Reciprocal influences between the signalling pathways regulating proliferation and steroidogenesis in adrenal glomerulosa cells

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Abstract

The main regulators of aldosterone secretion in adrenal gland zona glomerulosa (ZG) cells are the hormones angiotensin II (Ang II) and adrenocorticotrophin (ACTH) and small increases in the extracellular potassium (K+) concentration. The action of these agonists is mediated by different signalling systems – ACTH is mediated by cAMP and activation of protein kinase A while Ang II and K+ activate two protein kinases, Ca2+-calmodulin-dependent protein kinase (CamK) and diacylglycerol-dependent protein kinase (PKC). Ang II, besides being one of the main agonists for the secretion of aldosterone, also stimulates proliferation of ZG cells, a process mediated by mitogen-activated protein kinases (MAPKs). Recent studies aimed at elucidating the molecular mechanisms underlying cell proliferation have shown that calcineurin is the principal regulator of MAPKs activity. The purpose of this review is to discuss experimental evidence of possible reciprocal influences between the signalling pathways regulating proliferation and steroidogenesis in ZG cells.

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Introduction

Aldosterone is the principal mineralocorticoid synthesized by the adrenal gland cortex; it plays a fundamental role in electrolyte and water balance due to its action on epithelial cells of the kidney collecting tubule and the distal colon, where it promotes sodium (Na+) absorption and potassium (K+) excretion. High aldosterone production, via alterations in mechanisms controlling its biosynthesis, is associated with sodium retention, expansion of plasma volume, and high blood pressure. In the heart, elevated aldosterone levels cause hypertrophy, fibrosis, and loss of tissue electrolyte balance which eventually cause cardiac muscle dysfunction and, consequently, threaten life. Thus, aldosterone synthesis is finely regulated to ensure an appropriate physiological function (Lumbers 1999, Young & Funder 2000).

Aldosterone is synthesized in zona glomerulosa (ZG) cells which are in the outermost part of the adrenal cortex. In vivo, its secretion is regulated by the hormone angiotensin II (Ang II) and changes in plasmatic K+ concentration, independent of the pituitary hormone adrenocorticotrophin (ACTH), which acts in the zona fasciculata regulating synthesis and secretion of glucocorticoids. In vitro, ACTH is a potent stimulator of aldosterone secretion in primary cultures of ZG cells (Muller 1998). Figure 1 illustrates the major signalling pathways and their relationships in the regulation of aldosterone biosynthesis and proliferation in adrenal ZG cells which will be discussed in this review.

Different signalling pathways control aldosterone biosynthesis

ACTH stimulates aldosterone production through Gt protein-coupled receptors that activate cAMP formation, and by cAMP-dependent protein kinase (PKA) activation (Graham-Smith et al. 1967, Fakunding et al. 1979, Sala et al. 1979, Yoshida et al.
In contrast, Ang II and small increases in K+ concentration increase intracellular calcium (Ca2+) by several different mechanisms (Fakunding & Catt 1980, Foster et al. 1981, 1997, Cirillo et al. 1993, which serve as the main intracellular stimuli in aldosterone secretion. The binding of Ang II to Gq/11-protein-coupled AT1 receptors, leading to the activation of phospholipase C (PLC) and simultaneous formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds and activates IP3-receptors localised in discrete domains of the endoplasmic reticulum (ER), opening channels for release of Ca2+ in the vicinity of the mitochondria. DAG binds and activates protein kinase C (PKC). Ang II-AT1 receptor activation also increases the flow of Ca2+ from the external space through voltage-dependent Ca2+ channels (T- and L-types) and voltage independent Ca2+ release activated channels (CRAC). Binding of adrenocorticotropic hormone (ACTH) to Gs-protein-coupled receptors leads to the activation of cAMP-dependent protein kinase A (PKA). On the other hand, increases in physiological concentrations of potassium (K+) open voltage-dependent Ca2+ channels (T- and L-types) without release of Ca2+ from the ER. Ang II, ACTH and small increases in K+ concentration induce aldosterone secretion by phosphorylation of the steroid acute regulatory (StAR) protein, an obligatory step in the transfer of cholesterol to the mitochondria. The three agonists, acting through different signalling pathways, activate aldosterone biosynthesis at a site before StAR activation. Additionally, Ang II acting on AT1 receptors induces cell proliferation through activation of mitogen-activated protein kinases (MAPKs) such as ERK1/2 and p38, which also promote aldosterone biosynthesis. Solid arrows indicate pathways that have been demonstrated. Dotted arrows denote possible signalling pathways and regulators.

