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Molecular genetics of Conn adenomas in the era of exome analysis

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Abstract

Aldosterone producing adenomas (APA) are a major cause of primary aldosteronism (PA), the most common form of secondary hypertension. Exome analysis of APA has allowed the identification of recurrent somatic mutations in *KCNJ5, CACNA1D, ATP1A1, and ATP2B3* in more than 50% of sporadic cases. These gain of function mutations in ion channels and pumps lead to increased and autonomous aldosterone production. In addition, somatic *CTNNB1* mutations have also been identified in APA. The *CTNNB1* mutations were also identified in cortisol producing adenomas and adrenal cancer, but their role in APA development and the mechanisms specifying the hormonal production or the malignant phenotype remain unknown. The role of the somatic mutations in the regulation of aldosterone production is well understood, while the impact of these mutations on cell proliferation remains to be established. Furthermore, the sequence of events leading to APA formation is currently the focus of many studies. There is evidence for a two-hit model where the somatic mutations are second hits occurring in a previously remodeled adrenal cortex. On the other hand, the APA-driver mutations were also identified in aldosterone-producing cell clusters (APCC) in normal adrenals, suggesting that these structure may represent precursors for APA development. As PA due to APA can be cured by surgical removal of the affected adrenal gland, the identification of the underlying genetic abnormality by novel biomarkers could improve diagnostic and therapeutic approaches of the disease. In this context, recent data on steroid profiling in peripheral venous samples of APA patients and on new drugs capable of inhibiting mutated potassium channels provide promising preliminary data with potential for translation into clinical care.

Introduction

Arterial hypertension (HT) is a worldwide health problem which affects ~25% of the global population [1], resulting in an estimated 9.4 million deaths or approximately 12.8% of all deaths (Global Health Observatory data, WHO). A vast and diverse array of drugs exists for the treatment of HT, such as diuretics, antagonists of the renin-angiotensin-aldosterone system, notably angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers, calcium channel blockers, vasodilators, β adrenergic blocking agents. Optimal blood pressure control, however, is still far from being achieved in up to two thirds of the hypertensive population. In a certain proportion of cases, HT can arise from a specific disease; endocrine hypertension, a frequent form of secondary arterial hypertension, emerges following a dysregulation of one or more hormones that are involved in blood pressure regulation. Primary aldosteronism (PA), also known as Conn's syndrome, is the most frequent form of secondary hypertension with estimates of up to 10% of cases in referred patients, 4% in primary care [2] and 20% in patients with resistant hypertension [3,4]. PA is mainly due to aldosterone producing adenoma (APA) and bilateral adrenal hyperplasias (BAH, or idiopathic hyperaldosteronism, IHA). The clinical picture of patients with PA consists of HT, a high aldosterone to renin ratio, which has become one of the major diagnostic tools for PA alongside different confirmation tests and adrenal venous sampling for subtype diagnosis, and variable hypokalemia and metabolic alkalosis [5]. PA is associated to an increased risk of cardiovascular complications, which occur beyond the effect of hypertension, such as coronary artery disease, heart failure, myocardial infarction, atrial fibrillation and renal damage. Different studies insist on the importance of screening most hypertensive patients for PA to either confirm or exclude the diagnosis [6,7]. Indeed, early PA diagnosis can improve prognosis and prevent the development of target organ damage.

Although the management of PA in hypertensive patients has come a long way and the treatment is much better established, the prognosis of unilateral PA depends on different criteria such as age, sex, BMI, age upon diagnosis and the duration of hypertension [7,8]. Indeed, younger patients and female patients show a better clinical outcome after adrenalectomy in comparison to older or male patients [7].

