60 YEARS OF POMC

Adrenal and extra-adrenal functions of ACTH

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Abstract

The pituitary adrenocorticotropic hormone (ACTH) plays a pivotal role in homeostasis and stress response and is thus the major component of the hypothalamo–pituitary–adrenal axis. After a brief summary of ACTH production from proopiomelanocortin (POMC) and on ACTH receptor properties, the first part of the review covers the role of ACTH in steroidogenesis and steroid secretion. We highlight the mechanisms explaining the differential acute vs chronic effects of ACTH on aldosterone and glucocorticoid secretion. The second part summarizes the effects of ACTH on adrenal growth, addressing its role as either a mitogenic or a differentiating factor. We then review the mechanisms involved in steroid secretion, from the classical Cyclic adenosine monophosphate second messenger system to various signaling cascades. We also consider how the interaction between the extracellular matrix and the cytoskeleton may trigger activation of signaling platforms potentially stimulating or repressing the steroidogenic potency of ACTH. Finally, we consider the extra-adrenal actions of ACTH, in particular its role in differentiation in a variety of cell types, in addition to its known lipolytic effects on adipocytes. In each section, we endeavor to correlate basic mechanisms of ACTH function with the pathological consequences of ACTH signaling deficiency and of overproduction of ACTH.

Key Words
- adrenal cortex
- zona glomerulosa
- zona fasciculata
- ACTH
- adrenocorticotropin
- MC2
- signaling
- cortisol
- glucocorticoid
- aldosterone
- ion channels
- cytoskeleton
- extracellular matrix

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Introduction

The adrenocorticotropic hormone (ACTH) (39 amino acids (a.a.)) results from PC1/3 cleavage of the proopiomelanocortin (POMC) precursor and may be further cleaved by proconvertase 2 to generate α-melanocyte-stimulating hormone (α-MSH) (a.a. 1–13 of ACTH) (Raffin-Sanson *et al.* 2003, Dores *et al.* 2014). ACTH is mainly produced in the corticotropic cells from the anterior pituitary, but is also produced in the brain, adrenal medulla, skin, and placenta (Vrezas *et al.* 2003, Bicknell 2008, Evans *et al.* 2012). As ACTH is the most potent stimulus of the adrenal cortex, most of the knowledge on its mechanism of action derives from studies on the adrenal cortex or ACTH receptor-expressing cells.

The adult adrenal cortex is divided into three zones. At the periphery, under the capsular of tight connective tissue, the thin zona glomerulosa (ZG) consists of small cells organized as loops around capillaries, then the zona fasciculata (ZF) occupies the major part of the cortex, with cells that change progressively from radial centripetal columns, separated by sinusoids to a less-organized

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network, becoming the zona reticularis (ZR). Cells from the adrenal cortex, as for all steroid-producing cells, are characterized by the presence of lipid droplets containing cholesteryl esters (CE) as precursors for steroidogenesis. These lipid droplets are scarce and small in ZG and becomes large and numerous in the outer fasciculata. In the cells from ZR, lipid droplets vary in size and shape (NussDorfer 1986, Vinson 2003). The adrenal glands are also highly vascularized (Vinson & Hinson 1992, Bassett & West 1997) and innervated by pre- and post-ganglionic sympathetic fibers, sensory fibers, and vagal fibers (Vinson *et al.* 1994, Holgert *et al.* 1998).

Overview of the ACTH–MC2R complex

The ACTH receptor, called melanocortin 2 receptor (MC2R), cloned in 1992 (Mountjoy *et al.* 1992), is a member of the family of five melanocortin receptors (MCRs), which include MC1, MC3, MC4, and MC5 that bind the MSH peptides. This distinct family of G protein-coupled receptors (GPCRs) act primarily through cAMP as a second messenger. MCRs are characterized by their unusually short sequence and the absence of highly conserved a.a. residues or motifs common to most GPCRs. MC2R is both the smallest MCR and the smallest known GPCR (297 a.a). Compared with other MCRs, MC2R is unique in that it binds ACTH only and does not possess affinities for other melanocortins (for reviews see Cone 2006, Dores 2009).

Another important progress in understanding how the ACTH–MC2R complex is able to stimulate cAMP production was the discovery of melanocortin-2 receptor accessory protein 1 (MRAP, often called MRAP1) by Metherell *et al.* (2005). In the absence of melanocortin-2 receptor accessory protein (MRAP), MC2R is non-functional (i.e. there is no production of cAMP, even if the receptor is correctly addressed to the cell membrane). We (Kilianova *et al.* 2006, Roy *et al.* 2007) and others have been able to decipher the mechanisms of expression and regulation of MC2R in melanocortin-2 receptor accessory proteins (MRAPs) expressing cells. The role of MRAP1 as well as the relative roles that the various forms of MRAPs identified thereafter has been documented in several recent reviews (Hinkle & Sebag 2009, Cooray & Clark 2011, Jackson *et al.* 2015, Clark 2016). Another particularity is that ACTH treatment of adrenocortical cells (4 h or more) increases the expression of MC2R (Penhoat *et al.* 1989, Mountjoy *et al.* 1994), as well as the level of MRAP and MRAP2 (Hofland *et al.* 2012). Short-term stimulation of MC2R-expressing cells with ACTH (15–60 min) induces MC2R desensitization and internalization through a PKA-dependent mechanism (Rani *et al.* 1983, Baig *et al.* 2001), possibly acting in synergy with PKC (Kilianova *et al.* 2006, Chan *et al.* 2011, Gallo-Payet & Battista 2014).

Studies on structure–activity relationships have determined that ACTH(1–16) is the minimal sequence required for ACTH binding to MC2R and downstream signaling (Kapas *et al.* 1996, Chen *et al.* 2007). In addition, some ACTH fragments not only lack activity, but act as competitive antagonists of full-length ACTH, as is the case for ACTH(7–38) (Kapas *et al.* 1996). The latter is now known as corticotropin-inhibiting peptide (CIP) (Li *et al.* 1978). However, ACTH(11–24) has been described as a competitive antagonist of ACTH(1–39) (Seelig *et al.* 1971, Kapas *et al.* 1996), whereas in another study, it has been reported to stimulate corticosterone production of ZF cells and aldosterone production of ZG cells, in addition to potentiating the effects of ACTH(1–39) (Szalay *et al.* 1989). The a.a. 6–9 (HFRW sequence) is essential for cAMP production and has been called the 'message sequence', whereas the a.a. 15–18 (KKRR sequence) essential for the binding of ACTH to MC2R has been called the 'address sequence' (Dores 2009). Mutations in the HFRW or KKRRP motifs of ACTH (Liang *et al.* 2013) in the *POMC* gene, or a non-functional PC1/3 in corticotropic cells (Seidah & Chretien 1999), abrogate the hypothalamo-pituitary-adrenal (HPA)-activating axis (Dores 2009, Dores *et al.* 2014). The properties of MC2R are reviewed by Peng Loh and Robert Dores in this issue (Cawley *et al.* 2016, Dores *et al.* 2016).

Illustrating the importance of these sequences in ACTH action, we discovered a mutation (p.R8C; HF*R*W > HF*C*W) that abolishes ACTH binding and cAMP production in MC1R-, MC2R-, and MC4R-expressing cells (Samuels *et al.* 2013). ACTH-R8C was found to be immunoreactive, but failed to bind and activate cAMP production in MC2R-expressing cells, whereas α-MSH-R8C failed to bind and stimulate cAMP production in MC1- and MC4-expressing cells. Discovery of this mutation indicates that, in humans, the $His^{6}Phe^{7}Arg^{8}Trp^{9}$ (HFRW) sequence is important not only for cAMP activation but also for ACTH binding to MC2R (Samuels *et al.* 2013).

Pathological consequences of MC2R deficiency for the adrenal cortex

Mutations in the *MC2R* gene are responsible for 25% of familial glucocorticoid deficiency (FGD) and mutations in the *MRAP* gene, encoding the MC2R accessory protein MRAP, are responsible for 20% of FGD (Meimaridou *et al.* 2013, Jackson *et al.* 2015). FGD is an autosomal

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recessive disorder resulting in cortisol deficiency, due to resistance of the adrenal cortex to the action of ACTH. Postmortem examination of adrenal glands from FGD patients demonstrated a disorganization of glomerulosa cells and almost completed absence of ZF and ZR, suggesting that MC2R and/or MRAP may be important for the development of adrenal zonation (Gorrigan *et al*. 2011).

Effects of ACTH on the adrenal cortex

Steroids produced by the adrenal cortex

The adrenal cortex produces several steroid hormones, the most important being cortisol (glucocorticoid), aldosterone (mineralocorticoid), and androgen precursors. All these hormones are essential for homeostasis as well as survival. Disorders of the adrenal glands lead to classical endocrinopathies such as Cushing's syndrome, Addison's disease, hyperaldosteronism, and the syndromes of congenital adrenal hyperplasia (CAH) (Miller & Auchus 2011).

