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Acute and Chronic Regulation of Aldosterone Production

Namita Hattangady[*,1], **Lawrence Olala**[*,1], **Wendy B. Bollag**[1,2], and **William E. Rainey**[1,†]

[1]Department of Physiology, Georgia Health Sciences University (formerly the Medical College of Georgia), 1120 15th Street, Augusta, GA 30912

[2]Charlie Norwood VA Medical Center, One Freedom Way, Augusta, GA 30904

Abstract

Aldosterone is the major mineralocorticoid synthesized by the adrenal. Secretion of aldosterone is regulated tightly by the adrenocortical glomerulosa cells due to the selective expression of CYP11B2 in the outermost zone, the zona glomerulosa. Aldosterone is largely responsible for regulation of systemic blood pressure through the absorption of electrolytes and water under the regulation of certain specific agonists. Angiotensin II (Ang II), potassium (K^+) and adrenocorticotropin (ACTH) are the main physiological agonists which regulate aldosterone secretion. The mechanisms involved in this process may be regulated minutes after a stimulus (acutely) through increased expression and phosphorylation of the steroidogenic acute regulatory (StAR) protein, over hours to days (chronically) by increased expression of the enzymes involved in the synthesis of aldosterone, particularly aldosterone synthase (CYP11B2). Imbalance in any of these processes may lead to several aldosterone excess disorders. In this review we attempt to summarize the key molecular events involved in and specifically attributed to the acute and chronic phases of aldosterone secretion.

Introduction

Aldosterone is the major mineralocorticoid involved in maintaining fluid and electrolyte balance in all mammals. In humans, excessive secretion of this hormone results in hypertension, contributes to cardiac fibrosis, congestive heart failure, and exacerbates the morbidity and mortality associated with these disorders (Gekle and Grossmann, 2009; Marney and Brown, 2007). Although the signal transduction processes regulating aldosterone production under physiological and pathophysiological conditions are as yet incompletely understood, ongoing research has identified several important pathways mediating steroidogenesis. Aldosterone production (equivalent to secretion in the case of this steroid hormone) is primarily regulated by angiotensin II (AngII), serum potassium, as well as adrenocorticotropic hormone (ACTH).

Steroidogenesis (Aldosterone Production)

In mammals, aldosterone biosynthesis occurs almost solely in the adrenal zona glomerulosa. Aldosterone is derived through a series of enzymatic steps that involve three cytochrome

†To whom correspondence should be addressed: William E. Rainey, Department of Physiology, Georgia Health Sciences University, 1120 15th Street, Augusta, GA 30912, wrainey@georgiahealth.edu, Tel: (706) 721-7665, Fax: (706) 721-7299.
*These authors contributed equally to the manuscript.

P450 enzymes and one hydroxysteroid dehydrogenase (Figure 1). The enzymes cholesterol side-chain cleavage (CYP11A1), 21-hydroxylase (CYP21) and aldosterone synthase (CYP11B2) belong to the cytochrome P450 family of enzymes. CYP11A1 and CYP11B2 are localized to the inner mitochondrial membrane, while CYP21 is found in the endoplasmic reticulum. Cytochrome P450 enzymes are heme-containing proteins that accept electrons from NADPH via accessory proteins and utilize molecular oxygen to perform hydroxylations (CYP21 and CYP11B2) or other oxidative conversions (CYP11A1). The fourth enzyme, type 2 3β-hydroxysteroid dehydrogenase (HSD3B2), is a member of the short-chain dehydrogenase family and is localized in the endoplasmic reticulum. Aldosterone and cortisol share the first few enzymatic reactions in their biosynthetic pathways (cholesterol to progesterone); however, adrenal zone-specific expression of CYP11B2 (aldosterone synthase) in the glomerulosa and that of CYP11B1 (11β-hydoxylase) in the fasciculata leads to the functional zonation observed in the adrenal cortex (Rainey, 1999).

Like all steroid hormones, the glomerulosa cell uses cholesterol as the primary precursor for steroidogenesis. The cholesterol needed for adrenal steroidogenesis can come from several sources, which include de novo synthesis from acetate or cholesteryl esters stored in lipid droplets or up take from lipoproteins by the low-density lipoprotein (LDL) receptor (for LDL) or scavenger receptor-BI (for high-density lipoprotein or HDL). Movement of cholesterol from the outer mitochondrial membrane, across the aqueous intra-membranous space, to the inner mitochondrial membrane must occur for CYP11A1 to access the molecule for cleavage to pregnenolone. Because steroid hormones are secreted upon synthesis, the initial reaction involving mitochondrial conversion of cholesterol to pregnenolone is tightly controlled and represents the rate-limiting step in aldosterone synthesis. This step is regulated by the expression and phosphorylation of steroidogenic acute regulatory protein (StAR) (Arakane et al., 1997; Fleury et al., 2004; Manna et al., 2009). Pregnenolone passively diffuses into the endoplasmic reticulum and is converted to progesterone by HSD3B2. Progesterone is hydroxylated to deoxycorticosterone by CYP21. Finally, aldosterone biosynthesis is completed in the mitochondria, where deoxycorticosterone undergoes 11β- and 18-hydroxylation, followed by 18-oxidation, which in humans can be mediated by a single enzyme, CYP11B2. Although the last step of cortisol production also involves the 11-hydroxylation of cortisol to 11-deoxycortisol by 11β-hydoxylase, this enzyme only poorly catalyzes the 18-hydroxylation reaction and does not catalyze the 18-oxidation.

There are several factors regulating aldosterone production in the adrenal zona glomerulosa. First, the selective expression of CYP11B2 in the glomerulosa creates a tightly controlled zone-specific ability to make aldosterone and limits production of the steroid outside of this relatively small adrenal zone (Domalik et al., 1991; Ogishima et al., 1992; Pascoe et al., 1995). In rats and mice CYP11B2 is expressed in a tight zonal pattern that circles the adrenal (Domalik et al., 1991; Ogishima et al., 1992). A recent study revealed a variation in human adrenal glomerulosa zonation characterized by the presence of relatively few subcapsular cell clusters expressing CYP11B2 (Nishimoto et al., 2010). This phenotype may relate to the relatively high sodium diet and resultant suppression of the renin-angiotensin system in most human populations. It is hypothesized that these CYP11B2-expressing cortical cell clusters may be the precursors to aldosterone-producing adenomas (APA) (Nishimoto et al., 2010). The absence of CYP17 in glomerulosa cells is a second mechanism resulting in diversion of the steroidogenic pathway toward aldosterone (Narasaka et al., 2001). Finally, the centripetal blood flow in the adrenal cortex, itself, prevents the precursors of aldosterone in the fasciculata cells from accessing CYP11B2 in the zona glomerulosa, thereby helping to maintain the functional specificity of the adrenocortical zones.