Figure 1 Model for signalling pathways, interactions and regulators in adrenal ZG cells. Ang II activates Gq/11-protein-coupled AT1 receptors, leading to the activation of phospholipase C (PLC) and simultaneous formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds and activates IP3-receptors localised in discrete domains of the endoplasmic reticulum (ER), opening channels for release of Ca2+ in the vicinity of the mitochondria. DAG binds and activates protein kinase C (PKC). Ang II-AT1 receptor activation also increases the flow of Ca2+ from the external space through voltage-dependent Ca2+ channels (T- and L-types) and voltage independent Ca2+ release activated channels (CRAC). Binding of adrenocorticotropic hormone (ACTH) to Gs-protein-coupled receptors leads to the activation of cAMP-dependent protein kinase A (PKA). On the other hand, increases in physiological concentrations of potassium (K+) open voltage-dependent Ca2+ channels (T- and L-types) without release of Ca2+ from the ER. Ang II, ACTH and small increases in K+ concentration induce aldosterone secretion by phosphorylation of the steroid acute regulatory (StAR) protein, an obligatory step in the transfer of cholesterol to the mitochondria. The three agonists, acting through different signalling pathways, activate aldosterone biosynthesis at a site before StAR activation. Additionally, Ang II acting on AT1 receptors induces cell proliferation through activation of mitogen-activated protein kinases (MAPKs) such as ERK1/2 and p38, which also promote aldosterone biosynthesis. Solid arrows indicate pathways that have been demonstrated. Dotted arrows denote possible signalling pathways and regulators.

In marked contrast to Ang II, small increases in K\(^+\) concentration depolarize the cell membrane directly and activate \( \text{Ca}^{2+} \) entry through voltage-dependent \( \text{Ca}^{2+} \) channels without release of \( \text{Ca}^{2+} \) from intracellular stores (Foster et al. 1982, Mlinar et al. 1995, Rossier et al. 1996, Foster & Rojas 1999); this activates CamK (Pezzi et al. 1996). Thus, Ang II elevates cytosolic \( \text{Ca}^{2+} \) at the expense of internal and external \( \text{Ca}^{2+} \) compartments, while K\(^+\) increases cytosolic \( \text{Ca}^{2+} \) concentration solely at the expense of the external space.

Several studies have demonstrated that these signalling pathways are interrelated. In bovine ZG cells, for example, ACTH inhibits the \( \text{Ca}^{2+} \) signal and Ang II-induced aldosterone secretion, a process dependent upon cAMP formation (Yoshida et al. 1991). By contrast, Ang II potentiates the effect of ACTH on cAMP production, and inhibits K\(^+\)-induced increase in intracellular \( \text{Ca}^{2+} \) concentration and aldosterone production (Balla et al. 1991, Apte et al. 1996).

In spite of the importance of these studies, it has still not been established exactly where cAMP and \( \text{Ca}^{2+} \) and their protein kinases come together, especially when both pathways are activated simultaneously.

**Steroid acute regulatory (StAR) protein and aldosterone biosynthesis**

The limiting step in aldosterone biosynthesis, as for all steroid hormones, is the conversion of cholesterol to pregnenolone, a reaction catalysed by the cytochrome P450\(_{\text{sec}}\) which is localized in the internal mitochondrial matrix (Stocco 2001). Steroid secreting cells store steroids in minimal quantities and thus the regulation of steroid secretion is linked directly to the level of steroid synthesis. ACTH, through cAMP and PKA activation, and Ang II, through increased \( \text{Ca}^{2+} \) concentration, may regulate the process slowly through the transcription of genes that encode enzymes participating in steroid hormone synthesis. However, transcription and translation mechanisms are not fast enough to account for steroidogenesis activation on the time scale observed. In adrenal ZG cells, for example, increased aldosterone production is clearly detectable within minutes in response to Ang II and ACTH (Foster & Rasmussen 1983, Foster et al. 2002) and returns to basal levels within minutes after withdrawal of the hormone. The ability to induce steroid hormone synthesis within minutes is facilitated by the transport of cholesterol from the external to the internal mitochondrial membrane, a process that depends upon de novo synthesis of steroid acute regulatory (StAR) protein (Crivello & Jefcoate 1980, Stocco 2001).

