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MINIREVIEW

Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought

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Steroid hormone biosynthesis in steroidogenic cells is regulated through trophic hormone activation of protein kinase A (PKA) signaling pathways. However, many examples of the regulation of steroid synthesis via pathways other than the PKA pathway have been documented. In some cases these pathways act independently of PKA activation whereas in other cases, they act synergistically with it. The current understanding of additional signaling pathways and factors, such as the protein kinase C pathway, arachidonic acid metabolites, growth factors, chloride ion, the calcium messenger system, and others capable of regulating/modulating steroid hormone biosynthesis, and in many cases steroidogenic acute regulatory protein expression, are discussed in this review. (*Molecular Endocrinology* 19: 2647–2659, 2005)

STEROID HORMONES ARE synthesized in steroidogenic cells of the adrenal, ovary, testis, placenta, and brain and are required for normal reproductive function and bodily homeostasis. Both the acute and chronic regulation of steroidogenesis are predominantly controlled by trophic hormones and occur on the order of minutes and hours, respectively. The acute response is initiated by the mobilization and delivery of the substrate for all steroid hormone biosynthesis, cholesterol, from the outer to the inner mitochondrial membrane where it is metabolized to pregnenolone by the cytochrome P450 cholesterol side chain cleavage enzyme (P450scc) (1). The chronic response involves the increased transcription/translation of the genes encoding the steroidogenic enzymes (2). The acute response to hormonal stimulation has an absolute requirement for *de novo* protein synthesis (3, 4). Whereas inhibition of protein synthesis blocks hormone-induced steroid synthesis it has no affect on the activity of the P450scc or the delivery of cholesterol to the outer mitochondrial membrane, but its delivery to the inner mitochondrial membrane is completely inhibited. Therefore, acute production of steroids requires a trophic hormone stimulated, rapidly synthesized, and cycloheximide-sensitive protein that is involved in the transfer of cholesterol from the outer to the inner mitochondrial membrane, the rate-limiting and regulated step in steroidogenesis. Several candidate proteins have been proposed for this process, and among them the steroidogenic acute regulatory protein (StAR) possesses all the necessary characteristics of the acute regulator (5, 6). The 30-kDa StAR protein was purified, cloned, and sequenced from MA-10 mouse Leydig tumor cells (5). StAR is predominantly associated with steroid-producing tissues and consists of a 37-kDa precursor form containing an N-terminal mitochondrial targeting sequence and several isoelectric forms of a 30-kDa mature protein (7, 8). Clinical studies on patients suffering from congenital lipoid adrenal hyperplasia, a condition in which virtually no steroids are synthesized, as well as studies on StAR null mice, have critically illustrated the indispensable role of StAR in regulated steroidogenesis (9, 10). In addition, the correlation between the tissue-specific expression of

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Abbreviations: AA, Arachidonic acid; Ang II, angiotensin II; AP-1, activator protein 1; ARTISt, AA-related thioesterase involved in steroidogenesis; CG, chorionic gonadotropin; CoA, coenzyme A; COX, cyclooxygenase; CREB, cAMP response element-binding protein; CYP17, 17α-hydroxylase/17,20-lyase; dbcAMP, dibutyryl-cAMP; EGF, epidermal growth factor; 5-HETE, 5-hydroxyarachidonic acid; 3β-HSD, 3β-hydroxysteroid dehydrogenase; LHR, LH receptor; P450scc, cytochrome P450 cholesterol side chain cleavage enzyme; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; PMA, phorbol 12-myristate 13-acetate; PRL, prolactin; SIP, steroidogenic-inducing protein; StAR, steroidogenic acute regulatory protein.

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the StAR gene and the capacity of those tissues to produce steroids, both during development and in the adult, indicates its specific role in steroidogenesis (11, 12). Even so, whereas the transcriptional and/or translational inhibition of StAR expression results in a dramatic decrease in steroid biosynthesis, approximately 10–15% of steroid synthesis appears to be mediated via StAR-independent mechanisms (6, 13, 14).

Trophic hormone stimulation of steroidogenic cells has been shown to result in the activation of G proteins that stimulate adenylate cyclase activity and produce increased intracellular levels of cAMP and activation of protein kinase A (PKA) (15, 16). PKA action results in the phosphorylation of proteins such as cholesteryl ester hydrolase as well as the phosphorylation of transcription factors including steroidogenic factor 1, GATA-4, and cAMP response-element binding protein (CREB)/cAMP response element modulator that function to activate genes involved in steroidogenesis, including StAR (17–19). Studies have demonstrated the importance of PKA in maintaining steady state levels of StAR protein, and posttranslational modification of proteins by PKA is also involved in the regulation of steroidogenic function (12). Two separate PKA phosphorylation sites in murine and human StAR, serine56/57 and serine194/195, were mutated to alanine, and significant decreases in StAR expression and steroidogenesis were obtained only with the serine194/195 mutation (20). Using hamster adrenal StAR cDNA expressed in COS-1 cells, it has recently been demonstrated that mutation of the PKA phosphorylation sites in the StAR protein inhibited steroid biosynthesis by 70–80% (21).

Therefore, it is unequivocal that cAMP is an important second messenger for trophic hormone-stimulated steroid biosynthesis. The well-known role of the cAMP/PKA pathway in regulating steroid hormone biosynthesis has been the subject of many excellent review articles (5, 11, 12, 22–24) and will not be elaborated upon in great detail here. However, whereas the cAMP/PKA pathway is undoubtedly the major signaling cascade regulating steroidogenesis, many recent studies have indicated that additional pathways are involved in this process as well. The purpose of this minireview is to summarize the findings of many laboratories, including our own, that have examined, in addition to the cAMP/PKA transduction pathway, multiple signaling pathways involved in steroidogenesis, and where evidence exists, the role of these pathways in regulating StAR expression.