The regulation of glomerulosa aldosterone biosynthesis is divided into two key events in the steroidogenic pathway (Clark et al., 1992; Muller, 1998). Acutely (minutes after a stimulus), aldosterone production is controlled by rapid signaling pathways that increase the movement of cholesterol into the mitochondria where it is converted to pregnenolone. This has been called the “early regulatory step” and is mediated by increased expression and phosphorylation of StAR protein (Figure 2) (Arakane et al., 1997; Cherradi et al., 1998; Fleury et al., 2004; Manna et al., 2009). Chronically (hours to days), aldosterone production is regulated at the level of expression of the enzymes involved in the synthesis of aldosterone (Figure 3) (Bassett et al., 2004b). This has been called the “late regulatory step” and is particularly dependent on increased expression of CYP11B2. The goal of this review article is to summarize our current understanding of the signaling pathways that regulate the early and late regulatory steps of aldosterone biosynthesis.

Acute Effects of AngII

As mentioned above, the initial enzymatic step in aldosterone biosynthesis is the hydrolysis of cholesterol to pregnenolone by the cholesterol side-chain cleavage complex found in the inner mitochondrial membrane. Thus, initiation of aldosterone production in response to agonists such as AngII, elevated extracellular potassium concentrations or ACTH requires two major steps: first, cholesterol mobilization from lipid droplets to the mitochondria is thought to require cytoskeletal rearrangements (Crivello and Jefcoate, 1979; Crivello and Jefcoate, 1980; Feuilloley and Vaudy, 1996) and activation of cholesteryl ester hydrolase by, e.g., extracellular signal-regulated kinase-1 and -2 (ERK-1/2) (Cherradi et al., 2003). Mobilization is followed by movement of the cholesterol from the outer to the inner mitochondrial membrane, a process requiring active StAR protein (Clark et al., 1994; Krueger and Orme-Johnson, 1983; Krueger and Orme-Johnson, 1988; Pon and Orme-Johnson, 1985; Pon and Orme-Johnson, 1986). As discussed below, StAR appears to be regulated both at the level of transcription (Jo et al., 2005; Manna et al., 2009) and post-translationally, in that phosphorylation appears necessary for its full activity (Arakane et al., 1997; Fleury et al., 2004; Manna et al., 2009; Stocco et al., 2005).

Both of these processes are triggered, in the case of AngII, by the binding of the hormone to the type 1 AngII (AT_1) receptor and the activation of several signaling pathways (Figure 2). One such pathway is a phosphoinositide-specific phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Barrett et al., 1989; Bird et al., 1993; Bollag et al., 1991; Farese et al., 1981; Ganguly and Davis, 1994; Hunyady et al., 1990; Kojima et al., 1984a). IP_3 is thought to initiate aldosterone secretion by eliciting a transient increase in the cytosolic calcium concentration and activating calcium/calmodulin-dependent protein kinases (CaMK). CaMK activity is clearly important in mediating aldosterone secretion, as inhibition of this enzyme decreases AngII-induced aldosterone secretion (Ganguly et al., 1992; Pezzi et al., 1996; Spät and Hunyady, 2004). On the other hand, DAG functions to stimulate protein kinase C (PKC), the activity of which has been suggested by some groups to underlie sustained aldosterone secretion from bovine glomerulosa cells (Bollag et al., 1990; Bollag et al., 1992; Bollag et al., 1991; Kapas et al., 1995; Kojima et al., 1984a). This idea is supported by the ability of phorbol esters (Betancourt-Calle et al., 1999; Kojima et al., 1985d), a DAG-generating enzyme (Bollag et al., 1990) and synthetic DAGs (Kojima et al., 1985d) to elicit aldosterone secretion and of a selective PKC inhibitor to decrease AngII-induced aldosterone secretion (Kapas et al., 1995). However, other groups have suggested an inhibitory role of PKC in the aldosterone secretory response as discussed below (Aptel et al., 1996; Hajnoczky et al., 1992; Rossier et al., 1995).

There are two additional pathways activated by AngII binding to the AT_1 receptor, through mechanisms that are as yet incompletely defined. The first is an increase in calcium influx, in part through CaM kinase II (Barrett et al., 2000; Fern et al., 1995; Lu et al., 1994; Yao et al., 2006) and GTP-binding proteins (Lu et al., 1996), with this enhancement acting synergistically with PKC-activating agents to stimulate secretion. Indeed, this calcium influx is essential for a continued aldosterone secretory response (Barrett et al., 1989; Ganguly and Davis, 1994), as well as for regulating PKC activity (Kojima et al., 1994), since inhibition of calcium influx with calcium channel antagonists decreases AngII-induced aldosterone secretion (Kojima et al., 1985c; Rossier et al., 1996; Rossier et al., 1998; Spät and Hunyady, 2004). Calcium influx occurs through voltage-dependent T- and L-type calcium channels, as well as store-operated calcium channels or capacitative calcium influx (Aptel et al., 1999; Burnay et al., 1994; Burnay et al., 1998; Spät and Hunyady, 2004). The activity of these voltage-dependent channels is maintained by appropriate membrane polarization, maintained by proper functioning of potassium channels, such as TWIK-related acid-sensitive K (TASK) channels (Davies et al., 2008; Nogueira et al., 2010b). Emptying of the endoplasmic reticulum store by IP_3 also results in capacitative calcium influx, as discussed above. The importance of this calcium signal to acute aldosterone secretion, functioning largely through activation of CaMK, is well appreciated and has been reviewed thoroughly (Spät and Hunyady, 2004).

On the other hand, the role of DAG effector enzymes such as protein kinase C (PKC), in modulating aldosterone synthesis remains somewhat controversial, with some reports showing that activation of the PKC pathway enhances aldosterone secretion, and others that this pathway inhibits aldosterone production (Hajnoczky et al., 1992; Kojima et al., 1984a; Kojima et al., 1983; LeHoux et al., 2001; LeHoux and Lefebvre, 2006; Lehoux and Lefebvre, 2007). Thus, some investigators have shown that mimicking the PKC and calcium influx signals with pharmacologic agents essentially reproduces AngII responses including aldosterone secretion (Bollag et al., 1990; Kojima et al., 1984a; Kojima et al., 1985c) and the protein phosphorylation pattern (Barrett et al., 1986). These results suggest that the aldosterone secretory response to AngII requires both a PKC activation event and a calcium influx stimulus. In contrast, there is evidence to indicate that the DAG signal and its effectors oppose aldosterone secretion acutely in rat glomerulosa cells, with inhibition of PKC by pretreatment with the non-selective inhibitor staurosporine enhancing subsequent AngII-induced aldosterone secretion (Hajnoczky et al., 1992). This result has been confirmed in experiments with the selective PKC inhibitors bisindolymaleimide I and Gödecke 6976 (LeHoux et al., 2001). Other experiments have observed essentially no effect of DAG-mimicking phorbol esters on more chronic aldosterone secretion from the human adrenocortical carcinoma cell line, H295R (Bird et al., 1995b). One possible explanation arises from the lack of selectivity/specificity of phorbol esters as mimics of the DAG pathway and/or PKC inhibitors as blockers of the pathway (see below). Clearly, additional studies are required to define the role of DAG-mediated signaling in AngII-induced aldosterone synthesis.

