Mitochondrial regulation of mineralocorticoid biosynthesis by calcium and the StAR protein

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Introduction

In mammals, all steroids are synthesized from a common precursor, cholesterol. In contrast to polypeptide hormone-secreting cells such as pancreatic or anterior pituitary cells, steroid-producing cells cannot store mature, ready-to-be-delivered hormone in secretory granules because, as a result of their lipophilicity, steroids can diffuse more or less freely across organelar and cellular membranes. To circumvent this difficulty, instead of accumulating end products, steroidogenic cells esterify large amounts of cholesterol after endogenous biosynthesis or internalization of low- or high-density lipoproteins (LDL or HDL) and package it into intracellular lipid droplets (1). As a corollary to this accumulation of precursor, the cells must be endowed with a system permitting a rapid recruitment of cholesterol, followed by immediate activation of the steroidogenic cascade. This requirement for the ability to generate acute steroidogenic responses appears particularly important in tissues such as the two outer zones of the adrenal cortex, which are involved in reactions to stress (glucocorticoids) and in blood pressure control (mineralocorticoids), although relatively prompt secretory responses to organotropic hormones are also needed in the testis and ovary.

The zona glomerulosa of the adrenal cortex produces aldosterone under the control of two main physiological activators, the octapeptide hormone angiotensin II (Ang II) and extracellular potassium (K\(^{+}\)) (2). In vitro, these agents are able to elicit steroidogenesis within 1–2 min and these kinetics are closely matched by a fast activation of the calcium messenger system within the same time frame in glomerulosa cells (3). In vivo, when Ang II is infused in man, the onset of the increase in plasma aldosterone is observed within 10 min (4). Clearly, the glomerulosa cell is endowed with a system allowing it rapidly to transduce the elicited intracellular messenger changes into cholesterol metabolism. The present article reviews our current knowledge on the mechanisms of cytosolic- and mitochondrial-calcium mediated acute aldosterone biosynthesis in glomerulosa cells, in the light of the recent discovery of the steroidogenic acute regulatory (StAR) protein and of its role in other steroidogenic tissues (5).

The calcium messenger system in adrenal glomerulosa cells

In the adrenal zona glomerulosa cell, both extracellular K\(^{+}\) and Ang II cause a sustained increase in cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{c}\)) that is necessary and sufficient for the acute response of mineralocorticoid production (3, 6, 7). Distinct mechanisms underlie the [Ca\(^{2+}\)]\(_{c}\) responses to Ang II and K\(^{+}\): release of calcium from intracellular stores and capacitative calcium influx for the hormone, influx through voltage-gated, T- and L-type channels for K\(^{+}\) and the hormone. These mechanisms have been reviewed in detail elsewhere (2). We will focus here on the intracellular events occurring downstream of the generation of the calcium signal (Fig. 1). Although the precise intracellular target(s) of the calcium messenger are still poorly defined, it appears likely that mitochondria, which contain essential steroidogenic enzymes, constitute an important site for the regulation of steroidogenesis by calcium. As a consequence, the various pathways of calcium transport across mitochondrial membranes should have a crucial role.

Mitochondrial calcium homeostasis

In many mammalian cell types, basal mitochondrial free calcium concentration, [Ca\(^{2+}\)]\(_{m}\), has been found to be of the same order of magnitude as basal [Ca\(^{2+}\)]\(_{c}\), and any change in [Ca\(^{2+}\)]\(_{m}\) is immediately relayed into the mitochondrial matrix (8–10). In some cell types, an amplification in the matrix of the [Ca\(^{2+}\)]\(_{c}\), response to specific stimuli has even been observed, and has been attributed to the existence of microdomains in which mitochondria are located in close proximity either to inositol 1,4,5-trisphosphate-sensitive calcium stores (9, 11, 12) or to the plasma membrane (10, 13), reflecting a variability in the mitochondrial network among tissues.

The rapid mitochondrial calcium uptake occurs through a specific calcium carrier or uniporter (14, 15) (Fig. 1). Mitochondrial calcium uptake through the uniporter can be defined as a secondary active process that facilitates transmembrane calcium diffusion along
outwardly directed proton pumping performed by the respiratory chain. The transport activity of the uniporter can be modulated by numerous agents, among which the most widely used are magnesium and ruthenium red.

