrane voltage, expression of steroidogenic enzymes, and aldosterone synthesis, further evidence that angiotensin II-induced aldosterone production is mediated in part by Kir3.4.[31] Transduction of HAC15 cells with a lentivirus to overexpress KCNJ5 suppressed mRNA expression for StAR,

HSD3B2, CYP11B1 and CYP11B2 and blunted the angiotensin II-induced transcriptional activation of the CYP11B2 promoter, as well as basal and angiotensin II-induced aldosterone and cortisol secretion (Fig 2, top panel), membrane voltage, and intracellular Ca^{2+} concentrations. Downregulation of KCNJ5 using a shRNA-KCNJ5 lentivirus decreased the expression of the Kir3.4 protein, but did not alter membrane voltage, intracellular Ca^{2+} concentration or aldosterone biosynthesis.[31] This suggests that the Kir3.4 channels transfer potassium from inside to outside of the HAC15 cells continuously and that interruption by angiotensin II results in membrane depolarization, mobilization of calcium, and activation of signals resulting in transcription of the CYP11B2, thus increasing aldosterone production (Fig 2,3). Naringin blocks the effect of angiotensin II by activating the Kir3.4 potassium channel, increasing the transfer of potassium ions from the inside to outside and repolarizing the membrane. Overexpression of Kir3.4 maintains or increases the hyperpolarized state (Fig 3) and inhibits aldosterone secretion consistent with reports that activation of the Kir3.4 potassium channel in pituitary lactotropes by dopamine leads to hyperpolarization of the membrane and suppression of prolactin secretion.[27]

Aldosterone-producing adenomas and KCNJ5 mutations

Pioneering studies of aldosterone-producing adenomas using exome sequencing by the Lifton group [2] found two recurring mutations around the GYG motif of the *KCNJ5* gene in 8 of 22 patients studied. While the channel is normally very selective for potassium, the G151R and L168R mutations result in a decrease in the selectivity, allowing the passage of sodium. An inherited mutation of amino acid T158A was also found in a family presenting with severe hyperaldosteronism and bilateral adrenal hyperplasia which required treatment with bilateral adrenalectomy.[2, 33] Expression in HEK293 of the mutated KCNJ5 (G151R, L168R or T158A) with KCNJ3 (required to form the hetero-tetramer channel cells) was shown to result in loss of channel selectivity and membrane depolarization. Expression of the mutated KCNJ5 homo-tetramers also resulted in a similar loss of channel selectivity and membrane depolarization.[2] It was postulated that mutations of the selectivity filter that decrease selectivity for potassium and allow sodium to leak into the cells, would depolarize the membrane, increase calcium mobilization, thus stimulate aldosterone secretion and proliferation[2] (Fig 3).

Transduction of KCNJ5-T158A into HAC15 cells

The effects of the mutations on channel activity reported by Choi *et al*[2] were studied using transfection of the non-steroidogenic cell HEK293. We recently reported our studies of the T158A mutation of KCNJ5 on the regulation of aldosterone biosynthesis in HAC15.[34] Transducing the cell line HAC15 with the lentivirus KCNJ5 T158A resulted in a 5.3 fold increase in basal aldosterone secretion over cells transduced with empty lentivirus (Fig 2, bottom panel) and enhanced the stimulatory effect of angiotensin II and forskolin (an adenyl cyclase activator) on aldosterone synthesis. Forskolin-induced aldosterone secretion in the KCNJ5 T158A cells was greater than that by angiotensin II (Fig 1, bottom panel). Cortisol secretion was also increased, but to a significantly lesser extent than aldosterone. Synthesis of 18-oxocortisol was also increased in transduced HAC15 cells (Fig 1, bottom panel). Patients with APA with and without a KCNJ5 mutation generally have a marked increase in 18-oxocortisol, as well as aldosterone secretion in comparison to normal individuals and patients with idiopathic hyperaldosteronism.[35] In one family with familial hyperaldosteronism type 3 with the KCNJ5-T158A mutation the excretion of 18-oxocortisol was found to be very high and not suppressed by dexamethasone.[33] Levels of 18-oxocortisol have not yet been reported in other recently described patients with familial hyperaldosteronism type 3.[8–10]