Aldosterone is produced exclusively in the ZG due to the specific expression of P450 aldosterone synthase (P450aldo, CYP11B2), whereas cells from the ZF and ZR, which express P450c11β-hydroxylase (P450c11, CYP11B1), synthesize glucocorticoids (GC) (cortisol in humans, bovine, and dogs and corticosterone in rodents, except hamsters that produce cortisol). However, the ZR, through P450c17,20 lyase (CYP17A1), produces the androgen precursors, dehydroepiandrosterone (DHEA), its sulfated derivative DHEAS (which circulates at concentrations 1000 times higher than DHEA) and androstenedione, at least in humans and higher primates, but not in rodents (Vinson 2003, Arlt & Stewart 2005) (Fig. 1). The relative thickness of each zone is correlated with the efficacy and daily production of steroids (Rainey 1999).

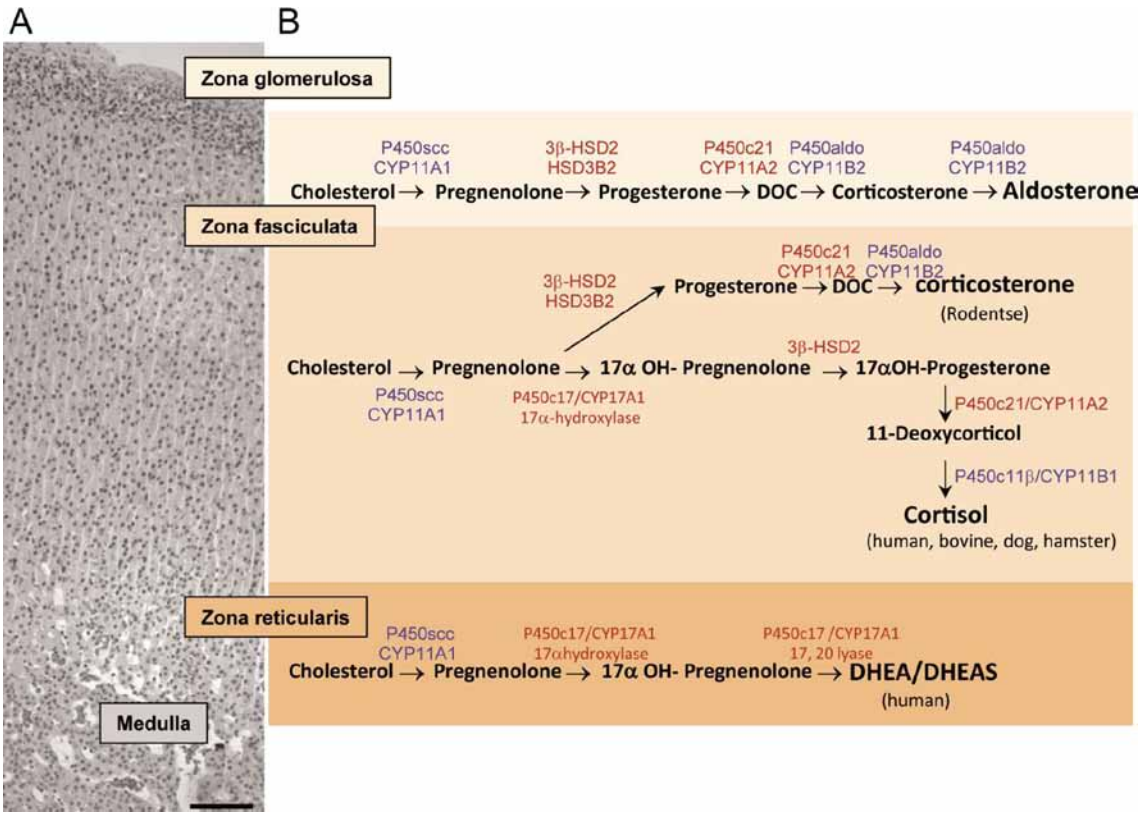

Figure 1
Steroidogenesis in the three zones of the adrenal cortex. (A) Hematoxylin- and eosin-stained section of an adult rat adrenal gland. Scale bar, 100 µm. (B) Free cholesterol is recruited in three enzymatic pathways, leading to aldosterone in zona glomerulosa; corticosterone or cortisol in zona fasciculata and zona reticularis; and dehydroepiandrosterone (DHEA), DHEAS, and androstenedione in zona reticularis. Cholesterol is cleaved in the inner mitochondrial membrane by P450 cholesterol side-chain cleavage enzyme (P450scc/CYP11A1) into pregnenolone. Further steps involve the enzymes indicated in the figure. The steps indicated in red take place in the mitochondria and the steps indicated in blue take place in the endoplasmic reticulum. Data from Arlt & Stewart PM (2005).

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Indeed, the amount of aldosterone needed to control salt balance is 100- to 1000-fold lower than that needed to control carbohydrate metabolism, and in humans, daily production of aldosterone is in the order of pmol/L (100–150 g/day), compared with the nmol/L range for cortisol/corticosterone (10–20 mg/day) and mol/L range for DHEAS (up to 20 mg/day) (Arlt & Stewart 2005).

Mineralocorticoids, such as aldosterone, stimulate sodium reabsorption, hence maintaining blood volume and pressure in sodium-depleted conditions. Excessive aldosterone secretion not only leads to hypertension and electrolyte imbalance, but is also associated with cardiometabolic complications (Funder & Reincke 2010, Briet & Schiffrin 2011). However, GC (cortisol, corticosterone, and cortisone) are implicated in a broad range of metabolic functions, including anti-inflammatory responses, stress response, and behavior (Chan *et al.* 2011, Corander & Coll 2011), increasing blood glucose concentrations through their action on glycogen, protein, and lipid metabolism (Arlt & Stewart 2005). However, chronically elevated GC levels alter body fat distribution, increase visceral adiposity, and are responsible for several metabolic abnormalities leading to metabolic syndrome (Dallman *et al.* 2004).

Role of ACTH in corticosteroid rhythmicity

Circulating GC levels are higher during the activity period (day for diurnal species and night for nocturnal species), and peak levels are linked to the beginning of the activity period (for rats, nadir in the morning and peak in the late afternoon). These circadian changes in ACTH and corticosterone are associated with circadian expression of steroidogenic genes and those involved in ACTH signaling (Park *et al.* 2013). In addition to the driving role established by the suprachiasmatic nucleus (SCN) (Chung *et al.* 2011, Ota *et al.* 2012), sensitivity of the adrenal glands to ACTH stimulation could be regulated through adrenal splanchnic innervation (Ulrich-Lai *et al.* 2006*a*) and by intra-adrenal circadian clockwork (Son *et al.* 2008). Interestingly, in rats, although the *Mc2r* gene is induced by ACTH, *Mc2r* mRNA is at its highest levels in the morning, when ACTH is minimal. By contrast, MRAP expression peaks in the evening, consistent with the circadian rhythm of ACTH. These data suggest that it is the circadian rhythm of MRAP, rather than of MC2R, that results in increased adrenal sensitivity to ACTH in the evening (Park *et al.* 2013). By contrast, the circadian rhythm of plasma aldosterone in recumbent normal subjects on a regular diet is independent of ACTH, but regulated by the activity of plasma renin (Williams *et al.* 1972). An exception is found in patients with aldosterone-producing adenomas, where short-term decrease in ACTH (by administration of dexamethasone) eliminates or markedly alters the circadian variation of plasma aldosterone, suggesting that patients with primary aldosteronism have a circadian rhythm of plasma aldosterone mediated by changes in ACTH (Kem *et al.* 1975).

Jet lag or sleep perturbations results in a transient mismatch between the internal circadian time and the external light–dark cycle. Over long periods, these changes are associated with increased body mass index and alterations in the levels of circulating insulin, glucose, and GCs (Van Cauter *et al.* 2008). Moreover, alterations in GC rhythmicity and dissociation of GC secretion from ACTH secretion occur during various pathological conditions, including Cushing's syndrome, metabolic syndrome, mood disorders, and even Alzheimer's disease (Bornstein *et al.* 2008, Chung *et al.* 2011, Russell *et al.* 2014).

Effects of ACTH on steroidogenesis

Under physiological conditions, cortisol and adrenal androgen secretion are controlled primarily by ACTH, although having a more complex action on ZG and aldosterone secretion. The response of adrenocortical cells to ACTH can be divided into two phases: the acute phase, which occurs within seconds to minutes, involves transcription-independent stimulation of adrenal steroid synthesis, although the more sustained phase affects not only steroidogenic capability, but also size and structural integrity of the gland, as evidenced by the atrophy observed after hypophysectomy or in POMC-deficient animals (Coll *et al.* 2004) (Chan *et al.* 2011, Corander & Coll 2011).

The acute response of ACTH involves mobilization and delivery of free cholesterol from lipid droplets to the inner mitochondrial membranes where it is metabolized by P450scc/CYP11A1 to pregnenolone – the first enzymatic step in the steroid hormone biosynthetic pathway. The transfer of free cholesterol from the outer to the inner mitochondrial membrane is triggered by phosphorylation and activation of the steroidogenic acute regulatory protein (StAR) (Stocco 2000, Jefcoate 2002), the rate-limiting protein of steroidogenesis. In ZG cells, such effects also involve calcium (Ca^{2+})- and calmodulin-dependent processes (Cherradi *et al.* 1996).