Another signaling system activated by AngII is phospholipase D (PLD), which can also increase DAG (indirectly) and presumably activate PKC (Bollag et al., 1990; Bollag et al., 2002; Zheng and Bollag, 2003). PLD, of which there are two well-characterized mammalian isoforms PLD1 and PLD2, hydrolyzes phospholipids, primarily phosphatidylcholine, to yield phosphatidic acid (phosphorylated DAG), which can then be converted to DAG by the action of lipid phosphate phosphatases (Bollag et al., 2007; Qin et al., 2010). Phosphatidic acid is a second messenger itself and is proposed to function as a slow-release reservoir of diacylglycerol for sustained cellular responses (Bollag et al., 1990; Bollag et al., 2007; Bollag and Xie, 2008a). Phosphatidic acid can also be deacylated by phospholipase A_2 to produce lysophosphatidic acid (LPA), a lipid message that activates G protein-coupled LPA

receptors. The released fatty acid can also be metabolized to additional lipid signals, such as eicosanoids and 12-hydroxyeicosatetraenoic acid (12-HETE), which has been reported to stimulate aldosterone secretion (Natarajan et al., 1990; Natarajan et al., 1988). Treatment of glomerulosa cells with exogenous PLD alone or in combination with the calcium channel agonist, BAY K8644, induces a sustained increase in aldosterone secretion without an increase in phosphoinositide hydrolysis (Bollag et al., 1990), suggesting that PLD activity may be sufficient to stimulate aldosterone secretion. PLD activity also appears to be necessary for AngII-induced aldosterone secretion, as demonstrated using the primary alcohol 1-butanol, which diverts production away from phosphatidic acid and DAG (Su et al., 2009) and instead forms phosphatidylbutanol (versus the control tert-butanol, which does not affect PLD-generated lipid signals). 1-Butanol was found to inhibit the AngII-induced increase in DAG and phosphatidic acid levels (Bollag et al., 2002), as well as AngII-elicited aldosterone secretion, in bovine adrenal glomerulosa cells (Bollag et al., 2002) and in H295R cells (Zheng and Bollag, 2003), whereas tert-butanol did not (Zheng and Bollag, 2003). More direct evidence arises from studies in which PLD was overexpressed using adenovirus-mediated transduction. Overexpression of wild-type PLD1 or PLD2, but not the lipase-inactive isoforms, increased PLD activity (Qin et al., 2010). However, only wild-type PLD2 enhanced AngII-stimulated aldosterone secretion (Qin et al., 2010), suggesting that PLD2 is the isoform mediating aldosterone secretion in response to AngII, although the lipid signal(s) mediating this effect is unclear.

Thus, as discussed above, DAG can be produced by both AngII-activated phospholipase C and PLD and, in turn, can activate DAG effector enzymes, which include PKC isoenzymes and protein kinase D (PKD), as well as Ras guanine nucleotide exchange factors (Ras-GRP1 through 3), which are upstream of the mitogen-activated protein kinase pathway involving Ras, Raf, MEK and ERK-1/2, and chimaerins (GTPase-activating proteins for Rac) (Brose and Rosenmund, 2002). PKC isoenzymes constitute a multigene family that belongs to the cAMP-dependent protein kinase A/protein kinase G/protein kinase C (AGC) family (Mellor and Parker, 1998). Members of the family play an important role in mediating numerous intracellular signaling events, including those involved in cell growth and differentiation, membrane trafficking, secretion, and gene expression (Mellor and Parker, 1998; Spät and Hunyady, 2004). The PKC family is divided into three groups based on their second messenger requirements: the conventional (also known as classical), novel, and atypical, with all members requiring acidic phospholipids such as phosphatidylserine for activation. Conventional and novel PKCs also require DAG and in addition conventional PKCs require calcium. Alpha, betaI, betaII and gamma comprise the conventional PKC subfamily, whereas novel PKCs include the delta, epsilon, eta, and theta isoforms. The novel isoforms have been shown to activate protein kinase D (PKD) via phosphorylating serines in the catalytic domain (Waldron and Rozengurt, 2003) (see below).

PKCs are also involved in regulating adrenal steroidogenesis through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on substrate proteins (Betancourt-Calle et al., 1999; Kapas et al., 1995; Lang and Valloton, 1987; Nakano et al., 1990; Natarajan et al., 1994). There is evidence to suggest that DAG activation of PKC coincides with the expression of the rate-limiting StAR protein (Manna et al., 2009) as well as StAR phosphorylation (Betancourt-Calle et al., 2001a). In turn, phosphorylation of StAR has been proposed to be required for its full activity (Arakane et al., 1997; Fleury et al., 2004; Manna et al., 2009). For example, using a mouse Leydig cell line, Stocco and colleagues (Manna et al., 2009) showed that activation of the PKC pathway can increase StAR expression but has a minimal effect on steroidogenesis, unless small concentrations of dibutyryl-cAMP are also included. Thus, these low doses of cAMP act synergistically with a PKC-activating phorbol ester to stimulate StAR phosphorylation and maximal steroid hormone production. Bollag and colleagues have also demonstrated that treatment of H295R

cells with the DAG-mimicking phorbol ester, phorbol 12-myristate 13-acetate (PMA) increases mitochondrial cholesterol levels and aldosterone secretion (Bollag et al., 2008), providing evidence for a possible role of PKC (and/or other DAG effector enzymes) in cholesterol transport and aldosterone production.

The 18kDa mitochondrial protein peripheral type benzodiazepine receptor (PBR), also known as translocator protein (TSPO), has also been implicated in steroidogenesis (Jefcoate, 2002; Papadopoulos et al., 2007). PBR has been proposed to functionally interact with StAR, among other proteins, to mediate mitochondrial cholesterol uptake into the mitochondria (Hauet et al., 2002). PBR is located on the outer mitochondrial membrane and is elevated in steroidogenic cells upon hormone stimulation (Hauet et al., 2002). Introduction of a null mutation of the PBR gene into R2C rat Leydig tumor cells inhibited pregnenolone production in response to 22R-hydroxycholesterol, a precursor that bypasses the need for StAR (Papadopoulos et al., 2007) supporting an important role for this protein in steroidogenesis. Recently, the DAG-sensitive PKC isozyme, PKC-epsilon was shown to mediate PBR gene expression (Batarseh et al., 2008) in MA-10 Leydig cells using PKC inhibitors and small interfering (si)RNA targeting PKC-epsilon, indicating a role for PKC-epsilon in regulating PBR expression (Batarseh et al., 2008). These results may provide insight into the possible role of the PKC pathway in mediating aldosterone secretion, although additional studies are certainly required to fully define the role(s) of this large family of serine/threonine protein kinases.

