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Abstract

Multiple elements of the cAMP/protein kinase A (PKA) pathway have been shown to be mutated or deregulated in various endocrine disorders, indicating a major role of this pathway in the regulation of hormone secretion, but also in tumorigenesis of endocrine cells. In particular, several alterations are known to be involved in the pathogenesis of adrenocortical adenomas associated with autonomous cortisol secretion.

In this review, recent findings on cAMP/PKA alterations causative for the pathogenesis of benign unilateral cortisol-producing adrenocortical adenomas are summarised. Special emphasis is given to latest reports on newly identified mutations in genes encoding PKA catalytic subunits and their functional characterization.

Adrenal Cushing syndrome

Endogenous cortisol excess, referred to as Cushing's syndrome (CS), is characterized by a number of clinical signs (e.g. muscle weakness, facial plethora, striae rubre, skin fragility, osteoporosis) and associated with substantial comorbidities (e.g. blood hypertension, hyper-coagulable state, diabetes mellitus, dyslipidemia, osteoporotic fractures, and psychiatric disorders) as well as increased mortality [1]. In particular, corticotropin (ACTH)-independent cortisol hypersecretion is mostly caused by benign tumours of the adrenal cortex, i.e. cortisol-producing adrenocortical adenomas (CPA) that are usually isolated and sporadic, or more rarely bilateral hyperplasia. Whereas only a subset of CPA is associated with overt Cushing's syndrome, some degree of cortisol excess (mild autonomous cortisol secretion=MACS) is present in up to 47% of patients with adrenal adenomas, depending on the diagnostic criteria applied. This condition might be associated with a range of phenotypes, such as metabolic syndrome and osteoporosis [2]. The molecular pathogenesis of CPA remained largely unknown until lately. In this review, I will provide an overview about the mechanisms involved in the pathogenesis of unilateral sporadic CPA, specifically focusing on recent findings about cAMP/protein kinase A (PKA) signaling pathway.

cAMP/PKA signaling pathway and adrenal cortex

The adrenal cortex plays an essential role in body homeostasis through the biosynthesis of steroid hormones. This physiological role relies on functional zonation, whereby *zona glomerulosa* synthesizes the mineralocorticoid aldosterone, *zona fasciculata* synthesizes the glucocorticoid cortisol (or corticosterone in rodents), and the *zona reticularis* produces androgens. The physiological stimulus for glucocorticoid and adrenal androgens synthesis and secretion, as well as adrenal cortex maintenance, is the pituitary hormone ACTH [3]. The actions of ACTH in the adrenal cortex is mediated by activation of the cAMP/PKA signalling pathway, via two temporally distinct responses (**Figure 1A**). The acute response leads to mobilization of cholesterol, the initial substrate for all steroidogenic pathways, from cellular stores and its delivery to the inner mitochondrial membrane where the cholesterol side chain cleavage cytochrome P450 (P45011A1) resides. The slower, chronic response to ACTH induces the

transcription of the genes encoding the steroidogenic enzymes [4]. ACTH acts *via* the G-protein coupled receptor (GPCR) melanocortin 2 receptor (MC2R), activating adenylyl cyclase and leading to cAMP production, which induces PKA activation [5]. Under basal conditions, PKA is a stable and inactive hetero-tetramer, consisting of two catalytic (C) and two regulatory (R) subunits. The human genome encodes three different C subunits (α, β and γ) and four different R subunits (Iα, Iβ, IIα, IIβ). PKA signalling is activated when two molecules of cAMP bind to each R subunit of the PKA heterotetramer, leading to its dissociation and release of the C subunits, which thereby become active. When activated, PKA phosphorylates specific nuclear factors, which bind to target promoters and facilitate steroidogenic gene transcription [5]. Among these transcription factors, steroidogenic factor 1 (SF-1), cAMP response element-binding protein (CREB), CRE modulator, CCAAT/enhancer-binding proteins, and activator protein 1 are well known for their implication in regulating the expression of the genes encoding for steroidogenic enzymes [6]. An increasing body of evidence points toward a regulatory role of the cAMP/PKA signaling pathway by targeting the C subunits to various C subunit binding proteins in the cytosol and nucleus. Moreover, recent identification of isoform specific amino acid sequences, motifs and three dimensional structures have together provided new insight into how PKA at the level of the C subunit may act in a highly isoform-specific fashion [7].