To avoid an accumulation of calcium inside the matrix, which may be toxic to the cell, three different mechanisms can promote mitochondrial calcium extrusion: the Na\(^+\)-dependent efflux, the Na\(^+\)-independent Ca\(^{2+}\)-efflux, and a non-specific calcium extrusion process, the permeability transition pore (PTP) (14–17) (Fig. 1). Mitochondrial calcium efflux requires a source of energy that is supplied by the concentration gradients of the coupled transported ions (H\(^+\) or Na\(^+\)). In adrenal glomerulosa cells, mitochondrial calcium efflux is believed to be predominantly mediated by an Na\(^+\)-dependent mechanism, through a Ca\(^{2+}\)/Na\(^+\) exchanger (18).

Although ions generally cross the inner mitochondrial membrane via specific transporters, there is the additional, third, mechanism through which mitochondria become permeable to small ions and molecules. It has been shown that calcium is extruded by this process, through a specific pore, the PTP (17). Permeability transition is characterized by a loss of membrane potential and leads to an osmotic swelling of mitochondria, mainly as a result of the entry of potassium ion along its electrochemical gradient. Although it is sometimes a source of irreversible injury, the permeability transition may have an important role in many mitochondrial functions that remain to be clarified. The PTP is activated by an increase in [Ca\(^{2+}\)]\(_{\text{m}}\) and the immunosuppressor, cyclosporin A, is known to inhibit the pore, presumably through its binding to a mitochondrial cyclophilin (19).

Mitochondrial calcium in glomerulosa cells

In view of the intramitochondrial localization of important steroidogenic steps, knowledge of the behaviour of [Ca\(^{2+}\)]\(_{\text{m}}\) upon stimulation with Ang II or K\(^+\) is a prerequisite to establishing a causal link between the calcium messenger and aldosterone production. Fluorescent probes have been used to monitor [Ca\(^{2+}\)]\(_{\text{m}}\) in various cellular systems (20–22). Recently, however, an elegant new approach for measuring [Ca\(^{2+}\)]\(_{\text{m}}\) in intact living cells has been designed by using the properties of the Ca\(^{2+}\)-sensitive chemiluminescent photoprotein, aequorin (23). A chimeric cDNA was constructed that contained the mitochondrial matrix targeting presequence of subunit VIII of cytochrome c oxidase fused in frame with aequorin, thus allowing the targeted expression of the protein in the mitochondrial matrix (8). Luminescence variations were calibrated in terms of [Ca\(^{2+}\)]\(_{\text{m}}\), the profile of which could thus be recorded in intact living cells during hormonal stimulation.

When this technique was applied to bovine adrenal glomerulosa cells, it was found that stimulation with...
either Ang II or K⁺ led to immediate increases in [Ca²⁺]m, concomitant with the [Ca²⁺]c changes (24). However, marked quantitative differences were observed in the [Ca²⁺]m responses to the two agonists: whereas the peak [Ca²⁺]c response to Ang II was amplified approximately 10 times inside the mitochondrial matrix, K⁺ induced a sustained [Ca²⁺]c response, which was relayed without amplification into the mitochondrial matrix as a plateau of [Ca²⁺]m. These observations are reminiscent of those reported in other cell types, suggesting a close proximity between calcium storage compartments of the endoplasmic reticulum and mitochondria (11). This confinement of components of the calcium messenger system (25) could lead to the formation of microdomains of high calcium concentration around mitochondria of glomerulosa cells upon activation with Ang II, following inositol 1,4,5-trisphosphate-mediated release of calcium, thus explaining the high amplitude of the [Ca²⁺]m changes. In contrast, although the intracellular calcium response to K⁺ – mediated by T- and L-type voltage-operated calcium channels – is also highly compartmentalized (26), mitochondria may not be directly exposed to similarly high [Ca²⁺]c.

Thus, in adrenal glomerulosa cells, the calcium signal elicited by physiological stimulators of aldosterone biosynthesis is indeed relayed inside the mitochondrial matrix. As a logical consequence, targets for mitochondrial calcium should be found within the mitochondrion.

**Mitochondrial calcium and steroidogenesis**

In permeabilized bovine glomerulosa cells, increasing ambient calcium concentration, within the range of physiological [Ca²⁺]c, changes, leads to a stimulation of steroidogenesis, and this effect can be abolished by ruthenium red – a specific blocker of the mitochondrial uniporter, which prevents calcium entry into the mitochondrial matrix (6). Moreover, it has been shown that increases in [Ca²⁺]c, are relayed into the mitochondrial matrix and that inhibition of the mitochondrial Ca²⁺/Na⁺ exchanger, which decreases the rate of calcium extrusion from the mitochondria, potentiates the calcium-induced steroidogenesis (24). In addition, Rossier et al. (26) have presented evidence suggesting that calcium entering the cell through voltage-operated T-type channels bypasses the cytosol to activate directly the intramitochondrial steps of aldosterone biosynthesis.