The PKD family of enzymes, composed of PKD1 (also known as PKC-μ), PKD2 and PKD3 (also known as PKC-n), represent DAG-activated serine/threonine kinases downstream of novel PKCs and a Src family tyrosine kinase pathway. An involvement of PKD family members has been proposed for a number of important cellular functions, including Golgi trafficking, hypertrophy, immune response, proliferation, migration, invasion and survival. (Guha et al., 2010; Lavalle et al., 2010), Importantly, PKD is activated in response to AngII in primary bovine adrenal glomerulosa cells (Shapiro and Bollag, 2004). Moreover, adenovirus-mediated overexpression of a constitutively active PKD1 mutant enhances, and a dominant-negative PKD1 mutant inhibits acute AngII-induced aldosterone secretion in these cells (Shapiro and Bollag, 2004). PKD performs a similar role to enhance chronic AngII-induced aldosterone secretion and aldosterone synthase expression in the H295R cell model (Romero et al., 2006), a result which was corroborated by a study showing that knocking down PKD levels with RNA interference decreased AngII-stimulated aldosterone secretion from these cells (Chang et al., 2007). Thus, PKD appears to mediate AngII-elicited aldosterone secretion acutely as well as to regulate aldosterone biosynthetic capacity of the adrenal. Also in the H295R cells, PKD activation in response to AngII resulted from PKC-epsilon-mediated transphosphorylation (Romero et al., 2006). While the mechanism by which PKD enhances aldosterone secretion is unclear, it is known that StAR gene transcription is influenced by several transcription factors that are targets for PKD, including cAMP response-element (CRE)-binding protein (CREB)/CRE modulator (CREM)/ activating transcription factor (ATF) family members and activator protein-1 (AP-1, Fos/ Jun) (Bassett et al., 2004a; Gonzalez and Montminy, 1989; Manna et al., 2003; Stocco et al., 2005). Indeed, Stocco and colleagues recently showed that PKD regulates StAR levels via CREB and AP-1 in MA-10 Leydig cells (Manna et al., 2011).

Acute Effects of Potassium

Similar to AngII, small increases in extracellular potassium levels also stimulate calcium influx, via depolarization of the glomerulosa cell plasma membrane and activation of the voltage-dependent calcium channels, transient T-type and long-lasting L-type. Also as with AngII, this influx is required for the response to potassium, since inhibition of calcium

influx abolishes potassium-stimulated aldosterone secretion (Capponi et al., 1984; Kojima et al., 1985a; Kojima et al., 1984b; Rossier et al., 1998; Spät and Hunyady, 2004). Related to calcium influx, an interesting finding is the fact that decreasing potassium levels to 2mM inhibits AngII-stimulated aldosterone production, presumably by inhibiting AngII-induced calcium influx (Kojima et al., 1985d). It can be speculated that this mechanism serves under conditions of low serum potassium levels to prevent AngII-stimulated aldosterone secretion, which could otherwise cause excessive excretion of potassium and subsequent severe, and possibly fatal, hypokalemia.

Other signaling pathways have also been proposed to be involved in potassium-induced aldosterone production, although controversy remains concerning these other events. For instance, some investigators have observed an ability of potassium to increase cAMP levels and others have not (Fujita et al., 1979; Ganguly et al., 1990; Kojima et al., 1985b; Tait and Tait, 1999). Potassium has also been reported to stimulate PLD activity through voltage-dependent calcium channels in bovine glomerulosa cells (Betancourt-Calle et al., 2001b), although whether and how this PLD activation contributes to potassium-elicited aldosterone production is unclear. Thus, there are still several areas related to the acute effects of potassium on aldosterone secretion in adrenal glomerulosa cells that still need to be studied and better understood.

Acute Effects of ACTH

ACTH is able to stimulate aldosterone production acutely, both *in vivo* and *in vitro*. It does so by binding to the ACTH receptor (MC2R) which activates adenylate cyclase via the heterotrimeric G protein, G_s. Adenylate cyclase produces the second messenger cAMP, thereby stimulating the activity of cAMP-dependent protein kinase or protein kinase A (PKA). PKA can then phosphorylate and activate StAR (Betancourt-Calle et al., 2001a), to increase cholesterol delivery to the inner mitochondrial membrane. In addition, PKA can regulate the transcriptional activity of CREB family transcription factors (Jo et al., 2005; Johannessen and Moens, 2007a; Manna et al., 2011). Since binding sequences for CREB/ATF transcription factors are found in the promoter of StAR (Bassett et al., 2000; Clem et al., 2005; Manna et al., 2002; Manna et al., 2003; Sher et al., 2007), PKA can also rapidly increase expression of StAR protein levels and further enhance acute steroid production. In addition to stimulating cAMP-induced PKA activation, ACTH is capable of promoting calcium influx, likely through PKA-mediated phosphorylation of L-type calcium channels (Sculptoreanu et al., 1993), thereby increasing cytosolic calcium concentration and further enhancing adenylate cyclase production of cAMP and aldosterone secretion (Gallo-Payet et al., 1996).

Finally, there may be some PKA-independent effects of cAMP on aldosterone secretion, in that, a recent report has suggested the involvement of the guanine nucleotide exchange factor, exchange protein directly activated by cAMP (Epac), in cAMP-mediated aldosterone production, via effects on CaMK activation (Gambaryan et al., 2006).

Chronic Effects of AngII

As noted earlier, the capacity for aldosterone production is also regulated through the chronic action of the same factors that acutely stimulate its biosynthesis. The chronic actions involve changes in the size of the zona glomerulosa as well as glomerulosa cell capacity to produce aldosterone. This review focuses on the second issue (glomerulosa cell differentiation) because of recent advances in our understanding of the mechanisms regulating steroidogenic enzymes.

AngII increases the expression of enzymes required for aldosterone synthesis, particularly CYP11B2. *In vivo* models have been crucial in describing such AngII effects. Sodium restriction experiments in rats indicate that activation of the renin-AngII system (most often by low sodium diets) induces the expression of CYP11B2 in glomerulosa cells without affecting that of CYP11B1 (Adler et al., 1993; Holland and Carr, 1993; Tremblay et al., 1992). This result confirms the capacity of AngII to specifically increase the production of aldosterone but not that of glucocorticoids (Holland and Carr, 1993; Tremblay et al., 1992). One explanation for the zone-specific effects of AngII is the greater expression of AT_1 receptors in glomerulosa versus fasciculata cells (Breault et al., 1996), and the inhibition of CYP11B2 expression by angiotensin inhibitors or AT_1 receptor blockers has been demonstrated (Kakiki et al., 1997). AngII can also increase CYP11B2 expression by increasing glomerulosa cell sensitivity to AngII through upregulation of its own receptor. This effect has been demonstrated *in vivo* in rats on a low-sodium diet (Du et al., 1996; Wang and Du, 1995); further studies have shown that pharmacological blockers of the AT_1 receptors, such as losartan (Du et al., 1996; Wang and Du, 1995) and angiotensin-converting enzyme (ACE) inhibitors (Lehoux et al., 1994; Wakamiya et al., 1994), reduce the effect of the RAS on the adrenal.