Aldosterone biosynthesis in the adrenal cortex

The human adrenal cortex is composed of three distinct zones that are characterized by their respective functions. Steroid hormones are synthesized following the sequential enzymatic breakdown of cholesterol by different cytochrome P450 enzymes as well as hydroxysteroid dehydrogenases, the particularity of each zone lies in the expression of specific steroidogenic enzymes and the ability to have each its own regulators. The most outer zone of the adrenal cortex, the zona glomerulosa (ZG), expresses aldosterone synthase (encoded by *CYP11B2*), which catalyzes the hydroxylation at the C11 position of the 11-deoxycorticosterone into corticosterone, furthermore, its hydroxylation at C18 into 18(OH)corticosterone followed by an oxidation of C18's hydroxyl group giving as an end result aldosterone. The main trigger for aldosterone biosynthesis is the activation of intracellular calcium signaling in the zona glomerulosa which is induced by either angiotensin II (Ang II) from the renin-angiotensin system or by extracellular potassium levels. The zona fasciculata (ZF) of the adrenal cortex mainly produces cortisol. In this process, the conversion of progesterone into 17-hydroxyprogesterone is catalyzed by the activity of 17α-hydroxylase which is not expressed in ZG cells. 17-hydroxyprogesterone undergoes a hydroxylation at C21 by the 21-hydroxylase enzyme, and finally a hydroxylation at position C11 by 11β-hydroxylase (encoded by *CYP11B1*) forming cortisol. The main regulator of cortisol production is the hypothalamus-pituitary-adrenal (HPA) axis primarily through the adrenocorticotropic hormone (ACTH).

Ang II is one of the major regulators of aldosterone secretion by ZG cells. The binding of AngII to its receptor (AT1) will lead to the activation of the Gαq-phospholipase C-mediated pathway, increasing inositol 1,4,5-triphosphhate (IP3) and 1,2-diacylglycerol concentrations. Ultimately, IP3 is responsible for increased intracellular calcium concentration due to calcium release from intracellular stores. AngII also inhibits the background TWIK-related acid-sensitive potassium channel (TASK), as well as GIRK4 and the Na+,K+ -ATPase, leading to a cell membrane depolarization [9]. This will lead to opening of voltage-gated Ca2+ channels, also increasing intracellular calcium concentrations.

The other main stimulator for aldosterone biosynthesis is the increase in extracellular potassium levels. In the normal ZG cell at a resting state, the cell membrane potential is hyperpolarized, the reason is that the membrane potential follows closely the equilibrium potential of potassium in these cells which largely express potassium channels. Small increases in extracellular potassium levels cause ZG cell membrane depolarization. The depolarization of the ZG cell membrane leads to the opening of voltage gated Ca^{2+} channels and an increase in intracellular calcium levels resulting in the activation of calcium signaling.

Calcium signaling acts by increasing the release of deesterified cholesterol from cytoplasmic stores, as well as cholesterol delivery to the outer mitochondrial membrane and then to the inner mitochondrial membrane by increasing the expression of the steroid acute regulatory protein (StAR). Calcium signaling also increases the expression of cofactors required for p450 cytochrome enzymes. Calcium/Calmodulin binding in the cytosol of the ZG cell induces the activation of protein kinases that regulate phosphorylation of transcription factors involved in *CYP11B2* transcriptional induction, mainly nuclear receptor subfamily 4 group A 1 and 2 (*NR4A1* and *NR4A2* coding for NUR77/NGF1B and NURR1 respectively) and the cyclic AMP-responsive element-binding protein (CREB).

Genetic abnormalities in APA

PA is due to inappropriate aldosterone production by the adrenal cortex in spite of the suppression of the renin-angiotensin system. In the last years, whole exome sequencing (WES) performed on DNA from APA led to the identification of recurrent somatic mutations in genes coding for ion channels (*KCNJ5* and *CACNA1D*) and ATPases (*ATP1A1* and *ATP2B3*). These genes are essential for regulating intracellular ion homeostasis and cell membrane potential. All these mutations promote an increased intracellular calcium signaling through cell membrane depolarization and opening of voltage-dependent calcium channels, or impaired intracellular calcium recycling, therefore leading to high aldosterone levels by constitutive expression of *CYP11B2*.