The above observations indicate that changes in [Ca²⁺]m are a prerequisite to Ang II- and K⁺-induced aldosterone biosynthesis. This does not preclude additional important metabolic effects of [Ca²⁺]m on other mitochondrial functions. In fact, calcium is known to stimulate mitochondrial dehydrogenases, leading to an increased supply of NADH (27, 28) (Fig. 1). NADH can be further transformed by mitochondrial transhydrogenases into NADPH (29), which is a necessary cofactor for several enzymes of the steroidogenic cascade (30). Indeed, in intact single rat glomerulosa cells, the increases in [Ca²⁺]c, occurring under Ang II or K⁺ stimulation are paralleled by increases in mitochondrial NAD(P)H (31–33). However, as an increase in NAD(P)H does not influence the rate of 25-OH conversion of cholesterol to pregnenolone, this effect of Ca²⁺ on NAD(P)H does not appear to be rate-limiting in the activation of steroidogenesis (34).

**The role of StAR in the acute regulation of steroidogenesis**

A number of review articles and commentaries on the involvement of the steroidogenic acute regulatory (StAR) protein in steroidogenesis have been published recently (5, 35–38). These reviews have essentially concentrated on the regulation of steroid hormone synthesis by agonists triggering the cAMP second messenger system and the interested reader is advised to refer to them for such details. In this review article, we will first briefly summarize the main characteristics of the StAR protein, before focusing on the involvement of StAR in steroidogenesis stimulated by Ca²⁺-mobilizing activators – specifically, aldosterone production in response to Ang II and K⁺.

The acute response of steroidogenic cells to hormonal stimulation is a rapid increase in the rate of steroid hormone biosynthesis. This enhanced rate of steroidogenesis results from an increased availability of the substrate, cholesterol, to the cytochrome side-chain cleavage enzyme (P450sc) located in the inner mitochondrial membrane. Numerous studies have clearly demonstrated that the delivery of cholesterol from the mitochondrial outer membrane to the inner membrane is the acutely regulated and rate-limiting step in this process (1, 39–42). Observations made initially by Ferguson (43) and Garren et al. (44), and confirmed thereafter in various laboratories (45–50), have indicated that the supply of cholesterol to the inner mitochondrial membrane is prevented by inhibitors of protein synthesis such as cycloheximide. These findings established that the hormone-induced transfer of cholesterol from the mitochondrial outer membrane to the cytochrome P450sc in the inner membrane involved de novo synthesis of a regulatory protein. Importantly, it was also observed that, after addition of cycloheximide to prestimulated adrenal cells, the rate of steroid synthesis declined very rapidly, suggesting that ‘a cycloheximide-sensitive and highly labile protein’ was required in the acute production of steroids (44). During the past three decades, many studies have attempted to identify and characterize this protein(s). Several candidates, including the sterol carrier protein 2 (SCP2), the steroidogenesis activator polypeptide (SAP), the peripheral benzodiazepine receptor (PBR) and its ligand, diazepam binding inhibitor (DBI), have been proposed.
to mediate the acute phase of steroidogenesis (5, 51–54). Although the reported ability of these factors to increase steroidogenesis has rendered them attractive, these proteins do not fulfill all the criteria required for an acute regulator of steroidogenesis. These candidates have been extensively described previously and will not be discussed here.

The protein now called steroidogenic acute regulatory protein was first described as a family of four 30-kDa mitochondrial proteins induced by trophic hormone or cAMP analogues in rat and mouse adrenal and Leydig cells (55, 56). These proteins are synthesized as a larger, phosphorylated cytosolic precursor of 37 kDa, which is targeted to the mitochondrion by an amino-terminal signal sequence, then imported and processed to its mature 30-kDa forms (Fig. 1). Two-dimensional gel electrophoresis analysis has shown that the four forms differed only in isoelectric points, and that two of them were the phosphorylated homologues of the two others. Importantly, the hormone-induced synthesis of these proteins paralleled steroid production in both a time- and a concentration-dependent manner, and their synthesis was sensitive to cycloheximide. Using similar techniques, this family of proteins has also been characterized in stimulated MA-10 mouse Leydig and R2C rat Leydig tumour cell lines (57, 58). The proposed functional form of these proteins is the 37-kDa precursor, its half-life being consistent with the rapid decline of steroidogenesis following inhibition of cytosolic translation by cycloheximide. A direct cause and effect relationship between StAR protein expression and steroidogenesis was made possible by the recent cloning of the murine cDNA encoding the StAR proteins (59).