In vitro cell models have been particularly useful in defining the intracellular signaling mechanisms that lead to the chronic effects of AngII. As with acute secretion, the best characterized pathway regulating AngII-induced chronic production of aldosterone is the PLC-mediated generation of DAG and IP_3, which increases intracellular calcium and acts via CaMK. Calcium signaling appears to be the primary regulator of CYP11B2 transcription. However, since inhibitors of calmodulin and CaMK cannot completely block AngII's stimulation of CYP11B2 (Pezzi et al., 1997), other signaling mechanisms are also likely involved. DAG-activated PKC, on the other hand, does not appear to increase CYP11B2 transcript levels, but does play a role in producing an aldosterone-secreting glomerulosa cell phenotype by inhibiting the expression of CYP17 (Bird et al., 1996; McEwan et al., 1999). This effect has been recently attributed to Fos-mediated repression of the CYP17 transcriptional activator, steroidogenic factor 1 (SF1) (Sirianni et al., 2010). On the other hand, there is evidence suggesting that certain PKC isoforms, such as PKC-epsilon may actually inhibit CYP11B2 expression via activation of ERK-1/2 (LeHoux and Lefebvre, 2006; Lehoux and Lefebvre, 2007). However, PKC-epsilon has been shown to activate PKD, a kinase that can increase CYP11B2 expression (Romero et al., 2006) (see below), indicating that the role of the PKC family in regulating chronic aldosterone production is as yet unclear.

AngII appears to increase CYP11B2 expression through the activation of its transcription. A number of studies involving promoter deletion and mutation analyses have revealed that cis-elements in the CYP11B2 promoter are essential for basal as well as AngII-mediated CYP11B2 promoter activity. These include three key regulatory cis-elements: one cAMP response element (CRE)/Ad1 and two distal cis-elements (Ad5 and NBRE) that are able to bind members of the nerve growth factor-induced clone B family of transcription factors (NGFI-B or NR4A family) (Bassett et al., 2004a; Bassett et al., 2004b; Bassett et al., 2000; Clyne et al., 1997; Nogueira and Rainey, 2010a; Romero et al., 2010; Szekeres et al., 2009). The over-expression of one of these transcription factors, NURR1 (NR4A2), has in fact been implicated in the development of aldosterone-producing tumors (Lu et al., 2004). Using multiple glomerulosa cell models, microarray studies have defined additional transcription factors that are regulated by AngII (Nogueira et al., 2009; Romero et al., 2007; Szekeres et al., 2010). The role of these newly defined factors requires further study. Conversely, other transcriptional regulators such as SF1 (also called AD4BP and NR5A1) have been shown to repress basal and AngII-stimulated CYP11B2 expression (Bassett et al., 2002; Ye et al., 2009). However, it appears that the relative level of SF1 may decide its ability to repress

CYP11B2 expression, since complete knock down of SF1 impairs the entire steroidogenic synthetic pathway (Ye et al., 2009). The underlying mechanism, however, is yet to be elucidated. As mentioned previously, yet another AngII-mediated mechanism regulating chronic aldosterone secretion seems to be via PKD (Romero et al., 2006). The pathway involved is undetermined in adrenal cells; although PKD activation of the transcription factor CREB has been demonstrated in kidney HEK 293 cells (Johannessen et al., 2007b) and in MA-10 Leydig cells (Manna et al., 2011). In the Leydig cell model PKD was shown to increase StAR levels via effects on CREB and AP-1 transcription factors (Manna et al., 2011). At the level of cholesterol uptake in glomerulosa cells, AngII has been shown to up-regulate the expression of LDL and HDL receptors (Pilon et al., 2003) and of enzymes involved in cholesterol synthesis (Liang et al., 2007).

Finally, AngII also increases aldosterone production by expansion of the zona glomerulosa via hypertrophy and hyperplasia. This effect has been demonstrated *in vivo*, in rats on low-sodium diets, which display glomerulosa cell hypertrophy and proliferation in AT_1R-dependent and -independent processes (McEwan et al., 1999). *In vitro*, primary cultures of bovine glomerulosa cells also showed similar responses to AngII (Tian et al., 1995; Tian et al., 1998). These effects may be attributed to the ability of AngII to induce the expression of cyclin D1 (Watanabe et al., 1996). Mitogenic and hypertrophic effects of AngII have also been demonstrated in epithelial cells, vascular smooth muscle cells (Berk et al., 1989; Geisterfer et al., 1988), fibroblasts, cardiac myocytes (Sadoshima and Izumo, 1993) and rat intestinal cells (Smith et al., 1994). It can thus be speculated that the predisposition to cardiovascular damage resulting from activation of the renin-angiotensin-aldosterone system can be attributed to AngII stimulation of not only aldosterone production pathways but also glomerulosa cell hyperplasia and pathological growth of cardiovascular cells. Worth noting is the fact that in mice with targeted deletion of the renin/AngII system, potassium can substitute for the effects of AngII to increase adrenal expression of CYP11B2 and synthesis of aldosterone (Chen et al., 1997; Okubo et al., 1997). This result suggests that the mechanisms of potassium and AngII stimulation of CYP11B2 expression likely overlap.

Chronic Effects of Potassium

Besides inducing early events to increase aldosterone production, potassium also regulates later/chronic events. It has been well documented that high potassium diets in rats increase the expression of aldosterone synthase (CYP11B2) and aldosterone production (Tremblay and LeHoux, 1993; Tremblay et al., 1991). A recent study in mice also reported a slight increase in the thickness of the zona glomerulosa and suggested the role of several genes in this process. These genes, including Mtus 1, Smoc 1 and Grp 48, were observed to be upregulated with 28 days of a high potassium diet, although the *in vitro* experiments did not completely parallel these microarray results (Dierks et al., 2010). However, attributing these observed changes solely to the chronic effects of potassium on aldosterone production is particularly challenging, since physiological potassium levels are tightly regulated by the renin-angiotensin system. Indeed, serum potassium levels following high potassium diets were not reported to be abnormally high. Other *in vitro* studies using primary cultures of rat glomerulosa cells and the human adrenocortical H295R cell line have demonstrated increased CYP11B2 mRNA levels, promoter activation and aldosterone biosynthesis in response to elevated potassium levels in the growth media (Bird et al., 1995b; Denner et al., 1996; Yagci and Muller, 1996b; Yagci et al., 1996a). These results may help to explain the finding that in transgenic mice with targeted deletion in the renin-angiotensin system, potassium can induce CYP11B2 expression in the adrenal as well as the synthesis of aldosterone (Chen et al., 1997; Okubo et al., 1997).