REGULATION OF STEROIDOGENESIS BY cAMP-INDEPENDENT SIGNALING EVENTS

As stated above, the cAMP/PKA signaling cascade is an important second messenger pathway for trophic hormone-stimulated steroid biosynthesis and StAR expression. However, a large body of evidence indicates that regulation of steroidogenesis can also be modulated through signal transduction pathways not involving cAMP. Indeed, several factors that do not require cAMP and/or protein synthesis have been demonstrated to potently stimulate steroidogenesis (25). These include growth factors, macrophage-derived factors, steroidogenic-inducing protein (SIP), chloride ions, and calcium (Ca^{2+}) messenger systems. It should be noted, however, that regardless of the stimulant, the cAMP-independent induction of steroidogenesis is quite modest when compared with the cAMP/PKA response, usually being less than 1% of that seen with the cAMP/PKA-dependent pathway. Even though the magnitude of response mediated by these factors on steroidogenesis is small, many of them are capable of potentiating the steroidogenic responsiveness of gonadal cells to gonadotropins or cAMP analogs, and, by doing so, they play important roles in regulating various testicular/ovarian functions. In addition, an overwhelming amount of data indicates that the testis produces a variety of regulatory molecules and that a local control system exists within this organ. This section of the review will focus on those events that play roles in the stimulation of the steroidogenic response via cAMP-independent mechanisms.

Several lines of evidence demonstrate that growth factors, especially epidermal growth factor (EGF) and IGF-I, can stimulate steroid synthesis and StAR expression in Leydig cells without altering the level of intracellular cAMP (Refs. 26–29 and Manna, P. R., and D. M. Stocco, unpublished observations). Although these factors are capable of stimulating steroidogenesis, their response is far lower ($<$1%) when compared with LH/human chorionic gonadotropin (hCG). The relevance of these factors has been demonstrated in the regulation of gonadal, especially testicular, development and function, including spermatogenesis (30–32). Treatment of mouse Leydig cells with EGF (2–8 h) has been shown to increase steroid biosynthesis and modulate the steroidogenic response of submaximal concentrations of hCG or cAMP analogs (27, 29, 33). In contrast, at longer times (8–48 h), EGF down-regulates LH/hCG receptors and thus limits steroidogenic responsiveness. Additional studies have also demonstrated that EGF does not affect intracellular Ca^{2+}, inositol 1,4,5-triphosphate (IP_3) or diacylglycerol levels (33). Likewise, treatment of IGF-I/insulin has been found to stimulate steroidogenesis and StAR expression in Leydig cells, and this process also does not require cAMP signaling (Refs. 27, 28, and 34 and Manna, P.R., *et al.,* unpublished observations). The temporal response pattern of IGF-I-mediated StAR protein expression was found to be significantly increased at 2 h, was maximal at 6 h, and thereafter started decreasing (12–24 h) with time. On the other hand, an increase in LH/hCG-mediated StAR expression was evident at approximately 30 min, increased to a maximal level by 4–6 h, and decreased by 24 h (6, 12, 5, 35). IGF-I was also found to potentiate the activation of Leydig cell steroidogenic responses me-

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diated by LH/hCG or cAMP analogs. Whereas IGF-I was capable of increasing StAR protein expression, it had no effect on StAR phosphorylation, a requirement in obtaining the optimal steroidogenic response (20). Moreover, IGF-I was shown to increase LH/hCG receptor protein and mRNA levels and the activity and mRNA levels of several steroidogenic enzymes (27). Recent studies in our laboratory demonstrate that the protein kinase C (PKC) pathway plays an essential role in IGF-I-mediated StAR expression and steroid synthesis, and that a specific PKC inhibitor, GF-109203X, markedly decreased the IGF-I-mediated steroidogenic response. EGF and IGF-I utilize the MAPK/ERK pathway, which has previously been implicated in steroid synthesis and StAR expression (29, 36). However, the role of MAPK/ERK in the steroidogenic response has been demonstrated to vary with cell surface receptor-effector coupling and in a species and tissue-specific manner (36, 37). We also observed that IGF-I phosphorylated CREB/activating transcription factor-1 and the activator protein-1 (AP-1) family member cJun/JunD, and that these events were found to be involved in regulating StAR gene expression. In addition, a PKA inhibitor, H-89, was capable of inhibiting the IGF-I-mediated steroidogenic response, indicating that endogenous cAMP most likely plays a role in IGF-I responsiveness as the latter had no affect on intracellular cAMP levels. These results imply that mechanisms of IGF-I action involve multiple signaling pathways in regulating steroid synthesis and StAR expression in mouse Leydig cells (Fig. 1).

Testicular macrophage-derived factors, IL-1 and TNFα, have been shown to be both stimulatory and inhibitory in controlling steroidogenesis in rat Leydig cells (38, 39). Verhoeven *et al.* (39) have shown that IL-1 markedly stimulates the production of progesterone and testosterone in immature rat Leydig cells. The stimulatory effect was evident within 2 h of IL-1 incubation either alone or with low concentrations of LH. On the other hand, treatment with IL-1 (6–12 h) in the presence of maximally effective doses of LH ($\geq$100 ng/ml) resulted in an inhibition of steroidogenesis. It was demonstrated that stimulation occurred at the level of the C-17,20 desmolase and involved a cAMP-independent pathway. Additionally, chronic effects of IL-1 (24–48 h) in Leydig cells were shown to be additive to those of GnRH, EGF, and arginine vasopressin, suggesting different mechanisms of action. In human adrenocortical cells, treatment with IL-3 and IL-6 (12–48 h) have been reported to stimulate cortisol secretion in a cAMP-independent manner, and the involvement of the cyclooxgenase and lipoxygenase pathways was demonstrated (40). In a series of studies, Pollard and colleagues have demonstrated the physiological role of macrophage-derived factor in developmental processes and in reproductive function (reviewed in Ref 41). In addition, a lipophilic factor from testicular macrophages was identified and shown to stimulate steroidogenesis in gonadal and adrenal cells (42, 43). In Leydig cells, this factor was capable of

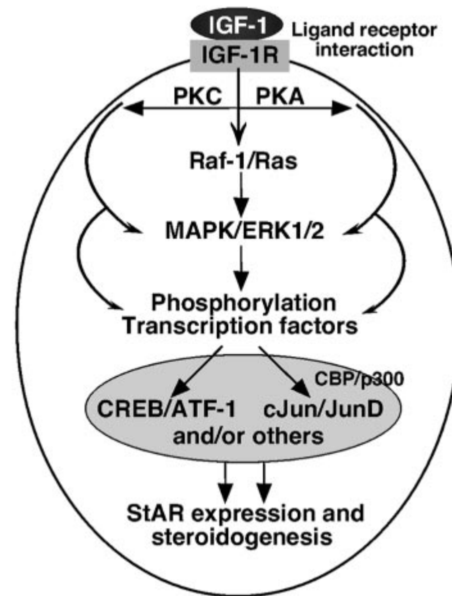

Fig. 1. A Schematic Model of IGF-I Signal Transduction Pathways Regulating StAR Expression and Steroidogenesis in Leydig Cells