In a large multicenter study from the European Network for the Study of Adrenal Tumors (ENS@T) that analyzed somatic mutations in APA from 474 patients [10], hot spot regions for mutations in *KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3* were sequenced. Somatic mutations were identified in 54.2% of APA, with *KCNJ5* being the most prevalent at 38 %, *CACNA1D* at 9.3%, *ATP1A1* at 5.3% and *ATP2B3* 1.7% of these mutations. However, *KCNJ5* mutations are more prevalent in Asian populations, with up to 76% of prevalence [11-15]. These observations were corroborated by a meta-analysis of clinical and genetic data from 1636 patients with APA showing an overall prevalence of *KCNJ5* mutations of 43%, with higher prevalence in patients from Asia [16]. Some aldosterone producing adenomas also carry somatic mutations in the gene that codes for β-catenin (*CTNNB1*), less common mutations have also been identified in *PRKACA* (encoding Protein Kinase cAMP-Activated Catalytic Subunit α) [17-19].

KCNJ5 codes for an inwardly rectifying K^+ channel, which is the G-protein-activated inward rectifier potassium channel GIRK4 (also known as Kir3.4). It is mainly expressed in the ZG of the adrenal cortex. *KCNJ5* mutations were found to be more frequent in female and younger patients, and the expression of GIRK4 in APA was found to be correlated to the mutation status [20]. GIRK4 is composed of 2 membrane spanning helices with one pore-forming region in between and N- and C- termini that contribute to the pore structure [21]. Choi et al identified two somatic *KCNJ5* mutations mapping to the selectivity filter of GIRK4 (p.Gly151Arg and p.Leu168Arg). In addition to these two mutations (the most prevalent mutations in APA), the majority of the *KCNJ5* mutations described are located within or near the selectivity filter, rendering the channel permeable to sodium, which leads to chronic cell membrane depolarization [22]. Transient transfection of *KCNJ5* mutants in HAC-15 resulted in a calcium-dependent increase in *CYP11B2* expression and aldosterone biosynthesis in the cells; the mutant GIRK4, however, did not induce any increase in proliferation but rather a reduced cell viability or sodium-induced cell death [23,24]. This leaves the question of the role of KCNJ5 mutations on the cell proliferation and APA formation unanswered in tumors where these mutations occur. *KCNJ5* mRNA expression is not affected by *KCNJ5* mutations, but APA harboring *KCNJ5* mutations show decreased GIRK4 protein expression when compared with adjacent ZG, allowing the differentiation from APA harboring other mutations or without mutations identified [20,25].

More than 20 mutations have been identified in *CACNA1D* (encoding the voltage-dependent L-type calcium channel subunit alpha-1D, Cav1.3) [26]. The Cav1.3 calcium channel consists of 4 repeat domains, each one consisting of six transmembrane segments, with a membrane-associated loop between S5 and S6 [27-29]. Mutations occurring in *CACNA1D* are gain of function mutations that lead to a decrease in the threshold of the voltage-dependent activation or impaired channel inactivation, which is followed by increased intracellular calcium concentrations and thereby an induction of aldosterone biosynthesis [28,29].

ATP1A1 and *ATP2B3* are members of the P-type family of ATPases and are composed of 10 transmembrane domains (M1 - M10) with intracellular N- and C- termini. *ATP1A1* codes for the Na(+)/K(+) ATPase alpha-1 subunit. Mutations in this pump lead to a loss of its activity and affinity to K^+ and to an inward proton or sodium leak, which has been proposed to induce aldosterone production through cell membrane depolarization and increased calcium influx [28,30]. Nevertheless, transient transfection of 2 of the described *ATP1A1* mutations in the adrenocortical cell line H295R did not result in modifications of basal cytosolic calcium levels, and barely increased potassium-stimulated calcium concentrations, in spite of depolarizing the cells and stimulating aldosterone biosynthesis. In these cells, Stindl et al found that there was an increased intracellular acidification, which was suggested to regulate *CYP11B2* biosynthesis [31].