It is not yet known how StAR transfers cholesterol to the internal mitochondrial membrane. It has been proposed that StAR is synthesized rapidly in the cytoplasm from a 37 kDa precursor that is phosphorylated and proteolytically processed during the transport of cholesterol to the internal mitochondrial membrane (Arakane et al. 1997, Artemenko et al. 2001). The regulation and activation of StAR phosphorylation has been studied mainly in cells stimulated through cAMP production, such as Leydig tumour cells (Manna et al. 1999), granulosa cells (lines rLHR-4 and rFSHR-17) (Seger et al. 2001) and Y1 cells (Jones et al. 2000). In bovine ZG cells, Ang II, ACTH and K\(^+\) all activate StAR expression and phosphorylation (Betancourt-Calle et al. 2001) in concentrations that produce comparable effects in aldosterone production. Although the three agonists initially act through different signalling pathways, it seems likely that their pathways merge at a common site for the activation of aldosterone biosynthesis. However, there are no experimental data regarding StAR expression and phosphorylation that explain the inhibitory effect of ACTH on Ang II-induced intracellular \( \text{Ca}^{2+} \) increase and aldosterone secretion (Yoshida et al. 1991), the potentiation effect of Ang II on ACTH-induced cAMP production (Baukal et al. 1994), or the inhibitory effect of Ang II on K\(^+\)-induced aldosterone production (Balla et al. 1991, Apte et al. 1996). Considering these three effects leads one to hypothesize that in ZG cells the different pathways converge at some point before StAR activation.
Mitogen-activated protein kinases and their relationship with steroidogenesis

Besides their effects on aldosterone secretion, Ang II and ACTH both have important effects on cell proliferation through the activation of mitogen-activated protein kinases (MAPKs). MAPKs are protein kinases whose function and regulation are highly conserved from yeasts to humans. They are activated by stimuli ranging from growth factors and cytokines to hormones and neurotransmitters, which act by activating both tyrosine kinase receptors and G-protein-coupled receptors. The basic arrangement consists of a G-protein (Ras) and three kinases aligned downstream. GTP-activated Ras binds to Raf and translocates it from the cytosol to the plasma membrane, where kinase activation takes place. Activated Raf then phosphorylates MAPK kinase (MEK) which, in turn, phosphorylates and activates MAPKs (MEKs). The MAPKs include extracellular-signal-regulated kinase ERK1/2, c-Jun N-terminal kinase and MAPK p38. These kinases are activated by dual phosphorylation of threonine and tyrosine residues recognised by MEK, which itself is activated by Raf, and also by dual phosphorylation of threonine and tyrosine residues. Following activation, MAPKs may translocate to the nucleus and regulate gene transcription factors related to cellular proliferation, or may regulate processes at the cytoplasmic or plasma membrane level (Widmann et al. 1999, Kolch 2000).

Ang II has been linked with hypertension, cardiac tissue remodelling and cardiac hypertrophy. Its effects are primarily due to Ang II-dependent activation of AT1 receptors, a process accompanied by stimulation of Ras and the sequence of kinases aligned downstream, culminating in MAPKs activation (de Gasparo et al. 2000). In recent years, there has been a great deal of investigation to elucidate the hypertrophic and proliferating effects of Ang II observed in a variety of its target cells, such as vascular smooth muscle cells (Geisterfer et al. 1988, Dubey et al. 1992, Touyz et al. 2001), cardiac fibroblasts (Sadoshima & Izumo 1993, Peng et al. 2002, Seta & Sadoshima 2003) and cardiomyocytes (Paradis et al. 2000, Thomas et al. 2002).