Overexpression of StAR cDNA in MA-10 cells, which have undetectable amounts of these proteins under basal conditions, resulted in a marked increase of steroid production in the absence of hormone stimulation, indicating a direct role for the StAR proteins in enhanced steroidogenesis. Soon thereafter, the essential role of StAR protein in the acute regulation of steroidogenesis was confirmed by studies on lipid congenital adrenal hyperplasia (LCAH), a disease resulting in a complete inability of the newborn to synthesize steroids (60, 61). Mitochondria from affected adrenal glands and gonads fail to convert cholesterol into pregnenolone and, as a consequence, cholesterol accumulates within the cells. The defects responsible for this disease are mutations in the StAR gene, which generate truncated and non-functional proteins (60). In addition to the first report on StAR mutations causing LCAH (60), many other examples of mutations in StAR resulting in this disease have been described (61–64), and perhaps it is not as rare as previously believed. To date, mutations in the StAR gene are the only known causes of LCAH, and have demonstrated the indispensable role of StAR in the production of steroids.

Importantly, StAR appears to be highly specific for steroidogenic tissues, namely adrenal cortex, ovarian granulosa and luteal cells and testicular Leydig cells. In all these tissues, activation of the adenyl cyclase–cAMP–protein kinase A pathway leads to an increase in levels of StAR mRNA and protein (37). Studies on StAR expression during development revealed that the protein is present in the developing adrenal and testis, but absent in the developing ovary. These observations demonstrated a spatial and temporal relationship between the presence of StAR transcripts and the capacity to produce steroid hormones (65). In contrast, StAR was not detected in the placenta and brain, an observation suggesting that in these tissues, steroidogenesis is regulated by a StAR-independent mechanism. In fact, a protein with significant homology to StAR, termed MLN64 (66), which displays steroidogenic activity and is expressed in brain and placenta (67), may be instrumental for steroid biosynthesis in those organs, suggesting the existence of a family of proteins involved in intracellular cholesterol trafficking (67).

The mode of action of StAR in promoting cholesterol transfer is at present unknown. While it was believed initially that transmembrane cholesterol transfer could be facilitated during the importation of StAR mediated by its N-terminal addressing sequence, the subsequent discovery that all the mutations that inactivate StAR are located at its C-terminal end (61) and that the biologically active domain of StAR resides in its C-terminus (68), strongly suggested that additional component(s) participate in StAR action. The peripheral-type benzo- diazepine receptor has been proposed as one such partner (69, 70).

The role of StAR in calcium-stimulated aldosterone biosynthesis

As already mentioned, in adrenal zona glomerulosa cells, mineralocorticoid synthesis is under the control of two major extracellular stimuli activating the calcium messenger system, Ang II and K⁺. Many studies have clearly demonstrated the crucial role of the Ca²⁺ messenger in the acute regulation of aldosterone biosynthesis (3, 7, 30, 71, 72). Indeed, the steroidogenic response of isolated adrenal cells to Ang II and K⁺ is highly dependent upon the extracellular Ca²⁺ concentration (73). Moreover, calmodulin and calmodulin-dependent protein kinase II antagonists have been shown to inhibit Ang II-stimulated aldosterone production in rat zona glomerulosa cells and in the human NCI H295 adrenal tumour cell line (74, 75). Finally, as indicated above, increasing ambient Ca²⁺ stimulates aldosterone output in permeabilized glomerulosa cells, a response that is prevented by ruthenium red (6), and clamping cytosolic Cu²⁺ within the range of the physiological concentrations results in increased pregnenolone and aldosterone formation (34).

In spite of these clearcut data, the intracellular molecular targets of the Ca²⁺ messenger in the activation of aldosterone biosynthesis were poorly
defined. Many questions concerning the possible role of Ca\(^{2+}\) in the regulation of cholesterol supply to the P450\(_{scc}\) within mitochondria remained unanswered. Elliott et al. (76) reported the appearance of several mitochondrial proteins in bovine adrenal glomerulosa cells in response to Ang II and K\(^+\) stimulation. The molecular weight and isoelectric points of these proteins indicated that they belong to the StAR protein family (77). StAR was also shown to be induced by Ang II, K\(^+\) and the calcium channel agonist, BayK 8644, in the H295R human adrenocortical tumour cell line (78), indicating that the regulation of StAR protein expression may represent a common mechanism through which the cAMP and the Ca\(^{2+}\) messenger systems control adrenal steroidogenesis. Interestingly, the calmodulin-dependent (CaM) kinase II inhibitor, KN93, has been shown to impair Ang II- and K\(^+\)-stimulated aldosterone synthesis in the H295R cell line, without inhibiting the expression of StAR induced by these agonists, thus suggesting that CaM kinase II is involved in a phosphorylation step that might be necessary for StAR activation (Fig. 1). In fact, four potential consensus sites for CaM kinase II phosphorylation have been identified in the primary sequence of the StAR protein (37) and phosphorylation of StAR modulates its steroidogenic activity, as shown by site-directed mutagenesis (79).