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StAR does not act alone but is part of a multi-protein complex, which includes translocator protein (TSPO) (Rone *et al.* 2009) but also arachidonic acid (AA) metabolites (Maloberti *et al.* 2007). Then, the various steps of steroidogenesis take place alternatively in mitochondria and in the endoplasmic reticulum (where the three cytochrome P450 enzymes and one hydroxysteroid dehydrogenase (3β-HSD) are localized) and in a zone-specific manner, as illustrated in Fig. 1 (Stocco *et al.* 2005, Miller & Auchus 2011). Of note, steroids (which are lipoplytic hormones) are immediately released after synthesis, in contrast to peptidic hormones, which are stored in secretory vesicles.

In ZF, chronic treatment with ACTH (from hours to days) increases the expression of a number of genes including those involved in cholesterol availability, through selective lipoprotein-derived cholesterol (Kraemer 2007, Hu *et al.* 2010) and synthesis of the enzymes required for steroidogenesis, including StAR (Fleury *et al.* 1998). These latter actions are mediated by various transcription factors, one of the most important being the nuclear receptor NR5A1/steroidogenic factor 1 (SF1) (required not only for the expression of most of the steroidogenic enzymes, but also for the development of the adrenal cortex) (Sewer & Waterman 2003, Schimmer *et al.* 2006, Schimmer & White 2010, Xing *et al.* 2010, Miller & Auchus 2011). Chronic treatment with ACTH also increases the volume of the adrenal glands and blood flow within it (Mazzocchi *et al.* 1986, Thomas *et al.* 2004). Chronic stress (which mimics chronic ACTH treatment) induces hyperplasia in the outer ZF and hypertrophy in the inner ZF, but reduces the size and properties of the ZG. These effects are associated with elevated corticosterone responses (Ulrich-Lai *et al.* 2006*b*).

Effects of ACTH on protection against reactive oxygen species accumulation

Intense steroidogenesis in ZF leads to oxidative stress due to lipid peroxidation, and to the production of reactive aldehyde metabolites such as isocaproaldehyde (Hornsby & Crivello 1983, Lefrançois-Martinez *et al.* 1999). This may explain the large quantity of endogenous anti-oxidant compounds (vitamin E, β-carotene, and vitamin C) (Hornsby & Crivello 1983) and the presence of enzymes implicated in detoxification of steroidogenesis by-products (Martinez *et al.* 2001) in the adrenal glands (Lefrançois-Martinez *et al.* 1999, Chinn *et al.* 2002). To prevent cell toxicity, these reactive oxygen species (ROS) are metabolized to isocaproic acid by a family of aldo-keto reductases (AKR), including *Akr1b8* and *Akr1b7* in mice and *AKR1B10* in humans (Lefrançois-Martinez *et al.* 1999, Pastel *et al.* 2012). These enzymes are highly expressed in the adrenal glands, and their levels of expression are correlated with the level of ACTH (Schimmer *et al.* 2007).

Another mechanism used by cells to circumvent the negative side effects of intense steroidogenesis is through induction of 24-dehydrocholesterol reductase (DHCR24) (a member of the flavin adenine dinucleotide (FAD)-dependent oxidoreductase family) (Sarkar *et al.* 2001). As for AKR1B7, in human and rat adrenocortical cells, SELective Alzheimer disease INdicator 1 (seladin-1) is more abundant in ZF/ZR than in ZG, and ACTH treatment increases its expression and its nuclear localization (Battista *et al.* 2009). Overall, chronic levels of ACTH increase transcription of the genes that encode the steroidogenic enzymes, but also those involved in ROS detoxification (such as AKR and seladin-1), thereby maintaining optimal steroid production and reduction of harmful lipid aldehydes (Lefrançois-Martinez *et al.* 1999).

Acute effect of ACTH on aldosterone secretion and consequences of chronic ACTH treatment

The role of ACTH in the ZG and in aldosterone secretion is subject to controversy and probably more complex than currently perceived. Indeed, *in vivo* studies suggest that ACTH is rather a weak stimulus of aldosterone secretion; however, based on *in vitro* studies, ACTH is the most potent stimulus of aldosterone secretion. Continuous intravenous administration of ACTH leads to a sustained stimulation of cortisol secretion but to a transient stimulation of aldosterone secretion, followed by a decrease in prestimulation levels by 72 h. By contrast, pulsatile infusion of ACTH leads to a stimulation of aldosterone secretion, which is maintained for up to 72 h (Seely *et al.* 1989). Moreover, aldosterone secretion is more sensitive to low doses of ACTH(1–24) than the secretion of cortisol or DHEA (Daidoh *et al.* 1995), especially in humans under conditions of low-sodium intake (Rayfield *et al.* 1973, Kem *et al.* 1975, Nicholls *et al.* 1975).

Moreover, sustained exposure to ACTH (2 days or more) leads to transformation of the ZG cells into ZF cells. From a mechanistic point of view, several mechanisms may explain this transient response of glomerulosa cells to ACTH. In primary cultures of bovine adrenocortical cells, a 2 h ACTH treatment was sufficient to increase 17α-hydroxylase (P450c17) and 11β-hydroxylase (P450c11) activity by 55-folds in mitochondria from

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ZF cells, although the latter was reduced by 50% in mitochondria from ZG cells, as for 18-hydroxylase activity (P450c11B2). In addition, in ZG cells from adrenal glands of ACTH-treated rats (6 days, 2UI/day), Ang II receptors and Ang II-stimulated aldosterone are markedly decreased (Aguilera *et al.* 1981), whereas the production of deoxycorticosterone and precursor steroids is conversely increased, indicating a blockade in the late step of aldosterone synthesis (Bird *et al.* 1996). These functional changes are accompanied by a morphological transformation of ZG cells into ZF-like cells (Manuelidis & Mulrow 1973, Hornsby *et al.* 1974, Muller 1978, Crivello & Gill 1983, Pudney *et al.* 1984). In particular, mitochondria changed from an elongated shape with lamellar and tubular cristae to a homogeneous population of round or ovoid mitochondria with ovoid cristae, as in ZF cells (Armato *et al.* 1974, Riondel *et al.* 1987) (Vinson 2003, Corander & Coll 2011, Hattangady *et al.* 2012, Gallo-Payet & Battista 2014).

Effect of ACTH on adrenal growth

The adrenal cortex is a very dynamic organ, in which secretory activities correlate with morphology and structure according to external stimuli or environmental conditions. For example, a sodium-deficient diet increases width and volume of ZG, without affecting ZF. A study conducted with adrenals from 61 surgical/autopsy patients from 1 day old to 92 years old has revealed that the ZG was well developed in human adrenals from newborn to the third decade. However, after 40 years of age, an important decrease in ZG was observed. ZG cells become scattered and both ZG and ZF are surrounded by a progenitor zone, which has the ability to differentiate bidirectionally into either ZG-topped columns or ZF-topped columns, according to secondary aldosteronism or to exposure to severe stresses. These authors suggest that the involution of ZG with age may be due to the current high-sodium/low-potassium diet in humans compared with earlier human populations even as recently as 50 years ago (Aiba & Fujibayashi 2011).

However, ACTH deficiency decreases, while ACTH treatment increases the volume of ZF (Rebuffat *et al.* 1989, Thomas *et al.* 2004). Knockout of the *Mc2r* gene in mice leads to neonatal lethality in most of the animals, possibly as a result of hypoglycemia. Animals surviving to adulthood have a marked atrophy of the ZF. However, the ZG remains fairly intact, although aldosterone secretion was significantly decreased (Chida *et al.* 2007). These results confirmed and extended the importance of the ACTH–MC2R complex in adrenal development, as in the production of corticosterone and probably aldosterone (Chida *et al.* 2007). Supporting this conclusion is the recent observation of high levels of expression of MC2R and MRAP in the undifferentiated zone, which contains stem cells (Gorrigan *et al.* 2011).

The mechanisms involved in adrenocortical remodeling are complex and sometimes redundant, with the aim of preserving or restoring homeostasis or coping with stress (Pihlajoki *et al.* 2015). There are indications that ACTH is involved in various aspects of the dynamic organization of the adrenal cortex, namely cell migration and proliferation. It is generally assumed that proliferation takes place either under the capsule (stem cell region), in the ZG itself, or in the outer part of ZF and that cell senescence occurs mainly in ZR (Wolkersdorfer & Bornstein 1998, Kim *et al.* 2009). To discriminate between the effect of ACTH on cell proliferation or on cell hypertrophy, Engeland and his group have used a 14-day chronic variable stress paradigm in adult male rats. They found that chronic stress induced hyperplasia in the outer ZF, hypertrophy in the inner ZF and medulla, and reduced cell size in the ZG. These effects were associated with elevated corticosterone responses to ACTH (Ulrich-Lai *et al.* 2006*b*). However, there are indications that proliferation is probably not mediated by ACTH, but rather by other POMC-related peptides. Indeed, *in vivo* immunoneutralization of circulating ACTH reduces corticosteroid levels, but increases mitogenesis (Estivariz *et al.* 1982); cell proliferation in the ZF in *Mc2r*-knockout mice is comparable to cell proliferation in wild-type mice (Chida *et al.* 2007), whereas in *Pomc*-knockout mice, the absence of cell proliferation results in the atrophy of adrenal glands (Coll *et al.* 2004, Karpac *et al.* 2005). Further in-depth investigations have revealed that the active domain of POMC-derived peptide is a small fragment, N-POMC (50–74) (also named γ3-melanocyte-stimulating hormone, γ3-MSH). This aspect is reviewed further in this issue by Andy Bicknel (Bicknell *et al.* 2001, Bicknell 2016).