ATP2B3 codes for the plasma membrane calcium-transporting ATPase 3 (PMCA3). Mutations of PMCA3 are found in the transmembrane domain M4 and result in the deletion of different amino acids in the region between Leu422 and Leu433. One mutation in particular, p.Leu425_Val426del, leads to reduced calcium export which is due to the loss of the physiological pump functions, and an increased intracellular calcium signaling due to the depolarization-activated Ca^{2+} channels [32]. Recently, a second mechanism explaining aldosterone production due to *ATP2B3* mutations was suggested. *ATP2B3* mutations induce an increase in calcium influx by the opening of depolarization-activated calcium channels and by a possible calcium leak through the mutated PMCA3 [32].

CACNA1H encoding the pore-forming $\alpha 1$ subunit of the T-type voltage-dependent calcium channel Cav3.2 has been recently shown to be involved in familial forms of PA in some cases associated with developmental disorders [33,34]. In addition, it was also described as germline mutation in a patient with APA [34]. This channel consists of a single polypeptide chain of four homologous domains (I-IV), each one containing six transmembrane spans (S1-S6) and cytoplasmic C- and N- Termini. Mutant Cav3.2 channels show significant changes in their electrophysiological properties, specifically a shift in activation towards more negative voltages and modifications of their inactivation properties. Consequently, the channels are activated at less depolarized voltages leading to activation of calcium signaling and autonomous aldosterone production [33,34]. A germline *CACNA1H* variant was identified in one patient with APA without somatic mutations and improvement after adrenalectomy [33,34]. This case suggest that *CACNA1H* might be a susceptibility gene for different types of PA, including APA.

The Wnt/β-Catenin signaling pathway has been shown to play an important role in the development of the adrenal cortex and in aldosterone biosynthesis [35]. This signaling pathway is constitutively active in ~70% of APA [36]. In unstimulated conditions, β-catenin is located in the cytosol, and is part of the axin complex along with adenomatous poliposis coli protein (APC), axin, Glycogen Synthase Kinase-3β (GSK-3β) and Caseine Kinase-1β. Eventually, β-catenin in this complex will be phosphorylated resulting in its degradation by the proteasome, and preventing its translocation to the nucleus and the activation of different Wnt target-genes. The activation of the pathway occurs through binding of Wnt ligand to its receptor Frizzled resulting in the inhibition of the phosphorylation of β-catenin, which dissociates from the axin complex and translocates to the nucleus where it induces the expression of Wnt target genes, most notably the transcription factors T-cell factor (*TCF*) and lymphocyte enhancer factor (*LEF*), through its actions as a transcriptional coactivator [35]. Mutations in the *CTNNB1* gene, encoding β-Catenin, have been described in 2-5% of APA [17,19]. The description of somatic *CTNN1B* mutations associated with higher expression of luteinizing hormone–chorionic gonadotropin receptor (LHCGR) and gonadotropin-releasing hormone receptor (GNRHR) in APA diagnosed during pregnancy or menopause suggested that pregnancy may reveal an underlying PA [37]. Other studies, however, showed high expression of GNRHR and LHCGR in more than 40% of APA [38,39], and the presence of *CTNNB1* mutations both in females and males [17,19]. Further studies are necessary to establish the mechanism of *CTNNB1* mutations in the development of APA.

To a much lesser extent, somatic *PRKACA* (encoding the catalytic α subunit of Protein Kinase A) mutations have been described in APA [18]. Rhayem et al identified somatic mutations of the *PRKACA* gene in two patients with APA by whole exome sequencing. The mutation p.Leu206Arg, previously identified in CPA [40-42], was found in one patient with PA and Cushing syndrome. The second mutation (p.His88Asp) was identified in a patient without cortisol hypersecretion [18] . This particular mutation was not associated with a gain of function, the mechanism underlying increased PKA signaling and tumorigenesis in cortisol producing adenoma. The role of these mutations on aldosterone secretion and their frequency in APA remains to be established.

CTNNB1 mutations and *PRKACA* mutations are also identified in cortisol producing adenomas (CPA). Other evidences for an overlap of genetic determinants of aldosterone and cortisol excess have been described, including the cortisol co-secretion observed in a subset of APA, notably those harboring *KCNJ5* mutations [43]. The complex mechanisms that would explain how the same mutations could end up in two different hormonal phenotypes remain to be discovered.