The signalling mechanism of the Ang II-type AT1 receptor has been traditionally linked to the activation of phospholipase C and its signalling molecules, IP3 and diacylglycerol, with the concomitant increase in intracellular Ca2+ concentration and activation of PKC. However, activation of the AT1 receptor by Ang II also involves tyrosine phosphorylation, and the key question to solve has been how AT1 receptors, which lack the intrinsic tyrosine kinase possessed by receptor tyrosine kinases such as platelet-derived growth factor receptor or epidermal growth factor (EGF) receptor, induce tyrosine phosphorylation, MAPKs activation, hypertrophy and proliferation. Recent investigations have demonstrated that a major pathway for Ang II-induced hypertrophy is mediated by AT1-dependent transactivation of EGF receptor (Shah & Catt 2003). For example, in neonatal cardiomyocytes infected with an adenovirus that expresses functional AT1 receptors, Ang II stimulated hypertrophy, EGF receptor phosphorylation and MAPK activation, all effects that are inhibited either by the MAPK kinase inhibitor PD98059 or the EGF receptor antagonist AG1478. In contrast, in non-infected cardiomyocytes, which express undetectable levels of endogenous AT1 receptors, Ang II failed to promote tyrosine phosphorylation of the EGF receptor (Thomas et al. 2002).

The mechanism by which Ang II transactivates the EGF receptor has recently begun to emerge. Intracellular Ca2+ and PKC have been associated with AT1-dependent transactivation of the EGF receptor; however the experimental results appear to be contradictory. For example, in vascular smooth muscle cells Ang II induced transactivation of the EGF receptor in a Ca2+-dependent manner (Eguchi et al. 2001); however, in cultured neonatal cardiomyocytes, Ang II induced transactivation of the EGF receptor, and MAPKs activation occurred independently of Ca2+ and PKC (Thomas et al. 2002). Also in neonatal cardiomyocytes, Ang II induced activation of the EGF receptor, and MAPKs activation occurred independently of Ca2+ and PKC (Thomas et al. 2002). The causes of the different responses to Ang II in these studies are not known, but might include different cell types and different species from which neonatal cardiomyocytes are prepared.

A major breakthrough in the understanding of the mechanism of EGF receptor transactivation was the discovery that cultured human macrophages secrete a heparin-binding EGF-like growth factor (HB-EGF) that upon binding to EGF receptors promotes mitogenic effects in fibroblasts.
and vascular smooth muscle cells (Higashiyama et al. 1991, Prenzel et al. 1999). Consistent with these findings, it was later demonstrated that HB-EGF mRNA is highly expressed in the hypertrophied left ventricle of spontaneously hypertensive rats (Fujino et al. 1998) and in rat hearts after myocardial infarction (Tanaka et al. 2002). Moreover, Ang II-induced EGF receptor transactivation in vascular smooth muscle cells and cardiomyocytes is mediated by HB-EGF and requires metalloprotease-dependent cleavage of pro-heparin-binding EGF (Saito et al. 2002, Thomä et al. 2002).

Whether Ang II-induced AT₁-dependent transactivation of the EGF receptor plays a role in adrenal ZG cells has not yet been documented; however, Ang II acting on the AT₁ receptor increases the incorporation of thymidine into DNA and increases cell proliferation in primary cultures of bovine adrenal ZG cells (Tian et al. 1995). Consistent with these effects, Ang II induces activation of Raf and ERK1/2 (Tian et al. 1998, Smith et al. 1999) in a time-dependent manner, which is detectable within 1 min, reaches a maximum at 5 min, and gradually returns to the basal level during the next 60 min. Subjecting the cells to a short treatment with the phorbol ester, phorbol myristylacetate (PMA), also phosphorylates and activates ERK1/2 in a time-dependent manner. ERK1/2 phosphorylation and activation reach a maximum at 5 min, which correlates with the time required for maximal phosphorylation and activation of ERK1/2 stimulated by Ang II, suggesting that PKC has a role in Ang II-dependent phosphorylation and activation of ERK1/2 (Tian et al. 1998).