The binding of IGF-I to its receptors results in activation of receptor tyrosine kinase activity and mediates biological functions through several mechanisms, including receptor autophosphorylation, receptor clustering, and phosphorylation of intracellular protein(s). This leads to the activation of a cascade of protein kinases including Raf-1/Ras and the MAPK/ERK1/2 or related kinases. These protein kinases, in turn, activate different transcription factors, including CREB/activating transcription factor 1 (ATF-1) and cJun/JunD. Phosphorylation of these transcription factors results in transcriptional regulation of the StAR gene and thus, steroid synthesis. Regulation of StAR expression can be influenced by CBP/p300, a factor known to interact with many transcription factors including CREB and cJun. The PKA and PKC signaling pathways can directly or indirectly activate transcription factors, and both of these pathways were found to be involved, at least in part, in IGF-I mediated steroidogenic response in mouse Leydig cells. CBP, CREB-binding protein; IGF-1R, IGF-I receptor.

increasing steroid production within 30 min, was half-maximal by 6–8 h, and maximal by 15–24 h (44). They also demonstrated that this factor was equipotent with hCG in stimulating Leydig cell steroid production and did not require new protein synthesis and did not alter StAR protein levels. Later, this factor was purified from testicular and peritoneal macrophages, and its physicochemical properties were found to be identical with those of 25-hydroxycholesterol (45). The physiological relevance of macrophage-derived intratesticular 25-hydroxycholesterol has yet to be determined.

In yet another example of cAMP-independent regulation of steroidogenesis, a proteinaceous factor isolated from human follicular fluid, SIP, was demonstrated to stimulate steroid synthesis in gonadal and adrenal cells (46, 47). In Leydig cells, SIP was capable of augmenting steroid production in a dose- and time-

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dependent manner, and its response was essentially equivalent to that observed with maximal doses of LH/hCG or dibutyryl-cAMP (dbcAMP) (47, 48). Interestingly, the steroid synthesized in response to these stimuli was further enhanced by SIP, indicating that SIP mediates its effects via a distinct signaling pathway other than those activated by LH/hCG or cAMP analog. Also, SIP was found to increase DNA synthesis and protein-tyrosine kinase activity in rat Leydig cells (49). That SIP was not acting through the cAMP-dependent pathway was demonstrated with the PKA inhibitor HA1004, which, in contrast, markedly decreased LH/hCG-stimulated steroid synthesis. The action of SIP, like LH/hCG-stimulated steroidogenesis, requires the *de novo* synthesis of proteins (47). However, it has been demonstrated that the StAR protein is not involved in SIP-mediated steroid synthesis, and the identity of these as yet unknown protein(s) remains a mystery.

A member of the cytokine/lymphokine/GH receptor family, prolactin (PRL), is known to play an important role in testicular function, including Leydig cell steroidogenesis and LH receptor (LHR) function (50, 51). Evidence is accumulating that PRL action is dependent on time, concentration, and the functional state of the Leydig cells, and both stimulatory and inhibitory effects of PRL on steroidogenesis have been demonstrated. Biphasic effects of PRL on steroid synthesis, StAR expression, and LHR expression have been demonstrated in mouse Leydig cells and consist of an inhibition within 24 h at higher doses (≥30 ng/ml) of PRL and a stimulation at 48–72 h with low doses (1–10 ng/ml) (51, 52). Similar biphasic responses on intracellular Ca^{2+}, LHR, PRL receptor, and Janus Kinase 2 mRNA levels were also obtained in response to PRL; however, in the presence of Ca^{2+}, PRL increased the levels of hCG-stimulated steroid production and StAR expression (52). The effect of PRL on steroid synthesis and StAR protein levels was confined to the hCG signaling pathway, as similar effects were not observed with cholera toxin or with forskolin treatment. Notably, the induction of hypoprolactinemia by bromocriptine results in suppression of LHR levels, and PRL treatment has been shown to increase Leydig cell numbers and LHR levels as well as LH-mediated steroid synthesis in hypophysectomized rats (53). Nevertheless, hyperprolactinemia in men is known to be associated with hypogonadism and impaired gonadal function, and in rats it induces an inhibition of Leydig cell steroidogenesis (54). Even though considerable progress has been achieved in the understanding of PRL signaling in the past few years, the exact mechanisms of PRL action remain obscure.

The involvement of chloride (Cl^-) ions in stimulating steroidogenesis in response to LH/hCG or cAMP analogs has been demonstrated in Leydig and adrenal cells (55, 56). In Leydig cells, omission of Cl^- potentiated steroid production and StAR expression when cells were stimulated with low doses of LH (≤1 ng/ml) or cAMP analog (≤0.1 mM), but not with maximal stimulating levels of these agents. This indicates the importance of Cl^- in the stimulation of steroidogenesis at concentrations of LH that had little to no effect on cAMP synthesis (55, 57). More specifically, MA-10 cells treated with dbcAMP (0.1 mM; but not with 1.0 mM) in a Cl^--free buffer exhibited 4- and 15-fold increases in progesterone production by 2 and 6 h, respectively, over the response seen in Cl^--free buffer. Whereas Cl^- omission increased StAR protein expression, it had no significant effects on P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD) levels. These findings provide evidence for the involvement of two separate pathways, one operating at physiological levels of LH (≤1 ng/ml) that involve Cl^- channels and cAMP-independent action and the other at concentrations of LH (≥100 ng/ml) that stimulate cAMP synthesis and are associated with cAMP-dependent mechanisms (57). In support of this, using patch-clamp studies it has been established that LH/hCG or cAMP analog stimulation can increase Cl^- conductance in Leydig cells (58). Furthermore, omission of Cl^- can increase steroid production in the absence of Ca^{2+}, suggesting noninvolvement of the latter in potentiating the effects of Cl^- ions. Nevertheless, Cl^- channels are linked to Ca^{2+} channels and are regulated by phosphorylation via PKA or PKC, and a complex relationship between these two ions in the steroidogenic process has been reported (55, 59). This phenomenon was also demonstrated in rat zona glomerulosa cells in which low levels of ACTH (10^{-12} M) were able to increase Cl^- current in a cAMP-independent manner whereas cAMP analog, forskolin, or 3-isobutyl-methyl xanthine could not (60). These investigators proposed the role of an alternate signaling pathway, involving Ras, based on the inhibition produced using specific antisera to this protein. Furthermore, Ras-GTPγS was shown to stimulate Cl^- channels, and a Cl^- channel blocker decreased aldosterone production in the presence of low concentrations of ACTH. This study demonstrated that at low ACTH levels, Cl^- channels and Ras regulate steroidogenesis through a cAMP-independent pathway. However, in Leydig cells, cAMP analog-stimulated phosphorylation of ERK1/2 has recently been demonstrated to be mediated by a PKA-dependent activation of Ras (61), and ERK1/2 signaling is known to play permissive roles in regulating steroidogenesis.