In isolated cells in culture, ACTH inhibits cell proliferation to favor steroid secretion (Hornsby & Gill 1977, Mattos *et al.* 2011). It is now relatively well accepted that ACTH is preferentially a differentiation factor controlling steroid secretion rather than a proliferation factor. However, ACTH favors cell survival when viability is compromised, a protective effect occurring only when the adrenal glands are intact. Indeed, quartering of

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the glands enhances basal apoptosis and, interestingly, abolishes ACTH-induced inhibition of apoptotic DNA fragmentation, without altering ACTH-induced corticosterone secretion. These data suggest that the global organ architecture is required for modulation of adrenal cell survival by ACTH (Carsia *et al.* 1997). In another study conducted in mice, adrenal atrophy was observed after 14 days of dexamethasone treatment: a condition that suppresses ACTH secretion. Such treatment induced an important decrease in adrenal weight and cellularity, due to inhibition of cell proliferation, induction of cell apoptosis, and progressive regression of the vascular network. These data support the concept that ACTH had a trophic action on the adrenal cortex through a dual mechanism involving antiapoptotic effect and effects on vasculature (Thomas *et al.* 2004).

Effect of ACTH on gene expression

All the above effects of ACTH have been confirmed by measurements of gene expression (Xing *et al.* 2011, Nishimoto *et al.* 2012, Rege *et al.* 2014). In this regard, the Y1 mouse adrenocortical cell line is a model that has been widely used to identify changes in gene expression after treatment with ACTH. This cell line shares many features with normal cells from the adrenal cortex (Rainey *et al.* 2004, Schimmer *et al.* 2006). For example, a 15K mouse cDNA microarray was used to identify genome-wide changes in gene expression after a 20 min ACTH treatment with effects measured 24 h latter. ACTH affected the levels of 1275 annotated transcripts, of which 46% were up-regulated. Not surprisingly, the transcripts up-regulated in response to ACTH are those implicated in steroid biosynthesis and metabolism, transcription factors involved in the expression of the steroidogenic enzymes, and signaling molecules involved in the hormonal regulation of steroidogenesis. The transcripts down-regulated in response to ACTH are associated with DNA replication, mitotic activity, nuclear transport, and RNA processing. Such results are consistent with the growth-inhibiting effects of ACTH that are observed in Y1 cells under the conditions used in this study (Schimmer *et al.* 2006).

The signaling pathways of ACTH action

Although several second messengers have been described, the primary events following ACTH binding to MC2R is adenylyl cyclase (AC) activation and cAMP production together with Ca^{2+} influx. Thereafter, cAMP can directly activate various protein kinases, including protein kinase A (PKA), protein kinase C (PKC), mitogen-associated protein kinase (MAPK), ion-channels, guanine nucleotide exchange factors, or transcription factors.

In addition to human or nonhuman adrenocortical cells, two cell lines have been widely used to investigate the most selective signaling pathways, namely the Y1 mouse adrenocortical cell line and the NCI-H295R cells, involved in aldosterone secretion (Rainey *et al.* 2004). Indeed, in NCI-H295R cells, the expression of CYP11B2/Ang II is high, but level of expression of MC2R is low, whereas in Y1 cells, the expression of CYP11B2 and Ang II receptors is low, but the expression of MC2R is high.

Cyclic AMP and Ca^{2+}: lessons from structure–activity relationships

Since the pioneering work of Lefkowitz *et al.* (1970), several studies have shown that cAMP and Ca^{2+} interact closely through positive feedback loops to enhance steroid secretion (Fakunding *et al.* 1979, Fakunding & Catt 1980, Kojima *et al.* 1985*a*, Gallo-Payet & Payet 1989). The question of whether Ca^{2+} influx is consecutive to cAMP production and/or Ca^{2+} and cAMP are associated with different domains of the ACTH molecule is not yet resolved. Indeed, there are arguments supporting the view that ACTH(1–10) can stimulate steroid secretion through Ca^{2+}, without detectable changes in cAMP, whereas ACTH(5–24) or forskolin increases cAMP, and when used together, the two fragments reproduce the effects of ACTH(1–24) (Li *et al.* 1989). ACTH does not induce a rapid and transient Ca^{2+} influx (such as Ang II, which acts through phosphatidylinositol 4,5-bisphosphate (PtIns(4,5)P2), but instead induce a slow, but sustained, Ca^{2+} influx. The latter is mainly mediated by PKA-dependent phosphorylation of L-type Ca^{2+} channels (Tremblay *et al.* 1991), as stimulation of aldosterone by ACTH is completely inhibited by verapamil, an L-type Ca^{2+} channel blocker (Kojima *et al.* 1985*b*, Gallo-Payet *et al.* 1996) (Hattangady *et al.* 2012, Gallo-Payet & Battista 2014). Some studies have shown that rat ZG cells are much more sensitive to extracellular Ca^{2+} than ZF cells (Schiebinger *et al.* 1985). However, in bovine adrenal glands, sensitivities to Ca^{2+} of ZF cells and ZG cells are similar. Specifically, ACTH and *O*-nitrophenyl sulfenyl-ACTH (NPS-ACTH) (an analog of ACTH that does not increase cAMP) increase intracellular Ca^{2+} and stimulate cortisol synthesis by bovine ZF cells at concentrations

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that produce little or no increase in cAMP synthesis (Liu *et al.* 2010).

At least in ZG cells, Ca^{2+} acts on almost all steps of steroidogenesis: Gs activation of AC, cholesterol ester hydrolase activity, activation of intramitochondrial cholesterol transfer, and expression of StAR and most steroidogenic enzymes (Cherradi *et al.* 1998). The role of calcium/calmodulin-dependent protein kinase (Ca^{2+}-CaMK) in adrenal aldosterone production has recently been confirmed, using both pharmacological and molecular approaches (Nanba *et al.* 2015). Nishimoto and coworkers (Nishimoto *et al.* 2012, 2013) have compared the transcriptional profiles of ZG and ZF in rats. Although similarities between early ACTH events in ZG and ZF were detected, important differences were identified. With the exception of *Cyp11b2* and the gene encoding Ang II receptor type 1, these authors identified genes encoding extracellular matrix proteins, Ca^{2+} and K^+ channels, as well as transforming growth factor beta (TGF-β), and members of the WNT/β-catenin and ACTH signaling pathways (Nishimoto *et al.* 2013).

Mechanisms regulating cAMP production and Ca^{2+} influx

Cyclic AMP levels and Ca^{2+} influx are regulated by multiple and sophisticated mechanisms. In particular, the intracellular concentration of cAMP is partly determined by (1) a balance between AC activation through the GTP-binding protein Gs and inhibition through the GTP-binding inhibitory protein Gi (Hausdorff *et al.* 1987, Begeot *et al.* 1988, Hausdorff *et al.* 1989); and (2) several isoforms of ACs (AC5/6, insensitive to Ca^{2+}, AC3, activated by Ca^{2+}, and AC4, activated by the βγ subunits of G proteins) (Shen *et al.* 1997, Côté *et al.* 2001). Studies of gene expression of the rat ZG indeed showed that AC3 and AC4 are selectively enriched in ZG (Nishimoto *et al.* 2013); (3) several isoforms of phosphodiesterases (PDEs), in particular cGMP-PDE2 (the highest concentrations being found in the ZG (McFarlane & Sowers 2003)) and PDE8, important in regulating corticosterone secretion in ZF cells (Tsai & Beavo 2011).

Effects of ACTH on electrical properties of adrenocortical cells

Adrenocortical cells are characterized by a very negative resting membrane potential ranging from –78 to –90 mV (thus similar to that found in excitable cells) and by the presence of several channels, including (1) voltage-dependent K^+ and Ca^{2+} channels; (2) two types of Ca^{2+} channels: the T-type or low-voltage-activated channels (referred to as $Ca_v3.x$ after the channels were cloned) and the L-type channels or high-voltage-activated channels ($Ca_v1.x$); (3) voltage-independent Ca^{2+} channels; and (4) background channels (such as TASK and TREK channels) (the 'tandem of P domains in a weak inwardly rectifying K^+ channel'). In addition, as excitable cells, adrenocortical cells are able to generate spontaneous action potentials (Matthews & Saffran 1973, Lymangrover 1980, Tabares & Lopez-Barneo 1986, Enyeart 2005, Guagliardo *et al.* 2012, Gallo-Payet & Battista 2014).

An important difference between ZG and ZF cells is their sensitivity toward K^+ ions (which are involved in cell depolarization, and therefore Ca^{2+} influx) and thus higher impact on aldosterone secretion, compared with corticosterone/cortisol secretion in ZF cells (Enyeart 2005, Guagliardo *et al.* 2012, Gallo-Payet & Battista 2014). Such observations could explain that, in humans, aldosterone secretion is more sensitive to low doses of ACTH(1–24) than the secretion of cortisol or DHEA (Daidoh *et al.* 1995), especially in conditions of sodium depletion (Rayfield *et al.* 1973, Kem *et al.* 1975, Nicholls *et al.* 1975). In rat and human ZG cells, binding of ACTH to its receptor induces a rapid membrane depolarization, in part due to blockade of K^+ channels (Payet *et al.* 1987, 1994). Simultaneously, depolarization transiently abolishes T-channel activity (Durroux *et al.* 1991) and increases the amplitude of the L-type current, through a cAMP-dependent or a PKA-dependent phosphorylation of these L-type channels (Durroux *et al.* 1991).