Clinical correlates of somatic mutations

The discovery of clinical or biochemical surrogate markers of somatic mutations in APA could be of benefit for the management of the disease. Different studies described the higher prevalence of somatic *KCNJ5* mutations in women and in young patients with APA

[10,16,44]. *KCNJ5* mutations were also associated to higher levels of plasma aldosterone and larger tumors [16], and with higher left ventricular mass index [45]. *CACNA1D* mutations were associated with smaller APA [10]. More recently, *KCNJ5* mutations were described as a predictor of better outcome in young patients with APA [46]. Promising data for the identification of the underlying APA genotype came from a study that analyzed steroid profiles in adrenal and peripheral venous plasma samples from APA patients by liquid chromatography–tandem mass spectrometry [47]. The authors identified a 7-steroid fingerprint in peripheral venous samples allowing to correct classify 92% of the APA accordingly to genotype. Additionally, specific steroid profiles were associated with *KCNJ5* mutations, in particular the presence of significantly higher hybrid steroids 18-hydroxycortisol and 18-oxocortisol. This approach may be translated into clinical care, allowing to identify the APA genotype from peripheral venous plasma samples before surgery. This could be useful for the selection of patients for adrenal vein sampling.

Heterogeneity of APA

In spite of the fact that the relation between aldosterone production and the presence of somatic mutations is well established, the impact of these mutations on nodule/APA formation and cell proliferation is still far from fully understood. APA present a highly pronounced molecular heterogeneity not only on a mutational status but also in terms of aldosterone synthase expression within the same APA. Recent studies showed the presence of different somatic mutations in different aldosterone-producing nodules from the same adrenal [48,49], suggesting that somatic mutations are independent events occurring in a previously remodeled adrenal cortex. In the same context, Nanba et al described one case of a patient that was diagnosed with PA and Cushing syndrome with double adrenocortical adenomas harboring each a *KCNJ5* and a *PRKACA* somatic mutation [50]. Furthermore, in APA exhibiting heterogeneity of aldosterone synthase expression, APA driver mutations were identified only in positive aldosterone synthase regions [51]. Interestingly, two different mutations were identified in the same APA, lying in two distinct positive aldosterone synthase regions [51]. These findings suggest that somatic mutations are second hits in APA development that emerge from specific mechanisms that remain to be elucidated. Supporting this hypothesis, our group described the occurrence of a germline *APC* mutation and a somatic *KCNJ5* mutation leading to the development of an APA in a young patient with severe unilateral PA, bilateral macronodular adrenal hyperplasia and Gardner syndrome [52], suggesting a two-hit model for APA development with the *APC* mutation driving nodule formation and the *KCNJ5* mutation being responsible for aldosterone hypersecretion.

Another theory was suggested by Nishimoto et al, in a study that describes aldosterone producing cell clusters (APCC) in being the origin behind APA development [53]. APCCs are structures of outer morphological ZG cells in contact with the capsule and inner ZF-like cells, staining positive for both CYP11B2 and CYP11B1 [53,54]. They are found in normal adrenal tissue and in adrenals with APA. APCC express important amounts of aldosterone synthase in normal and pathological conditions. In a later study, Nishimoto et al sequenced DNA from APCCs that were collected from normal adrenal glands and identified mutations in APA driver genes in up to 35% of the collected samples; specifically, mutations in *CACNA1D, ATP1A1* and *ATP2B3*. Interestingly, no mutations in *KCNJ5* were reported, which is the most frequently mutated gene in APA [55]. The authors suggest that APCCs could represent cellular precursors that could lead to APA with their specific mutations through unknown mechanisms. On the other hand, they propose that APCCs with *KCNJ5* could be rarer, or that APCCs that develop *KCNJ5* mutations tend to become APAs quite quickly and are hard to be witnessed before the APA development [55]. It was suggested that the sequence of events leading to APA development from an APCC occurs trough the development of structures called possible APCC-to-APA translational lesions (pAATL) [56]. pAATL are composed by an outer APCC-like portion and inner micro-APA (mAPA)-like portion. The genetic characterization of pAATL is complex. In one adrenal, a *KCNJ5* mutation was identified only in the mAPA-like portion of a pAATL, not observed in the APCC-like portion and was different from the mutation identified in an APA within the same adrenal. This suggests that the APA and the pAATL do not share the same origin and that the *KCNJ5* mutation leads to differentiation of the mAPA portion from the APCC. In a second adrenal, both portions of the pAATL carried an *ATP1A1* mutation indicating its clonal origin. Although the model whereby APA arise from APCC through pAATL and mAPA is intriguing, further studies are required to better clarify the suite of genomic events involved in this transition.