All mutations of the *KCNJ5* gene associated with APA discovered so far are located either in the selectivity filter or adjacent to the channel pore. The G151R or G151E affect the first glycine of the GYG motif, a constant feature of most K^+ channels[36]. Other mutations, T158A, del157 and I157S, probably work by affecting the separation or charge between R155 and E159 that form crucial salt bridges around the pore. The L168R is the most distal mutation discovered to date and may also disrupt the local salt bridge by introducing a positive charge.[37, 38] The effect of the KCNJ5 T158A mutation on sodium influx, measured using the cell-impermeant dye CoroNa Green, showed that KCNJ5-T158A cells had a 1.2 fold higher fluorescence for sodium than control cells.[34] KCNJ5-T158A cells loaded with an indicator of plasma membrane voltage $DiSBAC_2$ also showed higher plasma membrane voltage than in control cells. KCNJ5-T158A cells loaded with the intracellular calcium indicator Fluo-4 AM showed a 1.6-fold increase in comparison to control cells.[34] These results suggest that the KCNJ5-T158A mutation induced an increase in Na^+ influx, membrane voltage and intracellular Ca^{2+} accumulation (Fig 2). Similar results of stimulation of aldosterone biosynthesis were reported with transfections of the HAC15 cell with a mutated cDNA KCNJ5-G151R and KCNJ5-L168R.[7] Microarray studies indicated that transfection of the mutated KCNJ5 altered the expression of 36 genes by more than 2.5-fold.[7]

Transduction of the HAC15 cell with the KCNJ5-T158A mutant lentivirus resulted in a 17.7-fold increase in the mRNA expression of the CYP11B2 and a 5.8-fold for the CYP11B1 enzyme mRNA. The CYP17α mRNA decreased in cells transduced with the KCNJ5-T158A lentivirus, probably explaining why the increase in cortisol secretion by these cells was much less than that of aldosterone (Fig 2, lower panel). Inhibition of L-calcium channels with nifedipine or the calmodulin inhibitor W-7 decreased aldosterone production by 75% and 18%, respectively, indicating the role of intracellular Ca^{2+} in the regulation of aldosterone biosynthesis.

When mutations of the selectivity filter of the KCNJ5 channel were described, it was postulated that the membrane depolarization and mobilization of intracellular calcium resulted in cellular proliferation responsible for formation of the adenoma, as well as increased aldosterone secretion. However transduction of HAC15 with the KCNJ5-T158A lentivirus decreased proliferation as measured by three different methods without affecting apoptosis.[34] Transfection of the HEK293 cell line with a plasmid with the KCNJ5-G151R mutation and an eGFP marker showed that there were significantly less cells at 24 and 36 hrs after transfection expressing the eGFP that those transfected with the wild type KCNJ5. Transfection with KCNJ5-G151E, another mutation found in some families with hyperaldosteronism, produced even fewer marked cells.[9] Patients with familial hyperaldosteronism type 3 with the KCNJ5-G151R mutation exhibit adrenal hyperplasia while patients with KCNJ5-G151E mutations do not.[9] This suggests that calcium toxicity of adrenal cells in the KCNJ5-G151E is strong and only a small subset of cells proliferate to produce mild hyperaldosteronism. How the KCNJ5-G151R mutation is associated with adenoma formation when transductions of this clone in HAC15 cells decreases proliferation is not clear, however a potential explanation is suggested by the studies by Williams *et al*,[39] who demonstrated that a calcium sensor gene visinin-like 1 (VSNL1) is upregulated in APAs, particularly those harboring a KCNJ5 mutation. Transfection of VSNL1 cDNA increases CYP11B2 expression under basal and angiotensin II stimulation conditions. Silencing of VSNL1 with a siRNA increases apoptosis to ionomycin or when cells are also transfected with a mutated KCNJ5 plasmid.[39] Thus VSNL1 appears to exert a protective effect on mutated KCNJ5-induced apoptosis. This suggest that some mutations of the selectivity filter of the KCNJ5 channel result in calcium toxicity and inhibition of cell proliferation, but in adenomas and maybe in familial hyperaldosteronism type 3, the expression of VSNL1 and maybe other genes mitigate against a toxic effect of calcium,

resulting in increased proliferation and the development of an adenoma. Proof of this potential mechanism is lacking.