The time taken for phosphorylation of Raf differs substantially from that of its activation. Ang II induces activation of Raf within 1 min, peaks at 5 min and declines thereafter reaching basal levels after 60 min (Tian et al. 1998), which correlates with the time course of ERK1/2 phosphorylation and activation. However, phosphorylation of Raf is first seen 5 min after the hormone is added and remains constant for 60 min, suggesting that Ang II-induced phosphorylation of Raf is not associated with the activation of the kinase, but rather with its deactivation (Tian et al. 1998). On the other hand, incubation of the cells in Ca²⁺-free medium containing EGTA potentiates Raf activation in response to Ang II (Smith et al. 1999), indicating that external Ca²⁺ has a negative regulatory influence on Raf activation.

Studies on the role of MAPKs in aldosterone biosynthesis have provided important evidence for cross-talk between Ang II-dependent proliferation and steroidogenesis. In bovine adrenal ZG cells, Ang II increases cholesterol supply to the mitochondria by ERK1/2-dependent phosphorylation of cholesterol ester hydrolase (Cherradi et al. 2003), and diminishes the activity of the Na⁺/Ca²⁺ exchanger through MAPK p38, reducing Ca²⁺ loss from the cytosol, and contributing to the maintenance of the cytosolic Ca²⁺ concentration required for full aldosterone response (Startchik et al. 2002). However, the authors of this latter study did not determine if specific inhibition of MAPK p38 affected Ang II-induced aldosterone production.

In a recent, rigorous investigation using two cell lines derived from granulosa cells (rLHR-4 and rFSH-17), Seger et al. (2001) reported that the gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) activate ERK1/2 and also increase progesterone secretion. These authors obtained similar effects using forskolin and 8-Br-cAMP, confirming that the action of the two hormones is mediated by cAMP in both processes. However, incubating the cells with the MEK inhibitor PD98059 for 15 min prior to adding the hormone, which inhibits ERK1/2 activation, notably potentiated the effect of LH and FSH on progesterone production. Consistent with this effect, PD98059 dramatically potentiated StAR expression in response to LH/FSH and forskolin/8-Br-cAMP. These findings suggest that the potentiation of steroidogenesis by MEK inhibition occurs after PKA activation, and that the signal cascade after ERK1/2 activation may down-regulate steroidogenesis acting at the beginning of the cascade before StAR expression.

Experimental results on the effect of ACTH on MAPK activation are also scarce. In Y1 cells, for example, ACTH stimulates the phosphorylation and activation of MEK and ERK1/2 (Le & Schimmer 2001) while in bovine adrenal fasciculata cells, ACTH, which stimulates steroidogenesis via cAMP, does not activate ERK1/2 and completely blocks ERK1/2 activation induced by Ang II (Chabre et al. 1995). No information is available at present with respect to the effects of ACTH and K⁺ on MAPK in ZG cells.
Possible role of calcineurin in steroidogenesis and MAPK activation

Reports on Raf activation in ZG cells (Tian et al. 1998, Smith et al. 1999) suggest that Ca\textsuperscript{2+} and PKC regulate Raf activity through a phosphorylation–dephosphorylation mechanism. Experimental evidence supports this argument. First, it has been shown that PKA inhibits Raf kinase activity through phosphorylation of serine 259 (ser259). Release from its inactive state is brought about by dephosphorylation of this same serine, an indispensable step for efficient recruitment of Raf to the plasma membrane (Dhillon et al. 2002a, b, Kubicek et al. 2002). This dephosphorylation is blocked by okadaic acid (OA), an inhibitor of protein phosphatase PP2A, indicating that PP2A participates in Raf activation. Once Raf is in position on the cell membrane, it is activated by its dissociation from Raf kinase inhibitory protein (RKIP), a process dependent upon phosphorylation of RKIP by PKC at serine 153 (ser153) (Corbit et al. 2003).

Secondly, we have recently demonstrated (Foster et al. 2002) that OA inhibits ACTH-induced aldosterone production completely, but has only a modest effect on Ang II and K\textsuperscript{+}. These results led us to suggest that another phosphatase, distinct from PP2A and regulated by intracellular Ca\textsuperscript{2+}, could be involved in control of aldosterone secretion in response to Ang II and K\textsuperscript{+}. This phosphatase could be calcineurin (CnA), which is also expressed in adrenal cells (Widmaier & Hall 1987, Kubo & Strott 1989).