Conclusion

The role of each mutation in the regulation of aldosterone production is well studied, while the impact of these mutations on cell proliferation remains to be established. In the future it would be of relevance to distinguish additional biomarkers or the development of techniques that are able to identify somatic mutations in APA. This could be of interest since PA is the most frequent form of secondary hypertension and is curable by the surgical removal of the APA carrying adrenal if recognized early enough. An additional benefit is the possibility of developing new diagnostic and therapeutic approaches. This is particularly the case for the use of macrolides in the detection and treatment of APA with *KCNJ5* mutations [57]. A recent work has shown that macrolide antibiotics, including roxithromycin, are potent inhibitors of KCNJ5 channels carrying the most frequent mutations p.Gly151Arg and p.Leu168Arg. Use of clarithromycin in primary cultures from APA showed a significant inhibition of *CYP11B2* gene expression and aldosterone production [58]. These compounds could therefore be used to identify patients carrier of APA with KCNJ5 mutations and as targeted treatments in patients who are not candidates for surgery.

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Disclosures

The authors have nothing to disclose.

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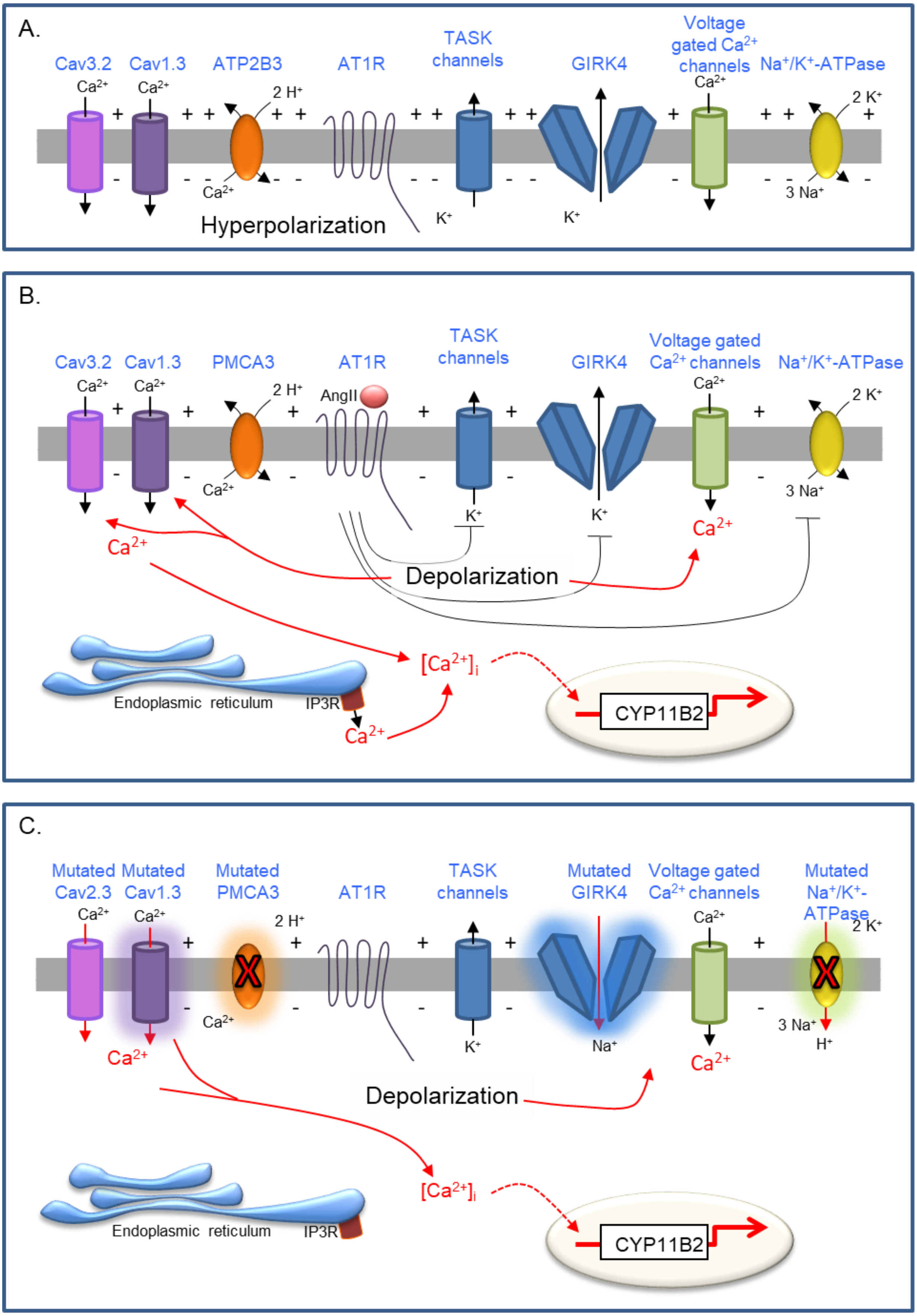
Figure legend