The role of the Ca^{2+} messenger system has been implicated in steroidogenesis in gonadal and adrenal cells. Hormonal response in steroidogenic cells involves alterations in intracellular cAMP and Ca^{2+} levels. Evidence is accumulating that LH/hCG, cAMP analogs, and GnRH or its agonists can directly stimulate steroidogenesis in Leydig cells (25), but the maximal effect of GnRH agonists in stimulating steroidogenesis was approximately 30% in comparison to LH/hCG. All of these compounds rapidly increase intracellular Ca^{2+} (within 1–5 min); however, the magnitude of the response is higher with LH/hCG and cAMP analog when compared with GnRH agonist (62–

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64). The increase in intracellular Ca^{2+}, either released from intracellular stores or by mobilization of Ca^{2+} from extracellular spaces, is known to play an important role in steroidogenesis. For example, the induction of steroid synthesis with GnRH agonists in ovarian (65) and Leydig (62) cells has been demonstrated, and in the case of ovarian cells, the mechanism was shown to involve Ca^{2+} and not cAMP. Studies have demonstrated that in Ca^{2+} free media at maximal stimulating doses of LH/hCG (≥100 ng/ml), steroid synthesis was decreased by approximately 50% at 6 h (55, 62, 64). Also, omission of Ca^{2+} from the extracellular medium diminished LH/hCG-stimulated steroid synthesis and StAR expression. Subsequent addition of extracellular Ca^{2+} to the incubation medium restored the LH/hCG-stimulated steroidogenic response. Importantly, the removal of extracellular Ca^{2+} inhibits cAMP analog-stimulated steroidogenesis, indicating a requirement for Ca^{2+} distal to cAMP formation. In addition, the Ca^{2+} ionophore A23187 was able to increase hCG-stimulated progesterone production and StAR expression by 4 h in an additive manner (64). Furthermore, the specificity of Ca^{2+} action on the hCG-induced steroidogenic response was demonstrated with the use of Ca^{2+} chelators or Ca^{2+} channel blockers. It has also been demonstrated that inhibitors of the Ca^{2+} binding protein, calmodulin, decrease steroidogenesis at the level of the mitochondria. In particular, the requirement for the entry of Ca^{2+} into the mitochondrial matrix after hormonal induction of steroidogenesis was demonstrated with an inhibitor of mitochondrial Ca^{2+} uptake, ruthenium red, which blocked Ca^{2+}-mediated steroid synthesis in adrenal cells (66). Also, the importance of mitochondrial Ca^{2+} in angiotensin II (Ang II)- and potassium (K^{+})-stimulated steroidogenesis has been reported in bovine adrenal glomerulosa cells. In the case of adrenal steroidogenesis, the stimulatory action of Ang II has been demonstrated to be associated with phospholipase C activity, the subsequent release of inositol 1,4,5-triphosphate, and the mobilization of Ca^{2+} (67). Similar to the role of Ca^{2+}, K^{+} is regarded as an important physiological regulator of adrenal steroid biosynthesis. K^{+} can act as a Ca^{2+} mobilizing agent through the opening of voltage-sensitive transmembrane Ca^{2+} channels (58). Treatment of H295R human adrenocortical cells with K^{+} for 48 h resulted in increases in aldosterone, cortisol, and dehydroepiandrosterone, and in the increased expression of P450scc and P450c17 mRNA levels (68). In addition, a Ca^{2+} channel agonist, BAYK8644, was able to induce steroidogenesis and StAR expression in adrenal cells (69, 70). In mouse Leydig cells, K^{+} has been shown to increase hCG-stimulated steroid synthesis and StAR expression by 4 h (64). Studies have shown that agents that affect intracellular Ca^{2+} levels inhibited the induction of the steroidogenic response mediated by K^{+}. These findings provide evidence for the importance of Ca^{2+} and/or K^{+} in potentiating the action of LH/hCG on the steroidogenic response (58, 64).

Collectively, it seems very clear that in addition to cAMP/PKA signaling, multiple and complex intracellular signaling events are operative in the regulation of steroidogenesis. It is highly likely that the interaction of one or more of these signaling pathways occurs as a result of cross-talk between them and results in the stimulation of steroid biosynthesis and StAR expression.

THE ROLE OF PKC IN STEROIDOGENESIS

The binding of trophic hormones to their specific receptors results in the formation of cAMP and the activation of the PKA pathway as indicated earlier. In addition, ligand interaction with these receptors can activate phospholipase C and trigger the activation of the downstream PKC pathway (71–73). The effects of PKC on steroidogenesis have been controversial as a consequence of much conflicting evidence on this topic. Since the characterization of PKC as an authentic phorbol 12-myristate 13-acetate (PMA) or 12-*O*-tetradecanoyl-phorbol-13-acetate receptor (74, 75), the involvement of the PKC pathway in steroidogenesis has been studied using this reagent in a variety of steroidogenic cells and has shown that PKC is inhibitory, stimulatory, or has no effect on steroid synthesis (76–80).