In general, perturbations of cAMP/PKA-dependent cascade (i.e. somatic mutations) may cause dysregulation of steroidogenesis, resulting in autonomous cortisol excess (**Figure 1B**), as well as alterations in adrenocortical cell proliferation.

cAMP/PKA signaling pathway and adrenocortical adenomas: old beliefs

For the association between activation of cAMP/PKA signaling and tumour formation in different endocrine tissues as well as hereditary syndromes, I rely on previous extensive reviews [8, 9]. I will focus on the link between abnormal cAMP/PKA activity and unilateral CPA. In brief, somatic mutations in the gene encoding the *α* subunit of the stimulatory G protein (*GNAS1*) have been first described in adrenal hyperplasia leading to CS in patients with McCune–Albright syndrome [10] or macro-nodular bilateral hyperplasia [11]. Moreover, germline and somatic mutations in the gene encoding the regulatory subunit of the PKA (*PRKAR1A*) have been identified in patients with CS due to primary

pigmented nodular adrenocortical disease (Carney Complex) [12]. These mutations cause a reduced expression of the RIα subunits or impair its association with C subunits, thus leading to constitutive PKA activation [13]. Finally, inactivating mutations in the genes encoding the cAMP-degrading phosphodiesterase 11A (*PDE11A*) and phosphodiesterase 8B (*PDE8B*) have been also reported in patients with adrenal hyperplasia and Carney Complex [14, 15]. It was also well known since early 2000 that also a good proportion of sporadic CPA are linked to one or another abnormality of the cAMP/PKA signaling pathway [16]. For instance, it was acknowledged already in previous studies that CPAs may harbor activating somatic mutations in either *GNAS1* or *PRKAR1A* gene [17]. More recently, according to whole-exome sequencing series, *GNAS1* mutations resulted to be found in up to 11% of unilateral CPA [18, 19]. Finally, very few cases of CPA have been associated with *PDE11A* and *PDE8B* genes defects [20, 21].

Genetic alterations in these driver genes, however, explained only a small fraction of CPA cases. The observation that a subset of adrenal adenomas is characterized by abnormal PKA activity, despite the absence of mutations in these candidate genes [22], suggested the presence of further unknown alterations in the cAMP/PKA signaling cascade in these tumors.

cAMP/PKA signaling pathway and adrenocortical adenomas: new insights from 2014

2014 marked a golden year in the elucidation of the pathogenesis of unilateral sporadic CPA. Somatic mutations in *PRKACA*, which encodes the catalytic subunit of PKA, have been described for the first time by whole-exome sequencing in about 30% of CPA [23]. The more frequent of the two mutations resulted in the substitution of a leucine residue at position 206 with arginine (p.Leu206Arg), while the second mutation caused insertion of a tryptophan residue between the amino acid 199 and 200 (Leu199_Cys200insTrp). These findings were simultaneously confirmed by three independent studies by other groups [18, 24, 25]. Functional experiments and an *in silico* analysis also revealed that *PRKACA* mutations caused constitutive PKA activation (i.e. PKA activation in the absence of cAMP) impairing the association between catalytic and regulatory subunits and thus providing a molecular explanation for the development of CPA [23, 26] (**Figure 1B**). Always in 2014, two novel mutations have been reported within a targeted analysis of the *PRKACA* gene in a large cohort of 149 CPA cases

(p.Cys200_Gly201insVal in three adenomas and p.Ser213Arg+p.Leu212_Lys214insIle-Ile-Leu-Arg in one) [27]. Notably, based on the patient cohorts published to date, *PRKACA* mutations are observed in 35-60% of CPA being clearly associated with higher circulating cortisol levels, younger age and a clinical phenotype of overt CS [28]. More recently, three further somatic mutations in *PRKACA* have been reported within a multicentric study investigating a large cohort of unilateral ACA (n=99), i.e. p.Trp197Arg, p.Glu32Val and p.245_248 [19]. Even if those mutations occur outside the known hot-spot region of *PRKACA* in exon 7, two of them are they located at the interface between the C and R subunits of PKA and all were associated with presence of overt CS. According to these findings, nowadays there are seven different mutations recognized in *PRKACA* gene, being responsible for unilateral CPA (**Figure 2A and 2B**). Considering all currently available whole-exome sequencing studies on CPA associated with overt CS or MACS, we can conclude that 57% and 12% of cases are related to mutations in *PRKACA* or *GNAS1* gene, respectively (**Figure 3**). On the other side, missense mutations in the gene encoding for beta catenin (*CTNNB1*) are reported in slightly more than half of CPA associated with MACS or inactive adrenocortical adenomas, but in only 9% of CPA associated with overt CS [18, 19, 24, 25].