Figure 1. Regulation of aldosterone biosynthesis in zona glomerulosa cells. (A) In basal conditions, zona glomerulosa cells are in a hyperpolarized state due to the activity of potassium channels at the cell membrane. (B) The binding of AngII to its receptor AT1R or the increase of extracellular K^+ concentration lead to inhibition of K^+ currents through TASK and GIRK4 channels, followed by cell membrane depolarization; AngII also inhibits the activity of the Na^+/K^+-ATPase pump (*ATP1A1*) activity. This depolarization leads to the opening of voltage gated calcium channels on the cell membrane increasing Ca^{2+} concentrations in the cytosol. AngII also induces, through inositol triphosphate (IP3), the release of Ca^{2+} from the sarco/endoplasmic reticulum. The increased intracellular Ca^{2+} concentration leads to the activation of the calcium signaling pathway, the major trigger for aldosterone biosynthesis. (C) In pathological conditions, mutations affecting specific ion channels (*CACNA1D*, *CACNA1H*, *KCNJ5*) and ATPases (*ATP1A1*, *ATP2B3*) lead to constitutively depolarized ZG cell membrane or directly to increased intracellular Ca^{2+} concentrations, constitutively activating Ca^{2+}signaling. The net result is an increased expression of *CYP11B2* and an autonomous aldosterone biosynthesis.


A.
Cav3.2
Cav1.3
ATP2B3
AT1R
TASK channels
GIRK4
Voltage gated Ca2+ channels
Na+/K+-ATPase
Ca2+
2 H+
2 K+
3 Na+
K+
Hyperpolarization
B.
Cav3.2
Cav1.3
PMCA3
AT1R
AngII
TASK channels
GIRK4
Voltage gated Ca2+ channels
Na+/K+-ATPase
Ca2+
2 H+
2 K+
3 Na+
K+
Depolarization
Endoplasmic reticulum
IP3R
[Ca2+]i
CYP11B2
C.
Mutated Cav2.3
Mutated Cav1.3
Mutated PMCA3
AT1R
TASK channels
Mutated GIRK4
Voltage gated Ca2+ channels
Mutated Na+/K+-ATPase
Ca2+
2 H+
2 K+
3 Na+
H+
K+
Na+
Depolarization
Endoplasmic reticulum
IP3R
[Ca2+]i
CYP11B2