PMA is a weak inducer of steroid synthesis in steroidogenic cells when compared with cAMP-mediated signaling, producing <1% of the maximal response (81–83). By itself, PMA has slight stimulatory effects on the synthesis of pregnenolone, progesterone, dehydroepiandrosterone sulfate, testosterone, and cortisol as demonstrated using adrenal, granulose, and Leydig cells (77, 84–86). The effects of PMA on steroid production were highly dependent on the types of cells used as well as on the nature of the steroidogenic stimulant with which it was coincubated.

PMA effects on steroidogenesis were investigated using freshly isolated rat granulosa and Leydig cells (77). Many studies have demonstrated that stimulation of primary cultures of rat Leydig cells with hCG, cholera toxin, and dbcAMP resulted in increases in testosterone production. However, when PMA (10 and 100 ng/ml) was added to these cells for 48 h, it inhibited 17α-hydroxyprogesterone, androstenedione, and testosterone production in a dose-dependent manner whereas the level of pregnenolone was increased. These data suggested that PMA inhibited 17α-hydroxylase/17,20-lyase (CYP17) activity in rat Leydig cells (77), and qualitatively similar results were seen in mouse Leydig (87) and H295R adrenocortical (68) cells. Recent studies from our laboratory using primary cultures of rat Leydig cells are consistent with those results in that PMA (10 nM, a dose equivalent to 62 ng/ml) reduced the production of testosterone but increased progesterone synthesis induced by dbcAMP (0.5 to 1 mM) or by LH/hCG at both 6 and 24 h of

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incubation, an observation ostensibly due to the inhibition of CYP17 (88). A comparable effect of PMA (10–167 nM) was demonstrated in LH-induced progesterone, androstenedione, and estradiol accumulation in theca cells from hen ovarian follicles (89). PMA (10–162 nM) itself resulted in no change in progesterone production but decreased the LH- or cAMP-induced levels of androstenedione and estradiol with acute (90) and chronic (89) treatments. The authors once again suggested that the target of PMA might be the inhibition of CYP17 activity, a result that may explain how theca cells in the ovary switch from androgen to progesterone production after the LH surge.

As mentioned earlier, PMA is able to induce steroid production only slightly in some steroidogenic cells (77, 84–86). We have recently determined that activation of the PKC pathway with 10–50 nM PMA for 6 h in MA-10 cells had little effect on steroid production but was able to significantly increase StAR expression to a level comparable to 0.5 to 1 mM dbcAMP (88). This seeming contradiction was explained when the posttranslational modification status of the StAR protein was investigated. It was found that whereas StAR protein expression was indeed significantly increased through activation of the PKC pathway, the protein thus produced was not phosphorylated. In contrast, activation of the PKA pathway, *e.g.* using 0.5 to 1 mM dbcAMP, results in the synthesis and phosphorylation of StAR and the production of high levels of steroids by 6 h of treatment. Thus, it appeared that whereas PMA was able to regulate expression of the StAR gene, the absence of steroid synthesis was a result of its inability to phosphorylate the StAR protein. This hypothesis was quickly corroborated when a subthreshold amount of dbcAMP (0.05 to 0.1 mM), incapable of inducing StAR expression or steroid production by itself, was added to the PMA-treated cells (Fig. 2). Under these conditions, a further increase in steroid production was seen, and the phosphorylation status of StAR was similar to that observed when cells were treated with maximally stimulating levels of cAMP analog (0.5 to 1 mM). Using Northern and RT-PCR analyses, it was determined that the levels of StAR mRNA were increased by PMA, indicating that the action of PKC occurred at the level of transcription. In addition, we also determined that PMA-induced PKC was able to activate MAPK/ERK1/2, resulting in the phosphorylation of CREB and the decreased expression of DAX-1 (Fig. 2) (88). Observations similar to those found with PMA were made in MA-10 cells using the Ca^{2+} ionophore, A23187. A23187 (1 μM) alone was able to induce significant amounts of StAR protein synthesis within 6 h with no increase in steroid production. When submaximal doses of dbcAMP (0.05 to 0.1 mM) were added to the A23187-treated cells, significant increases in steroid production were then observed. These findings allow us to make several conclusions. First, activation of the PKC pathway results in the transduction of signals that are able to increase the transcription and translation of StAR. Second, activation of PKC does not result in the phosphorylation of the StAR protein. Third, phosphorylation of StAR is an absolute requirement for its cholesterol-transferring function and thus, steroidogenesis. Fourth, the levels of intracellular cAMP and PKA activity required to obtain full steroidogenesis in the presence of activated PKC are quite low. In fact, the level of intracellular cAMP that can produce optimal steroid production in the presence of PMA is significantly below the level required to induce StAR expression and steroid synthesis. Essentially identical results were observed in studies with H295R cells. When stimulated with dbcAMP, Ang II, K^+, BAYK8644 (an L-type Ca^{2+} channel agonist), and PMA, each of these agonists was able to induce StAR expression, but only PMA was unable to increase aldosterone synthesis (69). In that study, the authors discussed the possibility that the StAR protein induced by PMA was not phosphorylated and thus not able to function in cholesterol transfer, a hypothesis corroborated by our recent findings. Therefore, it appears that, at least in some steroidogenic cells, the

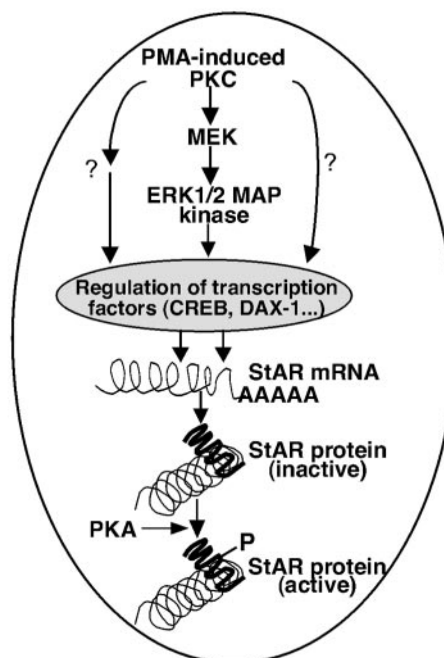

Fig. 2. A Schematic Diagram for the Interplay of the PKC Pathway and the Required Role of PKA Activity in StAR Regulation in Leydig Cells

PKC activation by PMA can regulate StAR transcription and translation by activation of positive regulatory transcription factors (*e.g.* CREB) and inhibition of repressors (*e.g.* DAX-1). The pathway results in activation of MEK/ERK1/2 either by direct or indirect regulation of transcription factors that are not clearly known (designated by ?). However, the StAR protein is not active because it cannot be phosphorylated by PMA. In the presence of submaximal doses of PKA activity, the PKC-activated StAR protein is phosphorylated and activated and thus able to transfer cholesterol and support steroidogenesis. By itself, this level of PKA activity does not significantly increase total StAR or result in steroidogenesis. MEK, MAPK kinase.