Interestingly, in a recent paper, our group also demonstrated a direct link between the presence of hot spot *PRKACA* mutations and reduced protein expression levels of two regulatory subunits of PKA in a sub-group of CPA cases. That specific expression also overlapped the different functional zones of the adrenal cortex, thus potentially indicating distinct roles of the different PKA regulatory subunits in the secretion of different hormones [29].

Considering other adrenocortical diseases associated with autonomous cortisol secretion, germline *PRKACA* amplifications were seen in different types of bilateral adrenal hyperplasia, i.e. both micro- and macro-nodular with various degree of CS severity [23, 30]. On the other side, taking into consideration adrenocortical neoplasia non typically associated with CS, somatic *PRKACA* mutations have been described only recently in one patient with unilateral adrenal hyperplasia [31] and, even more interestingly, as rare events in aldosterone-producing adrenocortical adenomas (APA) associated with Conn Syndrome [32]. Specifically, two *PRKACA* somatic mutations have been reported in 1.9% of APAs, i.e. p.Leu206Arg in a patient with associated biochemical CS and p.His88Asp in a patient

without cortisol excess. Thus, as cortisol co-secretion is known to occur in a subgroup of APAs [33], other molecular mechanisms are likely to be involved in their pathogenesis.

PRKACA alterations and other types of tumours

Since cAMP/PKA pathway is essential for the regulation of secretion of several other hormones despite cortisol, research groups investigated the presence of somatic *PRKACA* mutations in non-adrenal endocrine tumours. However, no *PRKACA* mutations had been found when specifically searched in either thyroid or pituitary functioning adenomas [34, 35]. Accordingly, we could hypothesise that the catalytic subunit Cα of PKA specifically plays a major role in the regulation of steroid production, but not of other hormones.

On the other side, *PRKACA* gene has been recently found to be altered in other types of tumours, thus opening new insight into its potential implication in tumorigenesis. In fact, somatic *PRKACA* variants (i.e. in-frame micro-insertions in exon 7 and 8) have been observed in a subgroup of 4 out of 41 sporadic cardiac myxomas [36] and have been functionally associated with increased PKA activity. Together with previous reports of *PRKAR1A* mutations in cardiac myxoma associated with Carney Complex, this study corroborates the importance of the PKA pathway in the tumorigenesis of cardiac myxoma.

Moreover, unexpectedly, three recent independent studies reported the presence of recurrent *DNAJB1–PRKACA* chimeric gene transcripts in fibrolamellar hepatocellular carcinoma [37-39]. This fusion gene produces an enzymatically active chimeric protein J-PKAcα and represents a driver genetic alteration for this disease. Thus, it could be possible that even other pathologies will be found to be due to *PRKACA* (or other PKA subunit) defects and that new class of medications that target cAMP signaling might be useful in fighting the corresponding tumors [40].

PRKACA mutations and adrenocortical adenomas: update from last two years

Although previous studies demonstrated a strong evidence for a causal role of first two described mutations in *PRKACA* [26], the mechanisms linking them to cortisol secretion and increased cell proliferation still remained under debate. A very recent study from our group reported a comprehensive functional characterization of all *PRKACA* mutations identified so far. The results indicated that in

addition to interfering with the binding of R subunits, *PRKACA* mutations cause major changes in the preference of PKA for its targets, leading to hyper-phosphorylation of several PKA substrates, most notably histone H1.4 at Ser36, which is required for and promotes mitosis [41]. The **Figure 2C** summarizes all experimental findings and biological effects associated with different *PRKACA* mutations reported until now. Of note, all variants described until now have been associated with the presence of overt CS expect one (d244-248), which has been reported in a CPA with mild cortisol excess [41].

In the last two years, there was also increasing interest in searching for suitable therapeutic inhibitors targeting cAMP/PKA pathway that might be suggested for pharmacological treatment of patients with persisting CS. In particular, kinetics and inhibition studies were conducted on the L206R-PKACalpha mutant, providing useful insights for the development of mutant selective inhibitors as potential therapeutics for cortisol excess [42].