Using the cytosolic Ca\(^{2+}\)-clamp technique, the effect of Ca\(^{2+}\) on cholesterol transfer from the outer to the inner mitochondrial membranes was investigated in freshly prepared bovine adrenal glomerulosa cells. It was shown that endogenous cholesterol transfer from the outer to the inner mitochondrial membrane and to functionally relevant membrane structures, the intermembrane contact sites, is indeed substantially stimulated by physiological concentrations of cytosolic Ca\(^{2+}\) within 2 h of stimulation (80). The supply of cholesterol to the P450\(_{scc}\) appears thus to be a Ca\(^{2+}\)-sensitive step in the early steroidogenic pathway. When bovine glomerulosa cells were challenged with the Ca\(^{2+}\)-mobilizing hormone Ang II, in addition to promoting intramitochondrial cholesterol transfer, the hormone stimulated the supply of cholesterol to the mitochondria (80). A striking feature of these studies was the marked increase in cholesterol content at contact sites, compared with that at inner membranes, in response to Ang II or to a high-Ca\(^{2+}\) clamp. This finding suggested that Ca\(^{2+}\) markedly enhances cholesterol availability in regions where the intermembrane space barrier is abolished.

Importantly, the activation of intramitochondrial cholesterol transfer was associated with a clearcut and specific increase of the mature form(s) of the 30-kDa StAR protein in the inner mitochondrial membranes of bovine glomerulosa cells, under stimulation of the calcium messenger system (81). The effect of increased [Ca\(^{2+}\)]\(_{c}\) was specific for StAR, as the concentrations of two additional key steroidogenic enzymes in the mitochondria, cytochrome P450\(_{scc}\) and 3β-hydroxysteroid dehydrogenase, were not affected under similar experimental manipulations (82). This was the first demonstration of a sub mitochondrial modulation of the StAR protein by Ca\(^{2+}\). Indeed, most studies that have investigated the regulation of the 30-kDa proteins involved in the acute steroidogenic response have used total cellular or mitochondrial protein extracts and have focused on trophic agents activating adenyl cyclase and cAMP production (55, 57, 58, 82, 83). Interestingly, although the StAR protein was present at contact sites, the increase in cholesterol induced in these structures by increases in [Ca\(^{2+}\)]\(_{c}\) was not associated with a concomitant increase in StAR content, indicating that the StAR protein is only transiently involved at contact sites. Moreover, the 37-kDa precursor of StAR was not detected in any of the sub mitochondrial fractions; this observation confirmed the short half-life of the StAR precursor, which is known to be rapidly processed by mitochondrial proteases (42), and indicated that the inner mitochondrial membranes constitute the final destination of the mature StAR protein. These biochemical data corroborated the morphological evidence on the localization of StAR in adrenal mitochondria that had been obtained by immunogold staining (84). Both intramitochondrial cholesterol transfer and the accumulation of StAR protein induced by increases in [Ca\(^{2+}\)]\(_{c}\) were highly sensitive to cycloheximide, a finding that strongly suggests that the blockade of StAR protein synthesis prevents the mobilization of cholesterol to the inner membranes. Taken together, these results provided strong correlative evidence that the increase in expression of StAR protein and its targeting to the mitochondrial inner membranes is linked to calcium-induced cholesterol redistribution from the outer to the inner membranes. Thus the current model of the mechanisms of calcium-induced activation of steroidogenesis would favour a dual site of action for Ca\(^{2+}\) (Fig. 1): in addition to an obligatory role for Ca\(^{2+}\) influx into the mitochondria, as demonstrated previously in permeabilized glomerulosa cells (6) and in glomerulosa cells treated with a blocker of the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger (24), the cycloheximide results imply an effect of cytosolic Ca\(^{2+}\) on StAR expression.

Conclusions
Aldosterone biosynthesis appears to be a good model for studying and understanding the mechanisms of the acute activation of steroidogenesis. For many years, the mediator(s) of cholesterol transfer to the intramitochondrial P450\(_{scc}\) enzyme have eluded intensive searches. An important breakthrough was brought by the discovery of the steroidogenic acute regulatory protein. Under the control of both the cAMP and the Ca\(^{2+}\) messenger systems, the StAR protein plays a major part in this system, although it is becoming increasingly obvious that some other cofactor(s) may be required.
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