CnA is a serine/threonine protein phosphatase controlled by intracellular Ca\textsuperscript{2+} and calmodulin, and plays a critical role in the signalling pathway controlling T-lymphocytes activation (Liu et al. 1991, Clipstone & Crabtree 1992, Clipstone et al. 1994). CnA is a heterodimer composed of a 19 kDa regulatory subunit and a 60 kDa catalytic subunit, which contains the binding site for calmodulin (Stemmer & Klee 1994, Sago et al. 1996).

The importance of CnA in the regulation of the immune cell function is based on the discovery that the immunosuppressants cyclosporine A (CsA) and FK506 are specific inhibitors of CnA in T-lymphocytes. CsA and FK506 form complexes with their respective intracellular binding proteins, cyclophilin A and FKBP12, which, in turn, bind to and inhibit CnA activity (Liu et al. 1991, Clipstone et al. 1994). Inhibition of CnA by CsA and FK506 blocks the dephosphorylation of activated T-cell transcription factors (NFATs) (Tocci et al. 1989, Loh et al. 1996, Timmerman et al. 1996, Batiuk et al. 1997).

NFATs are a family of proteins (NFAT 1–4, Rao et al. 1997) which reside in the cytoplasm in a phosphorylated form. When the intracellular Ca\textsuperscript{2+} concentration increases upon stimulation, CnA becomes activated and interacts directly with NFATs resulting in their rapid dephosphorylation and subsequent translocation to the nucleus.

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It has recently been reported that activated CnA is critical in the development of cardiac hypertrophy. Activation of CnA results in dephosphorylation of NFAT3 and the subsequent interaction of NFAT3 with the cardiac embryonic transcription factor, GATA4, during the development of hypertrophy in primary rat cardiomyocytes. CsA and FK506 block the increase in intracellular Ca\textsuperscript{2+} and hypertrophy in primary myocytes treated with Ang II (Molkentin et al. 1998). Moreover, transgenic mice expressing the constitutively active form of CnA developed a dramatic increase in heart size relative to their nontransgenic littermates. After treatment of the transgenic mice with CsA the size of the heart was not significantly different from the heart of nontransgenic littermates (Molkentin et al. 1998). More recently, it has been demonstrated by RT-PCR analysis that the four members of the NFAT (1–4) family are expressed, at the protein level, in ventricular myocytes, and are able to translocate to the nucleus after infection of the myocytes with an adenovirus expressing the constitutive form of activated CnA, or after stimulation with endothelin-1 (Van Rooij et al. 2003). The CnA/NFAT signalling pathway has also been associated with vascular development (Graef et al. 2001) and skeletal muscle remodelling (Crabtree & Olson 2002), indicating that the NFAT family members are expressed not only in T-cells but also in other cell types in response to a variety of external signals. Whether NFAT proteins are expressed in adrenal ZG cells is unknown; however, it is plausible that CnA through an NFAT independent mechanism regulates aldosterone biosynthesis or adrenal ZG cell proliferation, acting over other substrates. CnA could dephosphorylate RKIP, causing its reassociation with Raf (Corbit et al. 2003); this would explain the deactivation of Raf activity observed in bovine adrenal ZG cells in response to Ang II (Tian et al. 1998). On the other
hand, there is evidence that CnA is expressed in ZG cells (Widmaier & Hall 1987, Kubo & Strott 1989). The steroidogenic effect of Ang II in rabbit (Morishita et al. 1990) and rat adrenocortical cells (Mazzocchi et al. 1993) is markedly inhibited by CsA, but dependent aldosterone secretion had little effect on ACTH, and it has been shown that CsA and FK506 completely inhibited Ang II- or PMA-induced potentiation of ACTH-dependent cAMP formation in bovine ZG cells (Baukal et al. 1994), suggesting that the mechanism of the Ang II-induced increase of ACTH-stimulated cAMP formation is mediated by CnA.