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PKC pathway can participate in the steroidogenic process by taking part in the expression of the StAR gene, but in the absence of StAR phosphorylation by PKA, the synthesis of steroids is not possible. This represents yet another example of the exquisite cross-talk that can occur between signaling pathways.

THE ROLE OF ARACHIDONIC ACID (AA) METABOLITES IN STEROIDOGENESIS

In addition to the role of the cAMP signal transduction pathway, studies over the past three decades have demonstrated a critical role for AA-mediated signal transduction in trophic hormone-stimulated steroid biosynthesis (24, 91–93). It has been reported that trophic hormone stimulation not only induces cAMP formation, but also results in the release of AA from intracellular stores. AA release occurs within 1 min of LH stimulation (94) and is dependent on hormone-receptor interaction and the concentration of LH/hCG binding sites on the cell surface (95). Recent studies suggested that this hormone-receptor interaction resulted in the activation of G proteins followed by the activation of phospholipase A2 (PLA2), which in turn catalyzed the release of AA from phospholipids (96). In addition to its direct effect on PLA2 activity, G protein activation also induced AA release through its ability to increase intracellular cAMP. In addition to the release of intracellular AA through the activation of PLA2, at least one additional AA-releasing pathway in steroidogenic cells has been demonstrated (97–102). Treatment of adrenal cells with cAMP induced the synthesis of a 43-kDa acyl-coenzyme A (CoA) thioesterase, which was named the AA-related thioesterase involved in steroidogenesis (ARTISt; also known as mitochondrial acyl-CoA thioesterase or MTE-1). ARTISt releases AA using arachidonyl-CoA as the preferred substrate and the AA released plays a role in steroidogenesis (97). Later studies from this group indicated that in addition to ARTISt, an AA-preferring acyl-CoA synthase (ACS4), an enzyme rapidly induced by hormone stimulation, was also involved in the release of AA from intracellular arachidonyl-CoA (100–102). They also reported that knock down of MTE-1 and ACS4 with RNA interference resulted in the inhibition of StAR mRNA and StAR protein expression and steroid biosynthesis in adrenal cells, indicating that these proteins may be involved in steroidogenesis by providing the AA that is required for StAR expression (102). Thus, the release of AA that is involved in the regulation of steroidogenesis through an alternate pathway has been demonstrated.

Regardless of the cellular source, AA release is critical for trophic hormone-stimulated steroidogenesis and StAR expression. Abayasekara *et al.* (103) reported that inhibition of AA release from phospholipids reduced LH (100 ng/ml) and stimulated testosterone production approximately 80% by 2 h in rat testicular Leydig cells, without affecting intracellular levels of cAMP. They also determined that AA acts at the rate-limiting step of steroidogenesis, the transfer of the substrate cholesterol to the inner mitochondrial membrane. Later studies clearly demonstrated that AA release was required for steroid biosynthesis and StAR expression and that inhibition of AA release inhibited the LH- or dbcAMP-induced steroidogenic response in MA-10 cells (104, 105). Consequently, expression of the StAR protein and steroid synthesis at 6 h were reduced by approximately 80% and 60%, respectively, when compared with 0.5 mM dbcAMP treatment. Importantly, the inhibitory effects were reversed by the addition of AA to the cell cultures with StAR protein, and steroid hormone recovering as concentrations of AA in the culture medium were increased. Qualitatively similar results were obtained with StAR mRNA expression and StAR promoter activity, demonstrating that AA regulates StAR expression at the level of transcription. EMSAs utilizing a StAR promoter segment (−96/−67 bp) showed that AA can also enhance the binding of nuclear protein(s) to the StAR promoter, suggesting the presence of an AA-responsive element in this region (106). This element has not yet been characterized, and it is also possible that AA or its metabolites induce or activate transcription factor(s) or co-activator(s) that bind to this region of the StAR promoter to enhance StAR gene transcription. Thus, the initial observations documenting the requirement for AA in stimulated steroidogenesis appears to result from the role of AA in regulating expression of the StAR gene. In addition to the AA-induced StAR-mediated regulation of steroidogenesis, other studies have demonstrated that AA alone can induce steroid synthesis in rat Leydig (107) and MA-10 (108) cells and, at least in the case of MA-10 cells, AA alone appears to have no effect on StAR protein expression (105). Given the effects of AA on steroid biosynthesis in Leydig cells, additional studies on the steroidogenic process were performed to assess the activities of specific steroidogenic enzymes in response to AA. When exogenous AA was added to rat Leydig cells in culture, a dose-dependent inhibition of testosterone synthesis was observed. Further examination of the steroidogenic enzyme activities indicated that AA had no affect on P450scc or 3β-HSD but that AA inhibited testosterone synthesis by inhibiting the activity of 17β-HSD (109). The mechanism of this inhibition is not yet known.

The mechanism of AA action in regulating steroidogenesis has been further explored. After AA is released, it is metabolized mainly through one of three enzymatic pathways, the cyclooxygenase (COX), the lipoxygenase, or the epoxygenase. It was reported that inhibition of either lipoxygenase or epoxygenase activity inhibited StAR protein expression and steroid synthesis (105). In MA-10 cells, inhibition of the epoxygenase pathway reduced dbcAMP (0.5 mM)-stimulated steroid production approximately 80% by 6 h (105). Previous observations in granulosa and adrenal

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cells also described a trophic hormone-stimulated release of AA and an increase in its metabolites produced in the epoxygenase pathway and suggested the involvement of these metabolites in steroidogenesis (93, 110, 111). Additional studies showed that stimulation of MA-10 cells in response to dbcAMP significantly increased the intracellular levels of 5-hydroxyarachidonic acid (5-HETE) and 5-hydroperoxyarachidonic acid (5-HPETE), AA metabolites produced in the lipoxygenase pathway (106). Importantly, when these metabolites (20 μM each, 6 h) were added to MA-10 cells to verify their effect on steroidogenesis, it was found that they enhanced dbcAMP-stimulated StAR protein expression. Similarly, steroid production was increased approximately 40% and 50% by 5-HETE and 5-HPETE, respectively, over the response seen with 0.5 mM dbcAMP (106). In rat Leydig cells, 5-, 12-, and 15-HETE were also demonstrated to increase testosterone formation (107). Also, 12-HETE has been shown to increase intracellular Ca^{2+} and aldosterone synthesis in rat glomerulosa cells (112). Furthermore, the metabolites of the lipoxygenase and epoxygenase pathways have been demonstrated to stimulate 3β-HSD and 17β-HSD activities and enhance the synthesis of testicular steroid hormones (113). These studies suggested that AA metabolites produced through the lipoxygenase and epoxygenase pathways are positively involved in StAR expression and steroid biosynthesis.