In another very recent study, a somatic missense mutation in the gene encoding the catalytic subunit B of the PKA *(PRKACB)* was identified for the first time in one case of sporadic CPA (p.Ser54Leu). This mutation was specifically shown to impair the formation of PKA I holoenzymes [43]. This finding further highlights a potential role of the *PRKACB* gene in the pathogenesis of autonomous cortisol secretion. In fact, a genomic amplification of the *PRKACB* locus was previously found to lead to Carney Complex in absence of *PRKAR1A* mutations [44].

Conclusion

In conclusion, cAMP/PKA signaling pathway has long been identified to play a key role in cortisol secretion from adrenocortical cells and mutations affecting different elements of this pathway have been described in several adrenal diseases associated with autonomous cortisol secretion. However, *PRKACA* mutations have been only recently recognized as the cause of approximately 50% of unilateral sporadic CPA. While their role in the constitutive stimulation of steroidogenesis is largely accepted and new pathogenic mechanisms have been recently added to our knowledge [26, 41], only little is still known about their proliferative effects specifically contributing to adrenal tumorigenesis. Moreover,

despite the novel insights acquired predominantly high-throughput sequencing approaches, additional studies are now required to better elucidate the pathogenesis of the remaining proportion of CPA. Further research in this field might also pave the way towards new perspective for therapeutic advances.

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Annotated references

1) Bathon K, Weigand I, Vanselow JT *et al.* **Alterations in Protein Kinase A Substrate Specificity as a Potential Cause of Cushing Syndrome.** Endocrinology 2019; 160:447-459.

The Authors investigated all somatic *PRKACA* mutations identified so far in Cushing's syndrome and found that not all mutations interfere with the binding of regulatory (R) subunits as previously hypothesized. Specifically, they investigated their consequences on substrate specificity by quantitative phosphoproteomics and observed that all three mutations analyzed (L206R, 200_201insV and d244-248+E249Q) cause major changes in the preference of PKA for its targets, leading to hyperphosphorylation of several PKA substrates, including most notably histone H1.4 at Ser36, which is required for and promotes mitosis.
These findings suggest that besides hampering binding to R subunits, *PRKACA* mutations act via altering PKA substrate specificity, shedding a light on the molecular events leading to Cushing's syndrome and provide an illustrative example of how mutations altering substrate specificity of a protein kinase might cause human disease.

2) Luzi NM, Lyons CE, Peterson DL, Ellis KC. **Kinetics and inhibition studies of the L205R mutant of cAMP-dependent protein kinase involved in Cushing's syndrome.** FEBS Open Bio 2018; 8:606-613

In this study, the Authors conducted kinetics and inhibition studies on the hot spot PRKACA mutation (L205R-PKACα mutant). They found that the L205R mutation affects the kinetics of both Kemptide and ATP as substrates, decreasing the catalytic efficiency for each substrate by 12-fold and 4.5-fold, respectively. They also determined the IC_{50} and K_i for the peptide substrate-competitive inhibitor PKI(5-24) and the ATP-competitive inhibitor H89. The L205R mutation had no effect on the potency of H89, but causes an impressive loss in potency for PKI(5-24).
These results provide important insights for the potential development of L205R-PKACα inhibitors as potential therapeutics in Cushing syndrome.

3) Tseng IC, Huang WJ, Jhuang YL *et al.* Microinsertions in PRKACA cause activation of the protein kinase A pathway in cardiac myxoma. J Pathol 2017; 242:134-139.

Cardiac myxoma is the most common cardiac tumour, occasionally developing in patients with Carney Complex, a syndrome characterized by cardiac myxoma, spotty pigmentation, and endocrine over-activity. Two-thirds of patients with Carney Complex, but not those with sporadic cardiac myxomas, harbour germline mutations in *PRKAR1A*. Here, the Authors identified *PRKACA* mutations in 4 out of 41 sporadic cardiac myxomas (9.7%). In contrast to the point mutations identified in adrenocortical adenoma, all mutations were in-frame microinsertions of 18-33 bp clustered in exons 7 and 8. The mutated PRKACA proteins lost their ability to bind to PRKAR1A, and thereby lead to constitutive activation of the PKA pathway. Together with previous reports of PRKAR1A mutations in syndromic cardiac myxoma, these observations demonstrate the importance of the PKA pathway in the tumourigenesis of cardiac myxoma.