Early studies conducted in Y1 cells do not support the concept that activation of voltage-dependent Ca^{2+} channels is an important mechanism for steroidogenesis (Coyne *et al.* 1996), as the steroidogenic response to ACTH was observed even in the presence of blockers known to affect both Ca^{2+} and K^+ channels or in a medium containing low calcium concentration, suggesting that extracellular Ca^{2+} is not critical for a steroidogenic response (Coyne *et al.* 1996). However, subsequent studies performed with ZF cells from bovine origin have shown that ACTH affects the activity of various channels. ACTH inhibits bTREK-1 channels, inducing depolarization, which in turn induces activation of T- and L-type Ca^{2+} channels. Mibefradil, a specific T-channel blocker, inhibits ACTH-induced cortisol secretion in fasciculata cells (Enyeart *et al.* 1993). This mechanism is independent of PKA but can be mimicked

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by exchange protein directly activated by cAMP (Epac)-specific cAMP analogs (Liu *et al.* 2008). Epacs also enhance the expression of both Cav3.2 and functional Ca^{2+} channels (Liu *et al.* 2010). The contribution of Ca^{2+} to genome-wide actions of ACTH has been explored in Y1 cells (Schimmer *et al.* 2007). Cells were treated with the Ca^{2+} ionophore A23187 (10 µM) for 24 h to promote Ca^{2+} influx and changes in transcript accumulation were profiled using the 1.7 K human cDNA array. One hundred and twenty nine transcripts were up-regulated and 127 were down-regulated by this treatment, and 45 of these matched transcripts were regulated by ACTH. Interestingly, most of the ACTH-regulated transcripts assigned to the Ca^{2+} signaling pathway by these criteria also fulfilled criteria for activation *via* the cAMP pathway (Schimmer *et al.* 2007), further indicating that Ca^{2+} and cAMP are not independent, but closely interconnected.

Secondary intracellular events and implication of extracellular matrix (ECM) and cytoskeleton

Although cAMP-PKA-Ca^{2+} mediates most of the effects of ACTH, a number of PKA-independent effects of cAMP, including involvement of the exchange protein, were directly activated by cAMP (Epac1/2) (Liu *et al.* 2010). Moreover, the observations that ACTH and/or cAMP induced morphological changes of adrenal cells from flat and adherent to round and loosely attached prompted many investigators to investigate how the cytoskeleton (in particular through reorganization of the actin filament network and its associated proteins) was implicated in ACTH responses (Feuillolley & Vaudry 1996, Côté *et al.* 1997, Hall & Almahbobi 1997, Sewer & Li 2008).

Over the years, some of these non-canonical pathways have been well documented. For example, it has been known for decades that ACTH stimulates arachidonic acid (AA) release through a cAMP- and PKA-dependent mechanism and its lipoxygenase products (Hirai *et al.* 1985) are part of a complex of proteins that participate in the activation of StAR (Kang *et al.* 1997, Wang *et al.* 2003), but also in the transport of cholesterol into mitochondria (Cooke *et al.* 2011). Breakdown of phosphatidylinositol 4,5-bisphosphate (PtIns(4,5)P2) has been reported, both in bovine ZF cells (Bird *et al.* 1990) and in rat ZG cells (Gallo-Payet & Payet 1989). However, the production of inositol trisphosphate induced by ACTH is not sufficient to release Ca^{2+} from intracellular stores, thus suggesting that diacylglycerol (the other second messenger resulting from PtIns(4,5)P2 breakdown) and subsequent PKC activation may have a role in ACTH-induced steroid secretion (Cozza *et al.* 1990) or in the functional zonation of the adrenal cortex. It has been shown that PKC-induced activin A suppresses ACTH stimulation of CYP17A1 in the ZG to favor steroidogenesis toward aldosterone secretion, thereby contributing to functional adrenocortical zonation (Hofland *et al.* 2013).

The contribution of cell–matrix interactions to intracellular events leading to steroidogenesis is now well documented (Cheng & Hornsby 1992), in which fibronectin and collagens favor steroid synthesis and laminin favors cell proliferation (Otis *et al.* 2007), chemotaxis, and haptotaxis (Feige *et al.* 1998). Binding of ECM components to their receptors, integrins, favors tyrosine phosphorylation of several focal adhesion proteins that facilitate spreading of cells on their substratum, in particular on fibronectin and collagens. The rounding up of the cells following ACTH stimulation is correlated with both a loss of focal adhesions and a specific decrease in paxillin phosphorylation. This latter effect is mediated by the phosphotyrosine phosphatase, SHP-2 (Rocchi *et al.* 2000), itself activated by PKA-dependent serine phosphorylation. This last step has been reported to be essential for cAMP-induced corticosterone secretion (Sewer & Li 2008, Cooke *et al.* 2011, Gallo-Payet & Battista 2014). Li and Sewer (2010) showed that these cytoskeleton-associated modifications may dictate the nature of the steroid production. These examples support the view that the morphological and functional responses to PKA activation in steroidogenic cells are closely related to cytoskeleton dynamics in interaction with ECM and integrins (illustrated in Fig. 2).

Involvement of MAPK pathways Initial studies performed with bovine and rat adrenocortical cells have shown that ACTH does not stimulate p44/p42^{mapk} activity under conditions in which Ang II is effective (Chabre *et al.* 1995, Gallo-Payet *et al.* 1999), although *in vivo* ACTH increases ERK1 (p44^{mapk}), but not ERK2 (p42^{mapk}) in ZG, but not in the inner zones (McNeill *et al.* 2005). In Y1 adrenocortical cells (Lotfi *et al.* 1997, Le & Schimmer 2001), NCI-H295R cells (Janes *et al.* 2008), and more recently MC2R-transfected cells (Sebag & Hinkle 2010, Roy *et al.* 2011), ACTH induces a rapid increase in p44/p42^{mapk} phosphorylation while also promoting a lower but sustained and concentration-dependent p38 MAPK phosphorylation. The c-Jun N-terminal kinases pathway, however, was not stimulated under the same conditions. Examination of the mechanism involved indicates that cAMP participates in, but does not reproduce, p44/p42^{mapk} activation by ACTH (Roy *et al.* 2011), as ACTH is more

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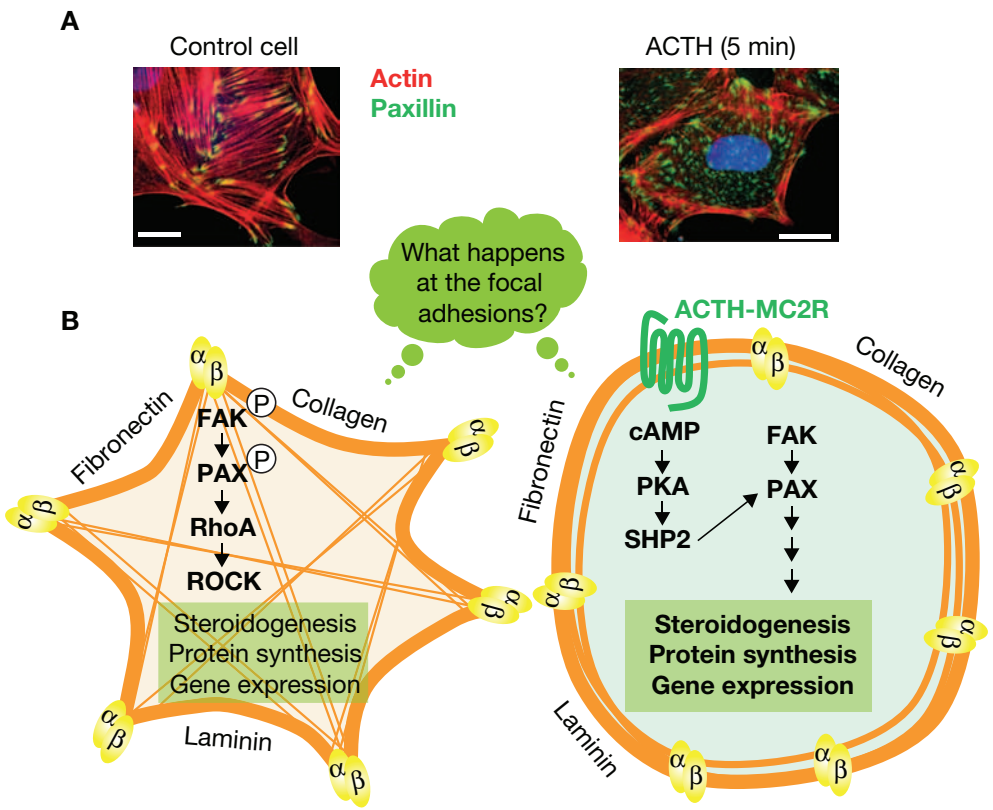

Figure 2
Involvement of the extracellular matrix (ECM) and the cytoskeleton in ACTH-stimulated rat adrenal glomerulosa cells. (A) Immunofluorescence labeling of actin filaments and paxillin of rat glomerulosa cells, incubated without (Control) or with 10 nM ACTH for 5 min. Cells were processed for immunofluorescence labeling, using phalloidin coupled to Alexa-Fluor 594 nm for visualization of F-actin (red) and with anti-paxillin antibody coupled to Alexa-Fluor 488 nm for visualization of paxillin (green). Merged images are illustrated. Scale bars, 13 µm. (B) Illustration of signaling pathways linked to ECM and cytoskeleton. In control conditions, binding of fibronectin or collagen to their integrins promotes strong cell adhesion, evidenced by the flat polygonal morphology, the thin stress fibers across the entire cell and by the presence of focal adhesion points revealed by paxillin labeling, as illustrated by the green fluorescent dots in the left part of panel A. ACTH induces a rapid but transient formation of a dense F-actin ring at the cell membrane, with disruption of the stress fiber network, as illustrated in the right part of panel A. These changes are accompanied by a dephosphorylation of paxillin at the plasma membrane and by the activation of the actin-associated kinases, such as the phosphotyrosine phosphatase, SHP2, which increase cell functionality.

efficient in increasing p44/p42^{mapk} phosphorylation than forskolin or cAMP analogs. Phosphorylated p44/p42^{mapk} was observed in the cytoplasm rather than in the nucleus, supporting the view that localization of p44/p42^{mapk} in the cytoplasm may be associated with cellular differentiation, such as steroid biosynthesis or hypertrophy or (Poderoso *et al.* 2008).