APA with KCNJ5 mutations. The level of Kir3.4 expression in the APA with a *KCNJ5* mutation is lower than that of normal human ZG.[30] Most patients with APA also have an increase in peri-tumoral nodules and zona glomerulosa hyperplasia, including aldosterone-producing cell clusters.[40–44] Patients with APA with KCNJ5 mutations tend to be younger females[2–5, 7, 9] except in Japan where there is no sexual preference[6] and have more severe hyperaldosteronism and higher lateralization index.[4, 45] The finding that the expression of Kir3.4 is lower in adenomas harboring a *KCNJ5* somatic mutation might contribute to increased proliferation as the cells would not have as much of a sodium-induced depolarization and calcium toxicity, but just enough intracellular calcium to stimulate proliferation. Some mutations like the KCNJ5-G151E that severely affect channel activity suppress cell proliferation so fewer cells survive, creating a mild hyperaldosteronism phenotype.[9, 30] However,other studies have higher expression levels of KCNJ5 in APA patients bearing a mutation.[6]

Conclusions and perspective

Kir3.4 is a subunit of the G-protein-activated inwardly rectifying potassium channel expressed in the zona glomerulosa of the human adrenal. Part of the mechanism for the stimulation of aldosterone synthesis by angiotensin II is the decrease in transcription of gene KCNJ5 for Kir3.4 and blocking channel activity. All of the somatic mutations in aldosterone-producing adenomas reported so far are in or next to the selectivity filter of the *KCNJ5* gene that codes for the Kir3.4 channel. The functional result is the increased mobilization of intracellular calcium and activation of the calcium calmodulin kinase signal cascade (Fig 3). Recently inactivating mutations in the sodium-potassium ATPase alpha subunit (ATP1A1) and the calcium ATPase (ATP2B3)[11] have been discovered in some APA. Together, these mutations are present in 50–70% of APAs studied. In the near future other mutations, perhaps of the calcium channels themselves, may be discovered that also cause unregulated aldosterone production in APA or IHA.

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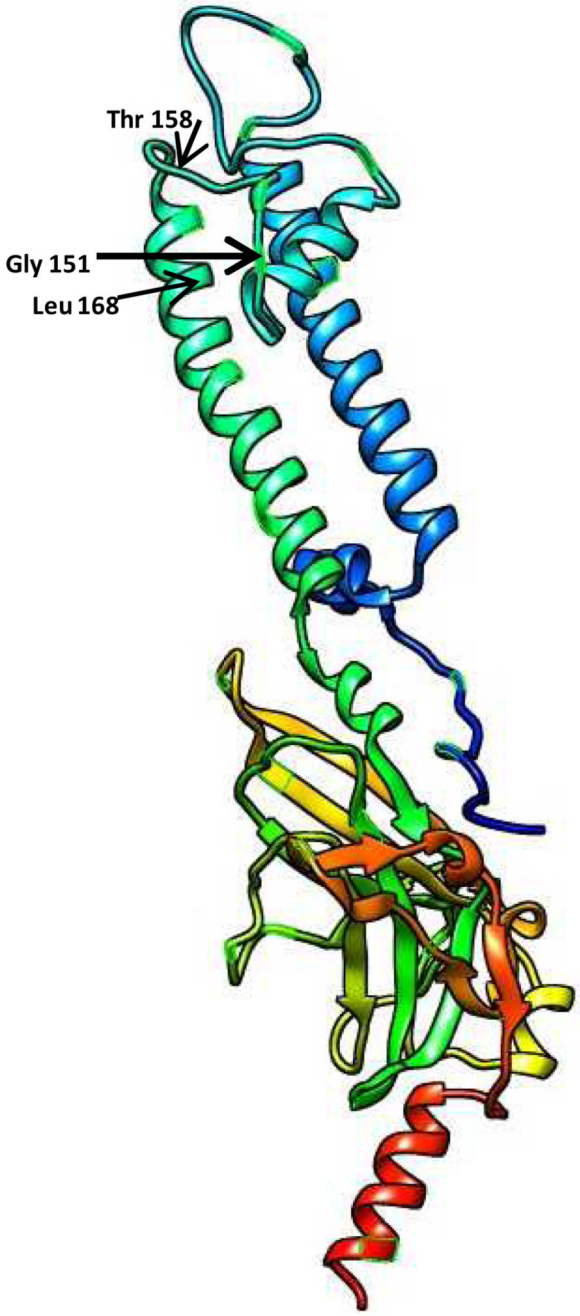

Fig 1.

Model of the crystal structure of the inward-rectifier potassium channel kir2.2 which has very high sequence homology in the selectivity filter with kir3.4. The location of glycine 151, threonine 158 and leucine 168 are shown. The model was based on the Phyre server.[23]