As mentioned earlier, the mechanism of potassium signaling in glomerulosa cells involves depolarization of the cells to allow extracellular calcium influx through the T- and L-type calcium channels. This increase in calcium influx upregulates CYP11B2 expression. Consistent with these findings, increasing calcium influx with the pharmacological calcium channel agonist BAYK8644 increases CYP11B2 mRNA expression in the H295R cell model (Clyne et al., 1997; Pezzi et al., 1997). Further, this potassium-induced calcium influx elevation is abrogated by the calcium channel blocker nifidipine (Denner et al., 1996; Yagci and Muller, 1996b), which also inhibits AngII-induced CYP11B2 upregulation. As in the case of AngII, potassium-induced calcium signaling occurs through the binding of calcium to the protein calmodulin. The calcium-calmodulin (CaM) complex activates several enzymes and kinases, many of which are expressed in a tissue-specific manner. Amongst the different CaM kinases (CaMK), types I and IV are more likely to be involved in chronic stimulation of aldosterone secretion by AngII and potassium (Condon et al., 2002). Immunohistochemistry was used to demonstrate that CaMKI expression is elevated in the glomerulosa of the human adrenal gland (Condon et al., 2002). The CaMK antagonist KN93 and the calmodulin inhibitor calmidazolium effectively inhibit potassium-induced CYP11B2 mRNA upregulation (Bassett et al., 2004b; Pezzi et al., 1997) and promoter activation (Bassett et al., 2004b; Condon et al., 2002).

Another similarity that potassium shares with AngII is the ability to activate several transcription factors, such as of NURR1, ATF1, ATF2 and CREB, which bind the proximal promoter of CYP11B2 at key cis-elements to enhance transcription. Activation of these transcription factors appears to be mediated through phosphorylation by potassium-activated CaMK (Bassett et al., 2004a; Bassett et al., 2004b; Bassett et al., 2000; Nogueira and Rainey, 2010a). Supporting these data is a recent study in which knock down of these transcription factors by siRNA technology reduced potassium-induced CYP11B2 promoter activity and mRNA levels (Nogueira and Rainey, 2010a). This idea is also supported by the observation that within the adrenal cortex, the transcription factor NURR1 has higher expression in the zona glomerulosa and in aldosterone-producing tumors compared to the adjacent zona fasciculata (Lu et al., 2004).

As mentioned earlier, a small but interesting effect of a high-potassium diet in mice is the observed slight increase in the thickness of the zona glomerulosa (Gao et al., 2009). A more significant increase in the thickness of the zona glomerulosa was found in rats on a high potassium diet for 2 to 7 weeks (Hartroft and Sowa, 1964). Also observed in the 2009 study by Gao et al. was elevated expression of Gpr48, a G protein-coupled receptor that has been implicated in increasing tumor invasiveness and metastasis in the HeLa cervical carcinoma cell line (Gao et al., 2006; Gao et al., 2009). Moreover, Gpr48 is also associated with down-regulation of cyclin-dependent kinase inhibitor p27 (Kip1) (Gao et al., 2006). It is hence tempting to ascribe the observed increased glomerulosa thickness to the effects of Gpr48. Chronic high potassium can thus regulate long-term aldosterone production, sodium retention and ultimately blood pressure via chronic mechanisms involving increased glomerulosa cell size and/or number as well as the cells' aldosterone synthetic capacity. However, a better understanding of chronic effects of potassium is required on the molecular pathways underlying secretion of aldosterone by adrenal glomerulosa cells.

Chronic Effects of ACTH

While thought of primarily as the regulator of adrenal cortisol production, ACTH is considered a secondary regulator of zona glomerulosa aldosterone production. It is clear that adrenal glomerulosa cells (both *in vivo* and *in vitro*) can acutely increase aldosterone production in response to ACTH. However, over time ACTH causes cultured glomerulosa cells to switch their phenotype to that of a cortisol-producing fasciculata cell (Crivello and

Gill, 1983; Hornsby et al., 1974). *In vivo* studies by Allen et al. demonstrated that ablation of the pituitary pre-proopiomelanocortin-secreting cells that produce ACTH, and the resultant low ACTH level, was accompanied by a steep decrease in the transcript levels of CYP11B1, but not of CYP11B2 (Allen et al., 1995). In agreement with this observation, treatment with ACTH causes an initial increase in mRNA levels of CYP11B2 in the first 3 hours; however, chronically, CYP11B2 expression decreases in response to ACTH *in vitro* in isolated rat adrenal cells (Holland and Carr, 1993). Similarly, chronic low-dose infusion of ACTH in human subjects results in an initial increase in plasma renin activity and plasma aldosterone levels during the first 12-36 h, but a slow decline in these values over the next several days (Fuchs-Hammoser et al., 1980). While the H295R adrenocortical cell lines express low levels of ACTH receptors, treatment of these cells with cAMP analogs preferentially increases the expression of CYP11B1 over that of CYP11B2 (Denner et al., 1996). The mechanism for chronic ACTH-mediated repression of aldosterone production and CYP11B2 expression remains unknown. An interesting observation has been that cAMP signaling reduces the sensitivity of adrenocortical cells to AngII by down-regulating the expression of AngII receptors (Bird et al., 1995a; Yoshida et al., 1991). Another possible mechanism for the reduction in aldosterone production with chronic ACTH stimulation could be via the hormone's direct induction of CYP11B1 and CYP17, the activities of which direct the precursors of the steroidogenic pathway away from the production of aldosterone, and towards that of cortisol (Bird et al., 1996).

Since CYP11B2 has a cAMP-regulatory element (CRE) in its 5′ promoter region (Rainey, 1999), the mechanism preventing glomerulosa cells from responding to ACTH with increased CYP11B2 and excessive aldosterone production is not clear, but two possible mechanisms have been suggested thus far. First, at least in bovine glomerulosa cells, there is high expression of the inhibitory guanine nucleotide-binding protein G_i. AngII signaling through the AT_1 receptor couples through G_i to inhibit ACTH-stimulated cAMP formation (Begeot et al., 1988; Hausdorff et al., 1987). Second, adrenal glomerulosa cells appear to express adenylyl cyclases 5 and 6, isoforms which are inhibited by a rise in intracellular calcium, a signaling mechanism common to AngII and potassium stimulation of aldosterone secretion (Shen et al., 2001) The above provide evidence for a supportive, but not an obligatory, role of ACTH in aldosterone production.

Lessons from Primary Aldosteronism

Primary aldosteronism (PA) is the major endocrine cause of hypertension and is characterized by normal or elevated aldosterone levels in the presence of low circulating plasma renin levels (Young, 2007). The most common cause of PA is idiopathic hyperaldosteronism (IHA), which occurs in approximately 60% of PA patients. The second most abundant cause of PA is unilateral adrenocortical adenoma, which is a curable form of PA that can be resolved by adrenalectomy. There are also rare forms of PA that include glucocorticoid-remediable aldosteronism (GRA), or familial hyperaldosteronism 1 (FH1), which is caused by unequal crossover between the CYP11B1 and CYP11B2 genes resulting in a hybrid gene with the CYP11B1 promoter driving CYP11B2. In these cases CYP11B2 expression and aldosterone secretion are regulated by ACTH in the adrenal zona fasciculata. Recently, several mouse models have been developed in an attempt to study PA.