Other ECM components which affect cell morphology and function In addition to the proteins mentioned above, other ECM components affect cell morphology and function. Among these are ephrins (EphA) and their receptors, which are mainly present in the ZG. Interestingly, the level of expression of EphA2 closely correlates with changes in the ZG phenotype, in particular it is increased in animals on a low-sodium diet (which increases ZG size), but decreased by ACTH treatment (which increases ZF size) (Brennan *et al.* 2008). Another family of extracellular matrix proteins, thrombospondins, is expressed in bovine adrenal glands, with thrombospondin 2 (TSP2) promoting cell attachment but preventing spreading of adrenocortical cells in primary culture (Feige *et al.* 1998).

Gap junction channels These channels facilitate direct exchange between adjacent cells, thus enabling propagation of signaling throughout neighboring cells. *In vivo* and *in vitro* studies have shown a strong positive correlation between ACTH-increased steroidogenesis of the adrenal glands and the expression of connexin 43 (α1Cx43), the main component of gap junctions in the

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adrenal cortex. However, there is an inverse correlation between Cx43 expression and cell proliferation in human adrenocortical tumors (Murray et al. 2003).

Adrenocortical pathologies associated with defective signaling pathways

Although mutations in genes encoding steroidogenic enzymes have long been described as the main cause of adrenal cortex pathologies, more recent molecular studies have shown that several intracellular mediators of ACTH action may also have an important impact on these pathologies, in particular in cortisol-producing adrenocortical tumors. For example, McCune–Albright syndrome is caused by mutations in the gene encoding the α-subunits of G proteins (*GNAS*); in Carney complex and in adrenocortical adenomas, inactivating mutations in the *PRKAR1A* gene (encoding the RIα subunit of PKA) lead to micronodular hyperplasias including a pigmented form referred to as primary pigmented nodular adrenocortical disease (PPNAD) (de Joussineau *et al.* 2012, Berthon *et al.* 2015, Lacroix *et al.* 2015); mutations in *PDE8B* and *PDE11A* have been found in adrenal hyperplasia, Cushing's syndrome, or in polycystic ovary syndrome (PCOS) (Horvath *et al.* 2006, Tsai & Beavo 2011, Leal *et al.* 2015). Decreased expression of cAMP-regulated aldose reductase (*AKR1B1*) is associated with malignancy in human sporadic adrenocortical tumors (Lefrançois-Martinez *et al.* 2004), or mutations in the components of the Wnt pathway are frequently found in adrenocortical tumors and carcinomas where β-catenin accumulates in the nucleus (El Wakil & Lalli 2011, Berthon *et al.* 2012).

FGD, characterized by the failure of the adrenal cortex to produce GC, was first shown to be caused by loss-of-function mutations in *MC2R*. After the discovery of the causative role of MRAP1 in FDG, more recent studies also identified another protein from the same family, MRAP2, which seems to be linked to obesity (Meimaridou *et al.* 2013, Jackson *et al.* 2015). Finally, it is important to consider extra-pituitary production of ACTH. In particular, recent studies indicate that cortisol secretion by adrenal glands in patients with macronodular hyperplasia and Cushing's syndrome is regulated by ACTH produced in hyperplastic adrenal glands by a subpopulation of steroidogenic cells (Louiset *et al.* 2013). Following this discovery that the hypercortisolism associated with bilateral macronodular adrenal hyperplasia appears to be ACTH-dependent, 'ACTH-independent macronodular adrenocortical hyperplasia (AIMAH)' has been renamed as 'primary macronodular hyperplasia (PMAH)' (Louiset *et al.* 2013).

From genomics to physiopathology

Recent studies have shown dysregulated microRNA (miRNA) expressions in adrenocortical tumors. In particular, miR-483-3p, miR-483-5p, miR-210, and miR-21 were found to be overexpressed, whereas miR-195, miR-497, and miR-1974 were found to be underexpressed in adrenocortical cancers (Ozata *et al.* 2011, Chabre *et al.* 2013). These dysregulated miRNAs are detectable in serum samples and may be candidate serum biomarkers for distinguishing between benign and malignant adrenocortical tumors (Patel *et al.* 2013).

Gene expression profiling of human adrenocortical tumors using cDNA microarrays have identified several candidate genes as markers of malignancy (de Fraipont *et al.* 2005). For example, PA represents the most common cause of secondary hypertension, characterized by dysregulation of aldosterone production (Cao *et al.* 2012, Monticone *et al.* 2012). The expression of aldosterone synthase (CYP11B2), MC2R, and their regulating transcription factors are increased in adrenal incidentaloma (AI)-hypertensive patients compared with normotensive patients and thus may be used to distinguish subclinical or atypical primary aldosteronism (PA) from AIs (Cao *et al.* 2012).

Recent information also connects PA and channel deficiencies (channelopathies). Two background K^+ channels have been associated with PA in rodents and humans: *KCNK3* (*TASK1*) and *KCNK9* (*TASK3*), one G-protein-activated inward rectifier K^+ channel 4 (GIRK4, encoded by the *KCNJ5* gene) and the voltage-dependent T-type Ca^{2+} channel (CaV3.2) (Chen *et al.* 2015). *TASK1* affects cell differentiation and prevents expression of aldosterone synthase in the ZF, whereas *TASK3* controls aldosterone secretion in ZG cells (Bandulik *et al.* 2014). Mice with single deletions of the *Task1* or *Task3* gene as well as *Task1/Task3* double knockout mice display partially autonomous aldosterone synthesis. These deletions also have a profound impact on adrenal zonation (Davies *et al.* 2008, Heitzmann *et al.* 2008). Indeed, deletion of *Task1* changed adrenal zonation and expression of CYP11B2, which was absent in the outermost ZG but was expressed to a large extent in the ZF. Furthermore, this expression pattern seemed to be restricted to females and to males before puberty. TASK channels maintain the membrane potential of ZG cells at a polarized ~70 mV by being constitutively open and acting as a K^+ leak channel. Decreased expression of TASK2 is also associated with a higher expression of miR-23 and miR-34, steroidogenic

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acute regulatory protein, and CYP11B2, thus enhancing aldosterone production (Lenzini *et al.* 2014).

Besides TASK channels, mutations occurring near the selectivity filter of the inward rectifying K^+ channel *KCNJ5* (Kir3.4) also result in PA (Choi *et al.* 2011). *KCNJ5* mutations are prevalent in sporadic APAs. These mutations interfere with the selectivity filter of GIRK4 causing Na^+ entry, cell depolarization, and Ca^{2+} channel opening, resulting in constitutive aldosterone production (Mulatero *et al.* 2013). Voltage-gated Ca^{2+} channels are also implicated in PA (Felizola *et al.* 2014). Indeed, calcium channel blockers can be efficiently used in the treatment of PA-related hypertension. The α-subunits of L-, N-, and T-type calcium channels have been analyzed in 74 adrenocortical aldosterone-producing adenomas (APAs) and 16 cortisol-producing adenomas using quantitative RT-PCR. Among these channel subunits, only CaV3.2 mRNA levels were significantly correlated with plasma aldosterone levels, CYP11B2 expression levels, and the presence of *KCNJ5* mutations in APA, suggesting that they are involved in Ca^{2+}-related aldosterone biosynthesis (Felizola *et al.* 2014).