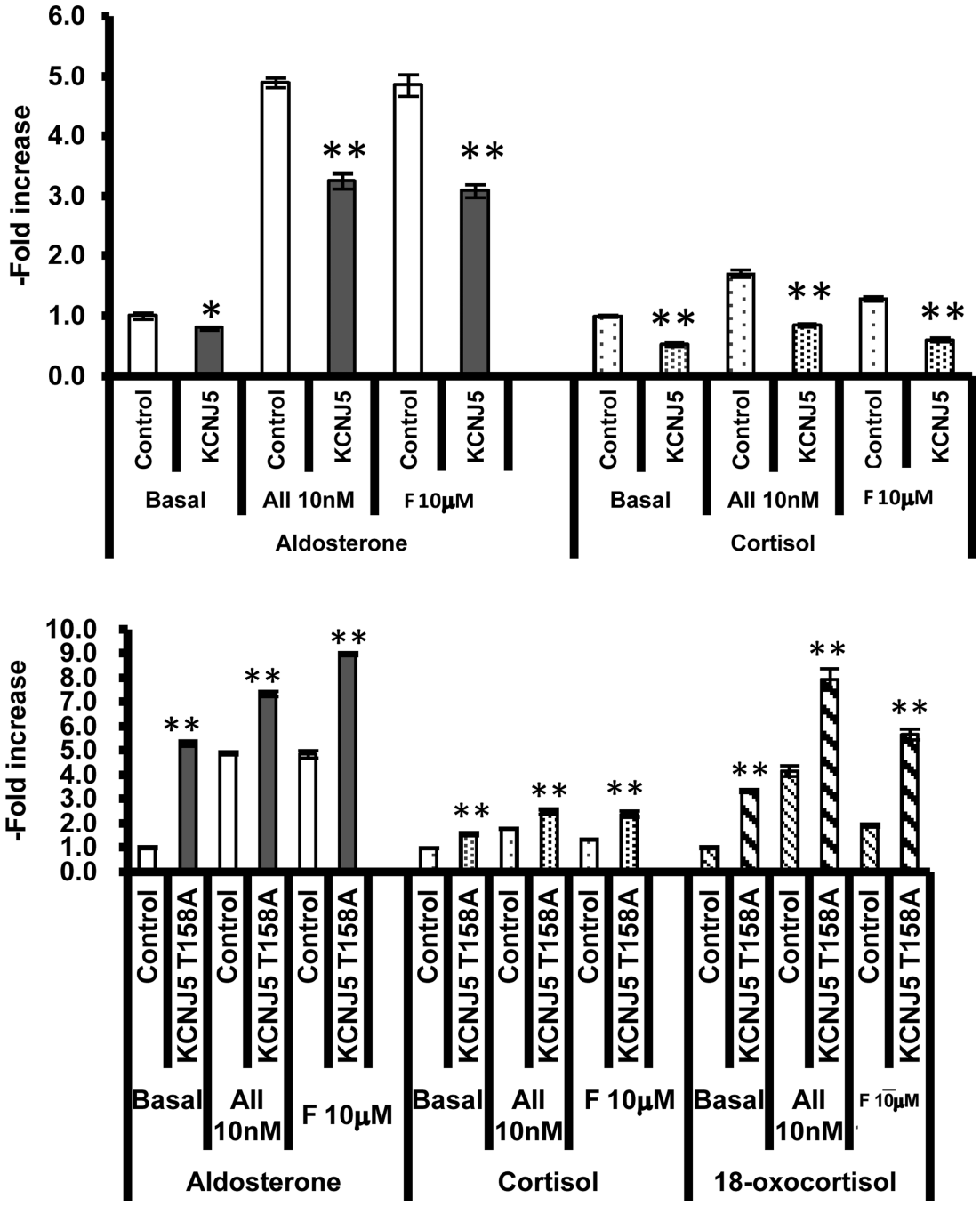

Fig 2.

Effect of KCNJ5 and mutated KCNJ5-T158A lentiviral transduction on steroid synthesis in the HAC15 cell. The upper panel shows the effect of transduction of HAC15 cells with wild type KCNJ5 lentivirus on aldosterone and cortisol secretion in cells under basal conditions and stimulated with angiotensin II (10 nM) and forskolin (10μM) for 24 hrs (*$p<0.05$, **$p<0.01$). Transduction of cells resulted in a decrease in basal and stimulated aldosterone and cortisol secretion (data redrawn from Oki *et al* by permission [31].

The lower panel shows the effect of transduction of HAC15 with a lentivirus with the mutated KCNJ5-T158A on aldosterone, cortisol and 18-oxocortisol secretion under basal and stimulation with angiotensin II (10nM) and forskolin (10 μM). There was a significant

enhancement of steroid secretion in the transduced cells with the KCNJ5-T158 lentivirus (data redrawn from Oki *et al* by permission) [34].