4) Weigand I, Ronchi CL, Rizk-Rabin M *et al.* **Differential expression of the protein kinase A subunits in normal adrenal glands and adrenocortical adenomas.** Sci Rep 2017; 7:49

In this study, the Authors linked the presence of somatic mutations in *PRKACA* gene to the loss of regulatory subunit IIβ protein expression at the tumour level and demonstrated the down-regulation of RIIβ to arise post-transcriptionally. They also found that the PKA subunit expression pattern observed in different adrenocortical tumours is also present in the different zones of the normal adrenal cortex. Their findings showed that different PKA subunits have a differential expression pattern in each zone of the normal adrenal gland, thus indicating potential specific roles of these subunits in the regulation of different steroid secretion.

5) Espiard S, Knape MJ, Bathon K *et al.* Activating PRKACB somatic mutation in cortisol-producing adenomas. JCI Insight 2018; 3.

This study identified for the first time a somatic mutation in the *PRKACB* gene, encoding the catalytic subunit β of PKA, in a single CPA from a patient with severe Cushing syndrome. Bioluminescence resonance energy transfer and surface plasmon resonance assays revealed that the mutation hampers formation of type I holoenzymes and that these holoenzymes were highly sensitive to cAMP. PKA activity was higher under basal conditions for the mutant enzyme compared with the wild type, while maximal activity was lower. These data suggest that at baseline the *PRKACB* mutant drove the adenoma cells to higher cAMP signaling activity, probably contributing to their autonomous growth.

These findings demonstrate that a *PRKACB* mutation can lead to an adrenal tumor and suggest another mechanism of PKA pathway activation in CPAs.

Legend to the Figures

Figure 1. Overview of cAMP/PKA signalling pathway in adrenocortical cells.

(A) Normal adrenocortical cells. Through binding of ACTH to the melanocortin type 2 receptor (MC2R), the α subunit of the G-protein leaves the βγ-complex and activates adenylyl cyclase (AC) which converts ATP into cAMP. cAMP binds the regulatory subunits of PKA which results in the dissociation of the PKA holoenzyme and the activation of the catalytic subunits of PKA. The signal is terminated by phosphodiesterases (PDEs), which degrade cAMP to AMP. Activated catalytic (C) subunits then phosphorylate several targets in the nucleus (including several genes involved in the steroid hormone synthesis) or in the cytoplasm (e.g. CYP11A1), initiating cortisol secretion of these cells.

(B) Adrenocortical cells from cortisol-producing adenomas with somatic mutations identified in the cAMP/PKA pathway. Activating mutations in *GNAS* (encoding the α subunit of the G-protein) lead to increased AC activation and hence increased cAMP levels. Inactivating mutations in *PDE* were shown to decrease the hydrolysis of cAMP into AMP and therefore preventing the termination of the PKA signal. Mutations in *PRKACA* were shown to inhibit the formation of PKA holoenzymes and the mutation in PRKACB was demonstrated to impair the formation of PKA I holoenzymes leading to over activated PKA signaling. Inactivating mutations in *PRKAR1A* lead to reduced RIα protein levels. All these mutations result into a constitutive activation of the cAMP/PKA pathway and an increase "autonomous" cortisol secretion.

Modified from [45] and [46]

Figure 2.

A) Location of somatic PRKACA mutations identified in cortisol-producing adenomas at the level of plain protein scheme. (1) [23] (2) *[27]*; (3) *[19]*.

B) Location of the identified PRKACA mutations in the crystal structure of the PKA holoenzyme. Note the clustering of the PRKACA mutations (in red) at the interface between the regulatory (R)

subunit (in grey) and the catalytic subunit (in yellow). The structure of the R subunit II type b2: Ca2 holoenzyme [protein data bank (PDB) entry 3TNP] was used as a template. (1) *[23]*; (2) *[27]*; (3) *[19]*. Modified from *[41]*.

C) Summary of currently available data about biological effects of various PKA catalytic (C) subunit alfa (α) mutants. Shown is a schematic representation of the experimental results obtained in different studies on PKA C**α** mutants compared to wild-type C**α** and the corresponding clinical phenotype. Modified from [29, 41].

Figure 3. Overview of general mutation status (i.e. somatic mutations in known driver genes: *PRKACA*, *GNAS* and *CTNNB1*) reported in unilateral sporadic adrenocortical adenomas in the literature.

Cortisol-producing adenomas (CPA) associated with overt Cushing syndrome (CS, n=100) or with mild autonomous cortisol secretion (MACS, n=43) in comparison with endocrine inactive adenomas (n=25).

Data taken from literature [18, 19, 23-25].