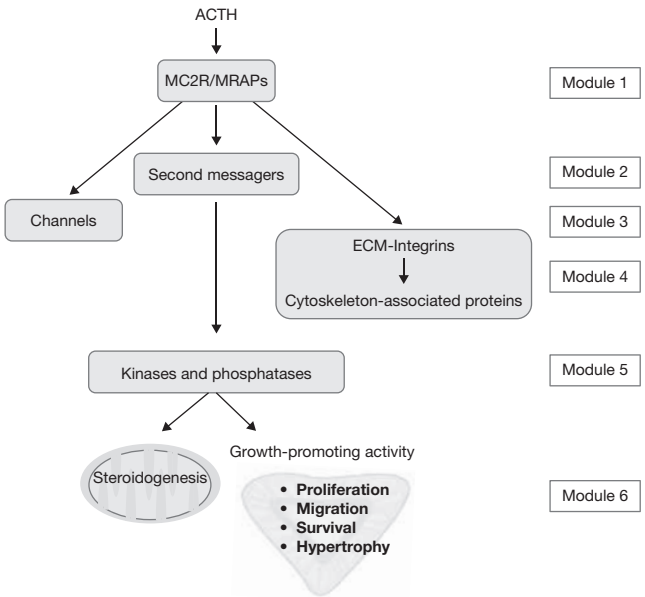

Figure 3
Overview of the main signaling modules implicated in the effect of ACTH on adrenocortical cells. Regulation of ACTH action on adrenocortical cells may occur at different levels that can be divided into modules: Module 1, ACTH binding to its receptor, MC2R; Module 2, production of second messengers; Module 3, modulation of membrane channels; Module 4, implication of the extracellular matrix and cytoskeleton; Module 5, activation of various kinases and phosphatases; and finally Module 6, proteins and enzymes engaged in steroidogenesis or trophic action. Each of these modules could be considered as independent signaling cascades that interact through some of their elements, as illustrated in Fig. 4.

Conclusion of ACTH and adrenal function

Although cAMP is still considered to be the main second messenger of ACTH action, and PKA the most important kinase stimulated by ACTH, each of the other ACTH effectors mentioned in this review are equally important modulators of ACTH response, as part of complex intracellular signaling platforms. The mechanism of action and regulation of StAR is an example of this complexity. StAR acts through a protein complex, the 'transduceosome' comprising, in addition to the TSPO, a voltage-dependent anion channel, a TSPO-associated protein 7 (PAP7), and protein kinase A regulatory subunit 1α (PKAR1A) (Miller & Auchus 2011, Manna *et al.* 2009, Rone *et al.* 2009). All pathways implicated in steroidogenesis and adrenal growth are closely interconnected and probably dependent on the extracellular matrix and the cytoskeleton (for a summary, see Fig. 3 and 4). For example, cell environment is important to dictate the nature of steroids secreted (cortisol vs DHEA) and even the activation of transcription factors (e.g., Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome, gene 1 (DAX1)) (Chamoux *et al.* 2002, Battista *et al.* 2005, Otis *et al.* 2007, Li & Sewer 2010). ACTH loses its protective effects when the adrenal architecture is disrupted (Carsia *et al.* 1997). The precise mechanisms of interactions between the ECM and integrin receptors with the cytoskeleton and intracellular kinases is beginning to emerge but is yet to be correlated with *in vivo* physiology.

Extra-adrenal actions of ACTH

Evidence for the presence of MC2R in tissues other than the adrenal cortex begins to emerge. In many instances, MC2R has the same properties in other tissues as in the adrenal cortex, namely acting as a differentiating factor and using the same main signaling pathways. Some examples of ACTH action in tissues other than the adrenal cortex are given below.

ACTH and adipocyte functionality

The demonstration of the presence of both MC2R (ACTH receptor) and MC5R (α-MSH receptor), in murine 3T3-L1 cells differentiated into adipocytes (Cammas *et al.* 1995, Noon *et al.* 2004, Moller *et al.* 2011), has confirmed earlier studies showing that ACTH stimulates lipolytic activity in mature adipocytes. Indeed, knockdown of *Mc2r* in

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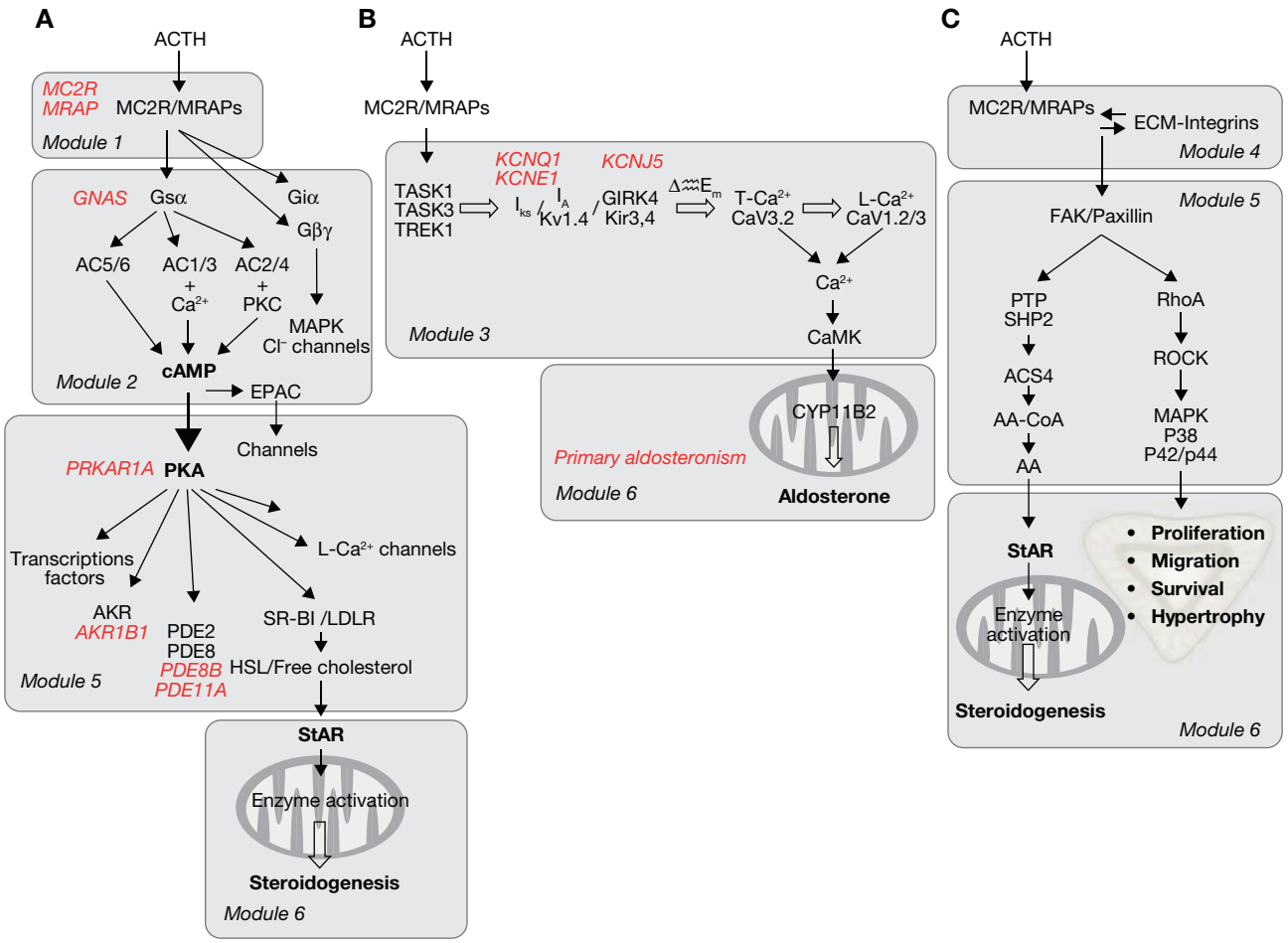

Figure 4

Illustrations of the main signaling cascades stimulated by ACTH, from binding to its receptor to cellular function in adrenocortical cells. (A) ACTH binds to MC2R and through interaction with MRAPs (Module 1) and initiates signaling, by activating Gs and various isoforms of ACs that increase cAMP. MC2R is also linked to G_i protein; activation of αi decreases the level of cAMP, whereas the release of βγ-subunits stimulates other effectors such as Mitogen-activated protein kinases (MAPK) cascade or cationic Cl^- channels (Module 2). Binding of cAMP to the regulatory subunits of protein kinase A results in the phosphorylation of several proteins, including steroidogenic acute regulatory protein (StAR) and the hormone-sensitive lipase. Protein kinase A (PKA) also regulates the level of expression of the receptors implicated in the uptake of cholesterol and genes encoding the steroidogenic enzymes (Module 5). The final output of this cascade is steroidogenesis, which is initiated in the mitochondria. cAMP also has a number of PKA-independent effects, including involvement of the exchange protein directly activated by cAMP (Epac1/2). cAMP also regulates its own intracellular level through activation of phosphodiesterases, in particular, PDE2 and PDE8 (Module 5). (B) Simultaneously, ACTH induces depolarization of the cell membrane inducing Ca^{2+} influx (Module 3). PKA also activates Ca^{2+} influx through L-type channels. The subsequent increase in intracellular calcium (Cai) activates Ca^{2+}-CaMK and steroidogenesis (Module 6). (C) Activated MC2R also interact with ECM and cytoskeleton-associated proteins (Module 4), modulating the phosphorylation and activation of a number of proteins that are involved in functional integrity of the cells. A decrease in paxillin phosphorylation and activation of the phosphotyrosine phosphatase, SHP2, itself activated by PKA-dependent serine phosphorylation is responsible for the rapid effect of ACTH on the rounding-up of adrenocortical cells in culture. SHP2 also induces dephosphorylation of specific substrate(s), including some involved directly or indirectly in steroidogenesis, such as the acyl-CoA synthetase (ACS4), which sequesters AA as arachidonyl-CoA (AA-CoA) (Module 5), hence participating in StAR activation and initiation of steroidogenesis (Module 6). Cytoskeleton-associated proteins and/or PKA are also implicated in the activation of the MAPK signaling, necessary to promote the trophic action of ACTH (Module 5). Clearly identified pathogenic mutations of key proteins are indicated in *red*. Among these mutations are loss of function of *MC2R* or *MRAPs*, activating mutations of the *GNAS* gene (encoding Gsα subunit), inactivating mutations of genes encoding the regulatory subunit of PKA (Ria) (*PRKAR1A*), encoding phosphodiesterases (*PDE11A* and *PDE8B*) or Aldo-keto-reductases (*AKR1B1*). Some mutations in voltage-dependent K^+ channels are directly involved in primary aldosteronism, in particular mutations of the *KCNJ5* gene encoding the potassium channel Kir3.4 (also called G-protein-activated inward rectifier potassium channel 4, GIRK4), and of the two genes *KCNQ1* and *KCNE1*, encoding the pore- and regulatory subunits of the slowly activating delayed K^+ current, I_{ks}. The resulting sustained Ca^{2+} influx increases activation of CYP11B2 and thus sustained increase in aldosterone secretion. Finally, the temporal integration of these signaling pathways may be coordinated at the levels of signaling microdomains, for example, through A kinase-anchoring proteins, or AKAPs (not illustrated).