Two recent studies using different mouse models, both with a deletion of genes encoding TWIK-related acid-sensitive K (TASK) channels, have provided interestingly different and complex primary aldosteronism phenotypes (Davies et al., 2008; Heitzmann et al., 2008). As mentioned earlier, TASK channels maintain the membrane potential of the glomerulosa cell at a polarized −70mV by being constitutively open and acting as a K^+ leak channel. Inhibition of these channels by the AT_1R or by increased serum K^+ levels depolarizes

glomerulosa cells and increases calcium influx to drive aldosterone secretion. In the study by Heitzmann et al., deletion of the TASK-1 channel resulted in a phenotype similar to the pathology of GRA, with characteristics such as salt-insensitive hyperaldosteronism, hypokalemia and dexamethasone-suppressible aldosterone secretion. The deletion of TASK-1 also seemed to change adrenal zonation and expression of CYP11B2, which was absent in the outermost zona glomerulosa but was expressed to a large extent in the zona fasciculata. Furthermore, this expression pattern seemed to be restricted only to females and to males prior to puberty. On the other hand, Davies and colleagues fournd that deletion of both TASK-1 and TASK-3 created a model with a phenotype resembling the pathology of IHA. Male mice showed increased aldosterone secretion that was not suppressible with a high-salt diet or the AT_1R blocker candesartan (Davies et al., 2008). Further studies will be required to understand the mechanism by which the different types of TASK channels interact and regulate adrenal function.

Besides TASK channels, Choi et al. have also identified both germ-line and somatic mutations that occur near the selectivity filter of the inward rectifying potassium channel *KCNJ5* (Kir3.4) to result in PA (Choi et al., 2011). The amino acid substitutions resulting from these mutations modified channel ion selectivity, such that the channel became permeable to both sodium and potassium, which led to increased depolarization of adrenocortical cells. This depolarization is believed to cause elevated intracellular calcium and thereby the production of aldosterone and cell proliferation (Choi et al., 2011). These findings are particularly relevant in that almost 40% of aldosterone-producing adenomas appeared to have such a mutation in KCNJ5. Additional studies will be needed to determine the exact mechanisms through which these mutations cause expansion of aldosterone-producing cells and formation of adenomas.

Summary

Aldosterone is an essential hormone with key roles in the regulation of electrolyte balance and blood pressure. Its normal physiological regulators include Ang II, K^+ and ACTH which can increase aldosterone secretion both acutely, by increasing StAR expression and phosphorylation, as well as chronically, by action on the steroidogenic pathway, mostly through increasing gene expression of CYP11B2. Dysregulation in aldosterone secretion, as is seen in primary aldosteronism, leads to pathologies such as hypertension and cardiovascular disease. Several animal and cell culture models are being developed to better understand aldosterone secretion under normal and pathological conditions. The recent development of unique mouse models of primary aldosteronism and the discovery of the KCNJ5 mutations as a cause of human PA have been particularly helpful in providing new clues to the mechanisms controlling aldosterone production and the adrenal glomerulosa cell phenotype. However, additional studies will be needed to define completely the detailed pathways that activate and repress aldosterone biosynthesis.

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Research Highlights

- Aldosterone is the major mineralocorticoid produced by the adrenal zona glomerulosa
- Glomerulosa cells are primarily regulated by angiotensin II, serum potassium and ACTH
- Aldosterone synthesis involves two key phases, acute and chronic
- Cholesterol delivery into mitochondria is the rate-limiting step in the acute phase
- Aldosterone synthase (CYP11B2) is the rate-limiting step in the chronic phase

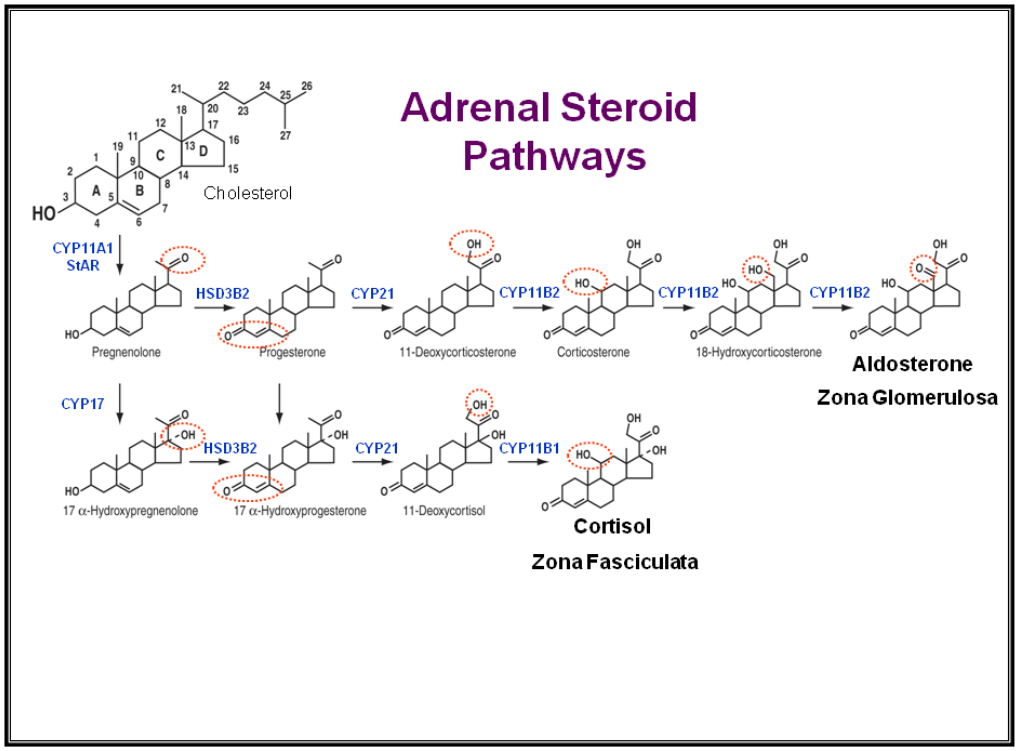

Figure 1. Adrenocortical steroidogenic pathways for the production of mineralocorticoids and glucocorticoids

The adrenal cortex produces zone-specific steroids as a result of differential expression of steroidogenic enzymes. In the initial step of steroidogenesis, steroidogenic acute regulatory (StAR) protein is needed for the rate-limiting step of movement of cholesterol to the inner mitochondrial membrane, where cholesterol is cleaved by cholesterol side-chain cleavage (CYP11A1) to pregnenolone. Further steps of the steroidogenic pathway include the enzymes 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), 17α-hydroxylase, 17,20-lyase (CYP17), 21-hydrolylase (CYP21), 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2).