As mentioned above, blocking AA release inhibited LH- or dbcAMP-stimulated StAR expression and steroid synthesis, but PKA activity still remained at high levels. However, this high level of PKA activity is unable to induce significant increases in StAR expression and steroid production in the absence of AA (105), suggesting that PKA phosphorylation alone does not represent the entire signaling pathway involved in trophic hormone-stimulated steroidogenesis. The inhibition of StAR protein expression and steroid production was reversed by addition of AA to the cell culture, indicating that the blocked signaling event is an AA-mediated pathway (105). Similarly, inhibition of PKA activity inhibited StAR protein expression but, in this case, the inhibition of StAR expression and steroid production could not be reversed by addition of AA. Also, AA alone had no significant effect on StAR protein expression and StAR-mediated steroid production. These studies indicated that AA transduces an obligatory signal from LH and cAMP to the nucleus through a pathway different from PKA phosphorylation. These findings demonstrate that both cAMP and AA signaling pathways are required and that they interact with each other in a synergistic manner for LH-stimulated steroidogenesis and StAR expression (114). One aspect of this interaction is the stimulatory effect of cAMP on AA release in rat Leydig and in MA-10 cells (93, 114). Another aspect is the dose-dependent enhancement of cAMP-induced steroid synthesis, StAR protein, StAR mRNA, and StAR promoter activity by AA (104–106, 114). The cooperation between these two pathways appears to increase the sensitivity of steroidogenesis to trophic hormone stimulation and, therefore, enhancing the signal in either of these pathways dramatically increases StAR expression and steroid hormone biosynthesis.

Whereas lipoxygenase and epoxygenase metabolites are involved in the AA-mediated signaling that enhances StAR expression and steroid production, additional studies from our laboratory suggested that COX2, one of the isoforms of COX, played an important role in regulating Leydig cell steroidogenesis by maintaining a tonic inhibition of StAR gene expression (115). Inhibition of COX2 activity using a selective COX2 inhibitor dramatically increased dbcAMP-stimulated StAR expression and steroid synthesis. Normally, a very low level of dbcAMP (*i.e.* 0.05 mM) is unable to induce significant increases in steroid hormone or StAR protein levels in MA-10 cells. However, by inhibiting COX2 activity, the effectiveness of subthreshold concentrations of dbcAMP was greatly enhanced and resulted in increases in StAR protein expression and progesterone production by 6- and 34-fold, respectively, by 6 h when compared with cells stimulated with dbcAMP. COX2 specificity was demonstrated when inhibition of COX1 activity using a selective inhibitor did not induce significant increases in StAR expression or steroid synthesis. Despite the impressive increase in steroidogenesis attributed to the reduction in COX2 activity, a minimal level of cAMP is always necessary. In the absence of cAMP, the COX2 inhibitor alone could induce neither detectable StAR protein nor a significant increase in steroid production. These observations suggested that the inhibitor itself had no direct stimulatory effect on steroidogenesis, but rather, resulted in a reduction of the tonic inhibition produced by COX2 and an increased sensitivity of steroidogenic cells to cAMP stimulation. This hypothesis was supported in studies on the expression of COX2 in COS-1 cells. Overexpression of COX2 reduced StAR promoter activity by 40% and abolished steroidogenic factor 1-enhanced StAR promoter activity in COS-1 cells (115). More recent studies demonstrated that expression of COX2 in MA-10 cells enhanced tonic inhibition of dbcAMP-stimulated StAR expression and steroidogenesis and that these events could be reversed by inhibition of COX2 activity.

The mechanism for the role COX2 activity plays in steroidogenesis is unknown. The increase in StAR protein expression and steroid production in COX2-inhibited cells was not due to an increase in PKA phosphorylation because PKA activity in inhibitor-treated cells remained at low levels (115). These observations indicated that a low level of cAMP or PKA phosphorylation is sufficient in stimulating StAR expression and steroid production if AA metabolism through the COX2 pathway is blocked. It has been previously reported that an AA metabolite produced by COX2 action, prostaglandin F2α, inhibits rat StAR gene expression through the AP-1 family member, c-Fos (116, 117). However, another member of the c-Fos subfamily,