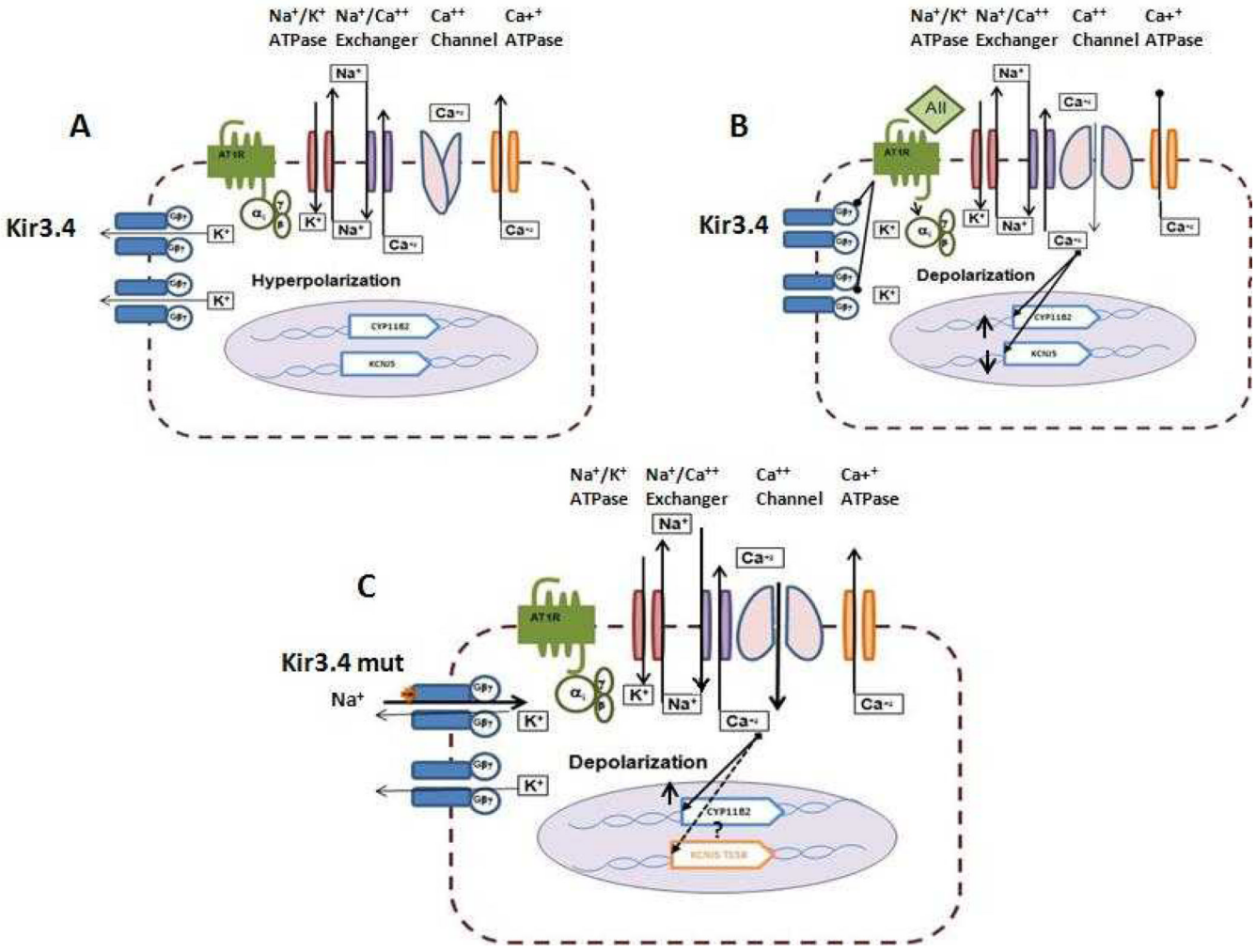

Fig 3.

Proposed mechanisms for the action of Angiotensin II on Kir3.4 and aldosterone secretion and for the effect of the KCNJ5-mutation on aldosterone secretion. A. Under basal conditions there is a continuous outflow of potassium through the Kir3.4 channel and in combination with other potassium channels the cell is hyperpolarized and aldosterone is formed in basal quantities. B. Upon stimulation with Angiotensin II there is a decrease in gene expression of the KCNJ5 (Kir3.4) and blocking of the activity of the channel with membrane depolarization, opening of the calcium channels and increase in intracellular calcium with activation of the calcium-calmodulin cascade resulting in increased expression of the CYP11B2 and aldosterone secretion. C. The KCNJ5 mutation (Kir3.4 mut) results in loss of selectivity of the channel with inflow of sodium, depolarization of the membrane, opening of calcium channels and ultimately increase gene transcription of the CYP11B2 and increased aldosterone secretion.