The hypothesis that CnA may participate in MAPK activation in ZG cells is strengthened by recent studies focused on establishing the molecular basis that underlies cardiac hypertrophy. These studies have found that CnA is associated with MAPK activation and intracellular Ca²⁺; CnA is therefore posited as the principal physiological regulator of cardiac hypertrophy. CsA and FK506 retard cardiac hypertrophy, collagen accumulation and CnA activity in animals submitted to blood pressure overload (Meguro et al. 1999, Lim et al. 2000, Bueno & Molkentin 2002). Transgenic mice that overexpress the dominant negative mutant of CnA, especially in the heart, display significantly less size and weight increase of cardiac muscle in response to blood pressure overload as compared with nontransgenic mice (Zou et al. 2001a). In mouse cardiomyocytes, Ang II activates CnA and induces phosphorylation and activation of ERK1/2, MAPK p38, and c-jun, effects which are completely inhibited by CsA and FK506, and in mice displaying hypertrophy due to chronic high blood pressure, CsA completely blocks the increase in heart mass (Murat et al. 2000).

The relationship between intracellular Ca²⁺, CnA activation and ERK1/2 has been elegantly demonstrated by Zou et al. (2001b). These investigators studied the effect of isoproterenol in neonatal rat cardiomyocyte primary cell cultures and observed that isoproterenol increased cardiomyocyte size and activated ERK1/2. The latter effect was completely suppressed in the presence of high EGTA concentration, demonstrating that ERK1/2 activation depends upon external Ca²⁺. Fluorescence experiments demonstrated that isoproterenol increased the beating velocity of cardiomyocytes and induced an intracellular Ca²⁺ elevation, which was accompanied by a transient increase in CnA activity, that became significant at 1 min, maximized at 5 min, and lasted up to 30 min. Isoproterenol phosphorylates and activates Raf, a process blocked by CsA, suggesting that CnA is engaged as a positive regulator of ERK1/2 activation.

Conclusions

In the last few years, considerable effort has been made to support the concept that the signalling pathways that mediate the steroidogenic function in adrenal ZG cells, in response to their agonists, interact either negatively or positively in their effect on aldosterone production. All these studies have measured either Ca²⁺ fluxes or cAMP formation, processes that occur mainly at the plasma membrane, and have assessed the final product which is aldosterone. That adrenal ZG cells secrete aldosterone has been extensively documented, but the underlying molecular mechanisms that couple the Ang II-AT₁ receptor activated by Ang II at the cell membrane with the mitochondrial events, remains to be understood. The key step in aldosterone biosynthesis, as for all steroid hormones, is the transfer of cholesterol to the internal mitochondrial membrane. This process is assisted by StAR, and it is not known whether the interaction of the Ca²⁺ and cAMP messenger system converge before StAR phosphorylation and activation. Therefore, it appears that further efforts should be made to identify the site or sites at which these two signalling pathways interact.

Ang II, besides being one of the main agonists for the secretion of aldosterone, also stimulates proliferation of adrenal ZG cells through AT₁-dependent activation of MAPKs. However, Ang II-dependent activation of AT₁ receptors exerts proliferating and hypertrophic effects in other target cells such as epithelial cells, vascular smooth muscle cells, fibroblasts and cardiomyocytes. A large and significant amount of experimental evidence indicates that activated AT₁ receptors transactivate EGF receptor and thereby activation of MAPKs.

Whether the AT₁-dependent activation of the EGF signalling pathway exists in adrenal ZG cells is as yet unknown. However, it is known that in adrenal fasciculata cells that also express AT₁ receptors, ERK1/2 is activated by receptor tyrosine kinase activation.
Experimental studies on the role of calcineurin (CnA) in cardiomyocytes have demonstrated that CnA mediates the effect of the Ang II-AT₁ receptor activated by Ang II. However, despite the evidence that CnA participates in adrenal steroidogenesis, not many attempts have been made to define its role and sites of action either on the signalling pathways that mediate steroidogenesis or on the signalling pathways that mediate proliferation.

Finally, the studies discussed in the present review raise several questions, the most important of which are: does the Ang II-AT₁ receptor transactivate the EGF receptor in adrenal ZG cells? What effect do ACTH and K⁺ have on MAPKs activity? Do Ang II and K⁺ activate CnA, and if so, how does this activity relate to aldosterone secretion and proliferation?

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