60 YEARS OF POMC

Adrenal and extra-adrenal functions of ACTH

Nicole Gallo-Payet1,2

1 Division of Endocrinology, Department of Medicine, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Quebec, Canada
2 Centre de recherche clinique Étienne-Le Bel of the Centre Hospitalier Universitaire de Sherbrooke (CHUS), Sherbrooke, Quebec, Canada

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.

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
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 & Metherell 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 <sup>W</sup> > HF<sup>C</sup>) that abolishes ACTH binding and cAMP production in MC1- and MC2R-expressing cells, whereas α-MSH-R8C 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<sup>6</sup>Phe<sup>7</sup>Arg<sup>8</sup>Trp<sup>9</sup> (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 MRA 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
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 complete 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).

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**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 (P450scoc/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).
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 2013). 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 steroidalogenic 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. 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).

Effects of ACTH on steriodogenesis

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 steroidalogenic 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 P450ccc/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 steroidalogenic acute regulatory protein (STAR) (Stocco 2000, Jeffcoate 2002), the rate-limiting protein of steriodogenesis. In ZG cells, such effects also involve calcium (Ca\(^{2+}\))- and calmodulin-dependent processes (Cherradi et al. 1996).
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 lipolytic 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. 2006b).

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 oxido-reductase family) (Sarkar et al. 2001). As for Akr1B7, in human and rat adrenocortical cells, SELLective 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
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, 2U/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. 2006b). 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
the glands enhances basal apoptosis and, interestingly, abolishes ACTH-induced inhibition of apoptotic DNA fragmentation, without altering ACTH-induced cortisol 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 Ca2+ 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 Ca2+: lessons from structure–activity relationships

Since the pioneering work of Lefkowitz et al. (1970), several studies have shown that cAMP and Ca2+ interact closely through positive feedback loops to enhance steroid secretion (Fakunding et al. 1979, Fakunding & Catt 1980, Kojima et al. 1985a, Gallo-Payet & Payet 1989). The question of whether Ca2+ influx is consecutive to cAMP production and/or Ca2+ 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 Ca2+, 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 Ca2+ influx (such as Ang II, which acts through phosphatidylinositol 4,5-bisphosphate (PIns(4,5)P2), but instead induce a slow, but sustained, Ca2+ influx. The latter is mainly mediated by PKA-dependent phosphorylation of L-type Ca2+ channels (Tremblay et al. 1991), as stimulation of aldosterone by ACTH is completely inhibited by verapamil, an L-type Ca2+ channel blocker (Kojima et al. 1985b, 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 Ca2+ than ZF cells (Schiebinger et al. 1985). However, in bovine adrenal glands, sensitivities to Ca2+ 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 Ca2+ and stimulate cortisol synthesis by bovine ZF cells at concentrations...
that produce little or no increase in cAMP synthesis (Liu et al. 2010).

At least in ZG cells, Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} and K\textsuperscript{+} channels, as well as transforming growth factor beta (TGF-\beta), and members of the WNT/\beta-catenin and ACTH signaling pathways (Nishimoto et al. 2013).

Mechanisms regulating cAMP production and Ca\textsuperscript{2+} influx

Cyclic AMP levels and Ca\textsuperscript{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\textsuperscript{2+}, AC3, activated by Ca\textsuperscript{2+}, and AC4, activated by the \(\beta\gamma\) 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\textsuperscript{+} and Ca\textsuperscript{2+} channels; (2) two types of Ca\textsuperscript{2+} channels: the T-type or low-voltage-activated channels (referred to as Ca\textsubscript{3,2} after the channels were cloned) and the L-type channels or high-voltage-activated channels (Ca\textsubscript{1,2}); (3) voltage-independent Ca\textsuperscript{2+} channels; and (4) background channels (such as TASK and TREK channels) (the ‘tandem of P domains in a weak inwardly rectifying K\textsuperscript{+} 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\textsuperscript{+} ions (which are involved in cell depolarization, and therefore Ca\textsuperscript{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\textsuperscript{+} 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\textsuperscript{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\textsuperscript{2+} and K\textsuperscript{+} channels or in a medium containing low calcium concentration, suggesting that extracellular Ca\textsuperscript{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\textsuperscript{2+} channels. Mibebradil, 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...
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\textsuperscript{2+} channels (Liu et al. 2010). The contribution of Ca\textsuperscript{2+} to genome-wide actions of ACTH has been explored in Y1 cells (Schimmer et al. 2007). Cells were treated with the Ca\textsuperscript{2+} ionophore A23187 (10 μM) for 24 h to promote Ca\textsuperscript{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\textsuperscript{2+} signaling pathway by these criteria also fulfilled criteria for activation via the cAMP pathway (Schimmer et al. 2007), further indicating that Ca\textsuperscript{2+} and cAMP are not independent, but closely interconnected.

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

Although cAMP-PKA-Ca\textsuperscript{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 (Feuilloley & 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 lipoygenase 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 (PtdIns(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\textsuperscript{2+} from intracellular stores, thus suggesting that diacylglycerol (the other second messenger resulting from PtdIns(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/p42mapk activity under conditions in which Ang II is effective (Chabre et al. 1995, Gallo-Payet et al. 1999), although in vivo ACTH increases ERK1 (p44mapk), but not ERK2 (p42mapk) 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/p42mapk 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/p42mapk activation by ACTH (Roy et al. 2011), as ACTH is more...
Efficient in increasing p44/p42mapk phosphorylation than forskolin or cAMP analogs. Phosphorylated p44/p42mapk was observed in the cytoplasm rather than in the nucleus, supporting the view that localization of p44/p42mapk 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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**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.
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 R1a subunit of PKA) lead to micronodular hyperplasias including a pigmented form referred to as primary pigmented nodular adrenocortical disease (PPNAD) (de Joussineau 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 FGD, 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 Ca2+ channel (CaV3.2) (Chen et al. 2015). TASK1 affects cell differentiation and prevents expression of aldosterone synthase in the ZE, 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 ZE. 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
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 (Kir3.4) also result in PA (Choi et al. 2011). Voltage-gated Ca2+ 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 Ca2+-related aldosterone biosynthesis (Felizola et al. 2014).

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
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 Gi protein; activation of ac decreases the level of cAMP, whereas the release of Pr 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++ influx (Module 3). PKA also activates Ca++ influx through L-type channels. The subsequent increase in intracellular calcium (Ca(i)) activates Ca++-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 aky-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 Gαs 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, Iaq. The resulting sustained Ca++ 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).
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 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. 1996b). 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. 1996a).

**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.
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 anti-epileptic 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 Ca2+ 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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Adrenal and extra-adrenal functions of ACTH

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