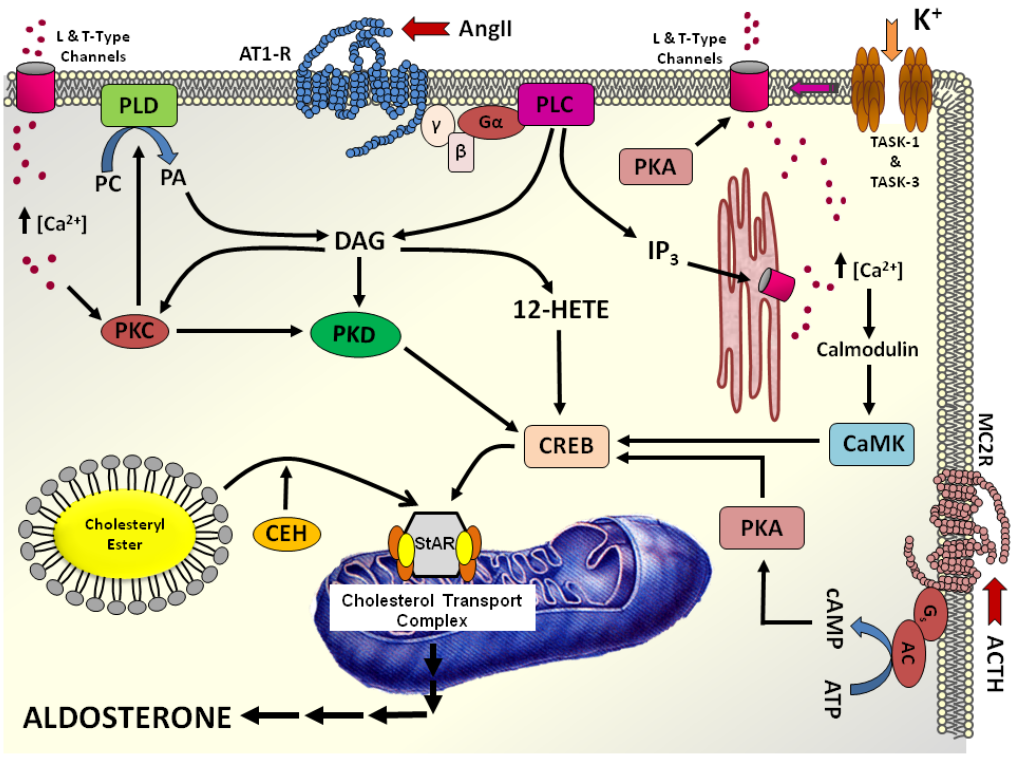

Figure 2. Acute actions of AngII, K^+ and ACTH on adrenal glomerulosa cell aldosterone production

AngII binds the AT_1 receptor to activate phosphoinositide-specific phospholipase C (PLC)-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds the IP_3R on the endoplasmic reticulum (ER), releasing calcium and raising cytosolic calcium concentrations. AngII also activates, in part through protein kinase C (PKC), phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) which can be metabolized to DAG by lipid phosphate phosphatases. Small increases in extracellular K^+ depolarize the glomerulosa cell, activating the voltage-operated L- and T-type calcium channels, increasing calcium influx. Increased intracellular calcium concentration activates calcium/calmodulin-dependent protein kinases I/II (CaMK), as well as PKC isoforms. Both of these pathways can modulate not only StAR phosphorylation, but also expression, likely in part through the StAR promoter binding of cAMP response element binding protein (CREB). The DAG/PKC pathway also activates protein kinase D (PKD) which can likewise phosphorylate (and activate) CREB. DAG can be hydrolyzed by DAG lipase to release arachidonic acid, which can be further metabolized by 12-lipoxygenase to 12-hydroxyeicosatetraenoic acid (12-HETE), which also induces the phosphorylation (and activation) of CREB. ACTH can also mediate aldosterone synthesis through binding to the melanocortin type 2 receptor (MC2R), thus activating through a heterotrimeric G_s protein, adenylate cyclase (AC). AC converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA) inducing a slow but sustained calcium influx through the L-type calcium channels. PKA also phosphorylates CREB, thereby increasing StAR expression. Cholesterol for aldosterone production arises from cholesteryl ester hydrolase (CEH)-mediated hydrolysis of cholesteryl esters synthesized *de novo* or obtained from lipoproteins and stored in lipid droplets. Free cholesterol is shuttled to the inner mitochondrial membrane by StAR likely in complex with other cholesterol transport proteins.

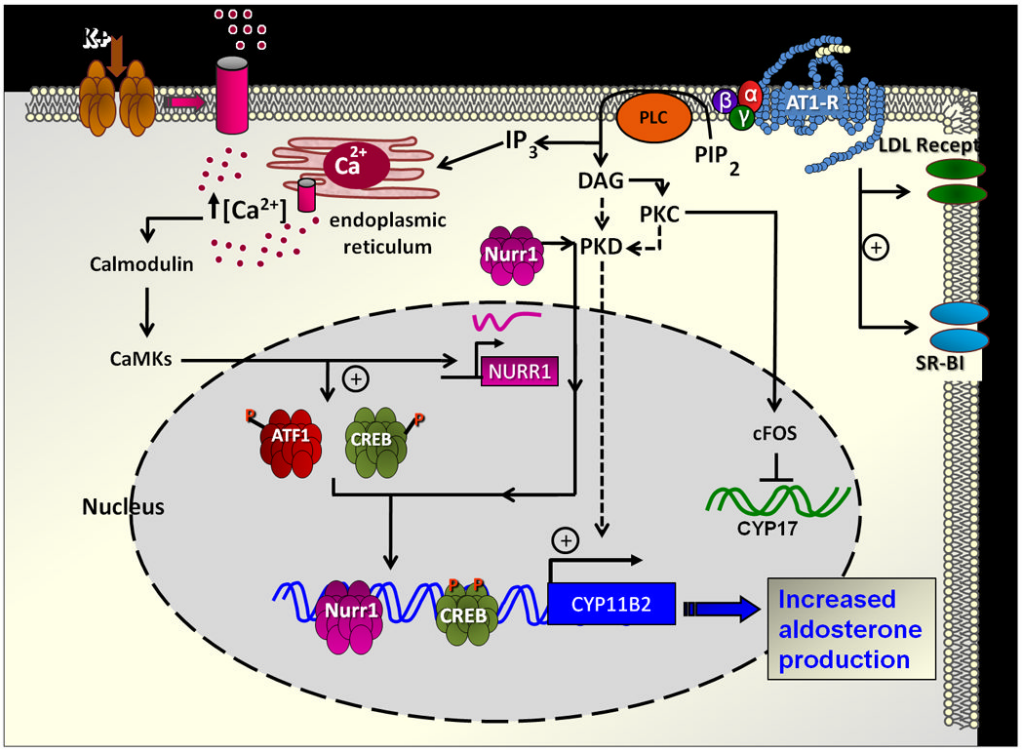

Figure 3. The chronic production of aldosterone is regulated by AngII and potassium (K^+)

AngII binds type 1 AngII receptors (AT_1-R) and activates phospholipase C (PLC) which causes hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) which inhibits transcription of 17α-hydrolyase (CYP17) through transcription factors such as cFOS. DAG may also increase the activity of protein kinase D (PKD), which has been shown to increase CYP11B2 transcription. IP_3 causes the release of intracellular calcium and the activation of calcium-calmodulin kinases (CaMKs). Small increases in extracellular K^+ also depolarize the glomerulosa cell, increasing calcium influx and activating CaMKs. CaMKs increase expression and/or phosphorylation and activation of transcription factors that increase CYP11B2 transcription. Further, binding of Ang II to the AT_1-R also increases the expression of LDL and HDL receptors, which increases cholesterol availability for steroidogenesis.