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3T3-L1 cells reduces lipid content and inhibits expression of differentiation regulators such as peroxisome proliferator-activated receptor (PPARγ2) (Noon *et al.* 2004, Betz *et al.* 2012). ACTH and α-MSH are also potent inhibitors of leptin expression (Norman *et al.* 2003). Studies from Iwen and coworkers (Iwen *et al.* 2008) indicate that chronic stimulation of white adipocytes with high doses of ACTH decreases insulin-induced glucose uptake as well as the expression of visfatin and adiponectin genes, whereas the pro-inflammatory cytokine, interleukin-6 (*IL-6*), and monocyte chemoattractant protein-1 mRNA levels are acutely up-regulated. Thus, ACTH could lead to dysregulation of energy balance, insulin resistance, and cardiometabolic complications when the pituitary–adrenal axis HPA is dysregulated or is under chronic inflammation (Iwen *et al.* 2008).

The role of melanocortins in the physiology of human adipocytes is yet to be fully elucidated. In *ex vivo* experiments with human adipocytes from obese subjects, high expression levels of MC1R, but only low levels of MC2R, have been detected (Smith *et al.* 2003). Nevertheless, MC2R is expressed in human mesenchymal cells (MSC) during adipogenic induction (Smith *et al.* 2003), suggesting that MC2R may have a role as a differentiating factor as in 3T3-L1 cells, but not in fully differentiated cells (Smith *et al.* 2003, Betz *et al.* 2012).

ACTH and matrix synthesis in mesenchymal cells

The expression of MCR in mesenchymal progenitor cell populations is also well documented (Evans *et al.* 2013). In particular, MC2R and MRAP are expressed in human and murine osteoblast cell lines, where they can play a role in differentiation through production of vascular endothelial growth factor (VEGF) (Zaidi *et al.* 2010). In murine osteoblasts, ACTH appears to be a regulator of bone mass, enhancing collagen production (Isales *et al.* 2010, Zaidi *et al.* 2010), an effect occurring in a dose-dependent manner through a transient increase in intracellular Ca^{2+}. Neither γ_2-MSH, a potent MC3R agonist, nor α-MSH, a potent MC5R agonist, duplicates the effects of ACTH, indicating the specificity of ACTH-MC2R action. Mouse aorta-derived mesenchymal progenitor cells also express both MC2R and MC3R. These progenitors respond to ACTH by increasing collagen matrix synthesis and intracellular Ca^{2+} and suggest a role in the maintenance and repair of the vascular extracellular matrix (Evans *et al.* 2013). The same study indicates that both macrophages and mesenchymal cells are relevant sources of local POMC peptides.

ACTH and thymus growth

ACTH directly controls thymic growth through MC2R, which is expressed in thymic epithelium. Adrenalectomized mice treated with ACTH under conditions repressing endogenous ACTH secretion exhibit an increase in the number of thymocytes and splenic naive T-cells compared with control animals. These results show that ACTH directly controls thymocyte homeostasis independently of circulating GC (Talaber *et al.* 2015).

Involvement in the skin In the skin, mRNA for *MC2R* and mRNAs for three obligatory enzymes of steroid synthesis, cytochromes P450scc, P450c17, and P450c21, have been detected in normal and pathological human samples (Slominski *et al.* 1996*b*). In fact, all components of the pituitary–adrenal axis have been detected in the skin, suggesting a role in regulating immune system or hair growth. However, this remains to be better explored for ACTH–MC2R complex, as these latter actions are best known to be mediated by α-MSH peptide (Schauer *et al.* 1994, Slominski *et al.* 1996*a*).

In mouse testis In fetal/neonatal mouse testis, the ACTH–MC2R complex is localized in Leydig cells, in which it stimulates androgen production. The mechanisms of action involve not only cAMP-PKA, but also AA (*via* phospholipase A2) and $p44/p42^{mapk}$ activation of StAR (Johnston *et al.* 2007).

In prostate cells In the prostate cell lines, LNCaP, PC3, and DU-145 cells, ACTH, through MC2R-induced cAMP, promotes concentration-dependent cell proliferation, suggesting that MC2R is involved in prostate carcinogenesis and that targeting MC2R signaling may provide a novel avenue in prostate carcinoma treatment (Hafiz *et al.* 2012).

ACTH also has a renoprotective effect in chronic kidney disease

In a rat model of tumor necrosis factor (TNF)-induced acute kidney injury, Si *et al.* (2013) found that ACTH gel prevented kidney injury, corrected acute renal dysfunction, and improved survival. Morphologically, ACTH gel ameliorated TNF-induced acute tubular necrosis, associated with a reduction in tubular apoptosis.

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ACTH and brain function

The idea that the adrenal cortex through corticosteroids may have a role in mood has been recently reviewed (Vinson & Brennan 2013). Indeed, changes in mood are a common consequence of chronic corticosteroid therapy. Corticosteroids are known for their capacity to generate both euphoria and depression in humans, even if these effects are still poorly understood. It is also known that ACTH/MSH neuropeptides affect social behavior, interact with opiate binding sites, and possess antiepileptic properties. ACTH/MSH peptides also possess neurotropic activities, stimulating regeneration of damaged nerve cells (de Wied 1990, Vinson & Brennan 2013).

Taken together, the data summarized above suggest that the ACTH–MC2R complex is involved in cell differentiation, not only in adipocytes, but also in a variety of tissues, from mesenchymal cell populations to adipocytes as well as in steroidogenesis in skin, testis, and prostate. Furthermore, a high level of ACTH or increased expression of MC2R could contribute to HPA, or to metabolic-related pathologies.

Conclusion: challenges and perspectives

As we have shown in this review, signaling pathways (i.e. second messengers and subsequent intracellular events) in interaction with ECM and integrins control cell fate decisions that ultimately determine the behavior of adrenocortical cells toward steroidogenesis, growth, and eventually aberrant physiology and pathological consequences (see summary in Fig. 3 and 4). Some of the examples given in this review indicate that the time-dependent production of these intracellular mediators may be important to consider in the final cell response. Yet, a transient vs a sustained production of cAMP or MAPK activation does not elicit the same final response. Furthermore, in addition to the well-described signaling cascades illustrated in Fig. 4, some other signaling pathways would deserve further exploration; in particular interaction of second messengers with the scaffold proteins, A kinase-anchoring proteins (AKAPs). AKAPs can target many signaling proteins to specific locations within the cell, creating preferential interactions on the scaffold. For example, AKAP79/150 can associate with K^+ voltage-dependent channels, ACs, or L-type Ca^{2+} channels. AKAPs can increase the rate at which signal transduction occurs or increase the magnitude of the signal response (Dessauer 2009, Greenwald & Saucerman 2011).

Computational models have been recently developed for the integration of quantitative data from complex systems that could be used as platforms to investigate the dynamic biochemical properties of cells. Studying the dynamics of pathway activity may provide prognostically relevant information different from the information provided by other types of biomarkers, due to their static nature (Hughey *et al*. 2010). Therefore, due to the complexity of the various interacting pathways involved in the regulation of adrenocortical functions (Figs 3 and 4), it would be interesting to develop similar models to explore the potential involvement of these pathways in specific adrenocortical pathologies. For example, alterations in one step could induce a switch activation from one function to another, resulting in the loss or gain of a physiological function, and thus in pathological situations (Lefrançois-Martinez *et al*. 2004, Horvath *et al*. 2006, Tsai & Beavo 2011, de Joussineau *et al*. 2012, Leal *et al*. 2015). The integration of various technologies (such as transcriptomics, proteomics, or metabolomics) combined with computational and mathematical models could be used to identify new therapeutic agents, drug targets, and novel biomarkers, as demonstrated for other paradigms in several recent publications (de Fraipont *et al*. 2005, Choi *et al*. 2011, Patel *et al*. 2013, Lenzini *et al*. 2014, Resendis-Antonio *et al*. 2015).

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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