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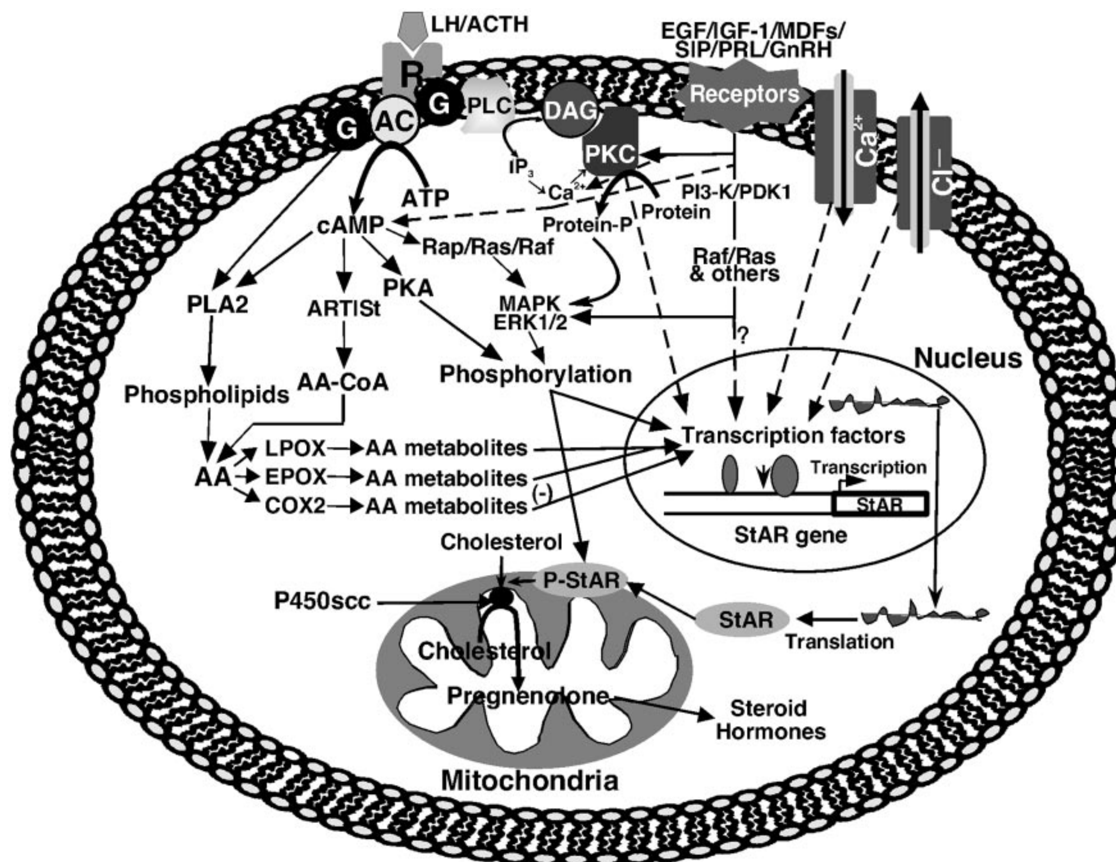

Fig. 3. Proposed Model Illustrating Multiple Signaling Pathways in the Regulation of StAR Expression and Steroidogenesis

Trophic hormone (LH/ACTH) interaction with specific membrane receptors results in the activation of G proteins (G), which, in turn, activate membrane-associated adenylyl cyclases (AC) that catalyzes cAMP formation from ATP. cAMP then activates PKA, which results in the phosphorylation of transcription factors regulating StAR gene transcription. CAMP-mediated signaling mechanisms predominantly regulate StAR expression and steroid biosynthesis in steroidogenic cells. The StAR protein regulates steroidogenesis by controlling the transport of cholesterol from the outer to the inner mitochondrial membrane, the site of the cytochrome P450scc enzyme. Conversion of cholesterol to pregnenolone is the first enzymatic step in steroid hormone biosynthesis. Ca^{2+} messenger system and Cl^{-} ions have been demonstrated to be effectively involved in potentiating trophic hormone-stimulated steroidogenesis and StAR expression. G protein or cAMP can also activate PLA2 that, in turn, catalyzes AA release from phospholipids. cAMP also activates ARTISt, an enzyme that catalyzes AA release from arachidonyl-CoA (AA-CoA). AA released from these sources is metabolized by one of three enzymes, lipoxygenase (LPOX), epoxygenase (EPOX), or COX. LPOX and EPOX metabolites play important roles in StAR expression and steroid synthesis. Conversely, COX2 metabolites produce a tonic inhibition of StAR gene expression. The PKC pathway was also found to be involved in regulating the steroidogenic response. Activation of the PKC pathway results in an increase in the transcription and translation of StAR, but not its phosphorylation. Thus, StAR induced through the PKC pathway is inactive in cholesterol transfer. EGF/IGF-I/macrophage-derived factors (MDFs) SIP/PRL/GnRH bind to specific membrane receptors and can stimulate the steroidogenic response via different signaling pathways. Furthermore, cAMP and/or different factors are capable of activating a cascade of protein kinases (Rap/Ras/Raf or related kinases) leading to the MAPK/ERK1/2 pathway that has been demonstrated to function in the regulation of StAR expression and steroid biosynthesis. PDK1, Protein-dependent kinase 1; PI3-K, phosphatidylinositol 3-kinase; P-StAR, phosphorylated StAR.

FosB, was found to be ineffective in regulating rat StAR gene expression, whereas expression was increased by c-Jun. This finding is in agreement with the opposing effects often seen in the regulation of a number of genes by the AP-1 proteins, Fos and Jun (118, 119). Recently, the specific involvement of Fos and Jun in transcriptional regulation of the mouse StAR gene was demonstrated by identifying an AP-1 binding site in the cAMP-responsive region (−151/−1 bp) of the mouse StAR promoter (120). Utilizing this promoter segment, c-Fos was demonstrated to increase basal but decrease cAMP-mediated StAR gene expression, although all Fos and Jun members influenced StAR's transcriptional regulation to varying degrees. In contrast, both basal and cAMP-stimulated rat StAR gene expression were repressed by c-Fos in Y-1 adrenocortical cells, as demonstrated utilizing the p–1862 bp StAR segment that contains three AP-1 recognition motifs. In fact, the contrary effects of c-Fos on basal StAR reporter activity (*i.e*. stimulatory in

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mouse *vs.* inhibitory in rat) could be due to the utilization of different StAR promoter segments and/or species specificity. Additionally, the observations with prostaglandin F2α suggest that inhibition of COX2 activity might reduce AA metabolites that inhibit StAR gene expression, resulting in an increase in steroid production. Further studies are needed to elucidate the COX2-dependent tonic inhibition of LH-stimulated StAR gene expression and steroid hormone biosynthesis.

In conclusion, the studies summarized here have attempted to describe the roles of multiple signaling events in controlling steroidogenesis. As a result of the studies listed here, it is not sufficient to concentrate solely on the cAMP/PKA pathway when examining the acute regulation of steroidogenesis. It is now abundantly clear that several signaling pathways all contribute to this process. Although complex in nature, the cross-talk that occurs among these pathways serves to regulate steroid production and, in many cases, this regulation occurs through the induction and/or inhibition of StAR expression. The complexity of the interactions of the signaling pathways controlling steroid biosynthesis and StAR expression are shown in Fig. 3. This process will undoubtedly become even more complex in years to come.

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