Up-Regulation of Basal Transcriptional Activity of the Cytochrome P450 Cholesterol Side-Chain Cleavage (CYP11A) Gene by Isoform-Specific Calcium-Calmodulin-Dependent Protein Kinase in Primary Cultures of Ovarian Granulosa Cells

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Intracellular calcium ions (Ca\(^{2+}\)) regulate steroidogenesis in the placenta, adrenal gland, testis, and ovary. Earlier data indicate that Ca\(^{2+}\)/calmodulin-dependent protein kinase (CamK) may mediate Ca\(^{2+}\)-dependent up-regulation of CYP11A (cholesterol side-chain cleavage). To examine this notion further, we assessed the expression and actions of isotype-specific CamK on in vitro transcription of the swine CYP11A gene promoter in primary cultures of ovarian granulosa-luteal cells. RT-PCR and oligodeoxynucleotide sequencing identified gene transcripts encoding CamKIII and IV in granulosa and theca cells and corpora lutea. DNA sequence homology with the cognate human and rat genes was 97 and 94% (CamKIII) and 96 and 88% (CamKIV), respectively. SDS-PAGE and isoform-specific immunoblotting corroborated expression of CamKIII (52 kDa) and CamKIV (60 kDa) proteins. To monitor transcriptional control, granulosa-luteal cells were transfected transiently with a putative 5'-upstream regulatory region of the homologous CYP11A gene (−2320 to +23 bp) from the transcriptional start site driving luciferase (CYP11A/luc). Coexpression of constitutively active CamKIV elevated basal transcription by 3.5 ± 0.2-fold (P < 0.001), whereas inactive mutant CamKIV and native CamKII had no effect. Forskolin, an activator of adenyl cyclase, stimulated expression of CYP11A/luciferase by 4.5 ± 0.9-fold (P < 0.001) and did not enhance transcriptional drive by exogenous CamKIV. Preliminary promoter-deletional analyses showed that a proximal 5'-fragment (−100 to +23 bp) regulated full responsiveness to CamKIV (4.5 ± 0.4-fold; P < 0.001). Threefold cotransfection of −100/+23 bp CYP11A/luciferase, active CamKIV, and a dominant-negative mutant of the cAMP-responsive element binding protein (10, 100, and 250 ng) inhibited CamKIV-stimulated transcriptional activity by 17, 47, and 48% (pooled SEM 2%) [P < 0.01]. The dominant-negative mutant of the cAMP-responsive element binding protein also repressed forskolin's stimulation of −100/+23 CYP11A/luciferase by 12, 38, and 52% (P < 0.01). Based on these ensemble outcomes, we postulate that endogenous CamKIV may serve as a Ca\(^{2+}\)-dependent effector mechanism to maintain basal CYP11A gene expression in ovarian granulosa-luteal cells. (Endocrinology 145: 5616–5622, 2004)
Reagents

Ovine FSH (National Institute of Diabetes and Digestive and Kidney Diseases; potency 94 × National Institutes of Health oFSH-S1) was obtained from the National Hormone and Pituitary Program, NIH (Bethesda, MD); porcine insulin and forskolin from Sigma-Aldrich Chemical Corp. (St. Louis, MO); Eagle’s MEM, Ham’s F12/Dulbecco’s MEM, Opti-MEM, penicillin/streptomycin, gentamicin, fetal calf serum, collagenase, DNase, T4 DNA ligase, and RNase inhibitor, murine leukemia virus reverse transcriptase, Ampli- wax PCR beads, and AmpliTaq Gold DNA polymerase from Perkin-Elmer Corp. (Brandsberg, NJ).

PCR oligonucleotide primer pairs were designed based on the known DNA coding sequences of CamKII and CamKIV in the human, mouse, and rat so that the expected PCR product would be 347 bp for CamKII and 271 bp for CamKIV. The sense and antisense primers for CamKII (GenBank accession no. U72973) were 5'-GCTGATGCCAGCCACTGTAAT-3' and 5'-TGTTGATGGGAAGTACGAGG-3', respectively. The sense and antisense oligonucleotide primers for CamKIV (GenBank accession no. XM_003965) were 5'-CCGATTACTGATCGACGC-3' and 5'-AACAGTGCTCCCTGCTGAC-3', respectively.

Granulosa cell culture

Porcine granulosa and theca cells were harvested from small and medium (1–5 mm)-sized follicles by fine-needle aspiration of ovaries obtained from immature swine (60–70 kg), as described previously (17–19). For subsequent RT-PCR and immunoblot analyses, cells (3 × 10^5 granulosa cells/dish or 1.5 × 10^5 theca cells/dish) were plated at 37°C in bicarbonate-buffered MEM with 3% fetal calf serum with antibiotics (penicillin, streptomycin, and gentamicin) overnight (18–24 h) in 10-cm dishes (Corning, Inc., Corning, NY). In transient transfection studies, granulosa cells (2.5 × 10^5/well) were plated in 24-well plates containing the same medium plus insulin (1 µg/ml), estradiol (0.5 µg/ml), and FSH (5 ng/ml) to permit cell anchorage for 48 h and partial steroidogenic maturation. The foregoing medium was replaced once after 24 h. Cells were harvested 24 h later by rinsing once with Dulbecco’s PBS and then lysed with either TrisReagent (Molecular Research Center, Inc., Cincinnati, OH, for RT-PCR experiments) or immunoprecipitation buffer (9.1 mM dithiothreitol, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% IEPGALCA-630, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride for immunoblot experiments).

RT-PCR

Semi-quantitative RT-PCR was used to detect mRNA encoding CamKII and CamKIV by reverse transcription (RT) of 2.5 µg total cellular RNA. Each reaction contained 1 µm of each dNTP, 2.5 µM oligo (dT)_15 primer and deoxynucleotidetriphosphates (dNTP) from Roche Molecular Biochemicals (Indianapolis, IN); and RNase inhibitor, murine leukemia virus reverse transcriptase, Ampli- wax PCR beads, and AmpliTaq Gold DNA polymerase from Perkin-Elmer Corp. (Brandsberg, NJ).

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Preparation of porcine CYP11A promoter-luciferase (luc) plasmid

A 2353-bp fragment (−2320 to +29) 5′-upstream to and inclusive of the transcriptional start site of the porcine CYP11A gene and nested deletion sequences have been described (17). In the present studies, fragments were recovered by BamHI/HindIII digestion and subcloned into cognate restriction sites in a promoterless vector upstream of firefly luciferase (p0Luc-IAV Link V4; a gift from Richard Day, University of Virginia) and polyadenylation tract (TK poly A, Stratagene, La Jolla, CA). The luc protein targets to the cytoplasm (Luc IAV, Promega Corp., Madison, WI). PCRs were performed using a thermocycler (Thermocycle Thermometry I, Barnstead-Thermolyne) and Taq polymerase to generate double-stranded DNA fragments containing −73 and −50 nucleotides 5′-upstream through 23 bp downstream of the transcriptional start site. BamHI and HindIII restriction sites were engineered into the DNA fragments for directional subcloning into p0Luc-IAV. The identity of each fragment was verified by DNA sequencing.

Transfection of porcine granulosa-luteal (GL) cells

Transfection conditions were optimized 24 h after the second medium change. Monolayer cultures were rinsed with serum-free MEM without antibiotics for 30–45 min before the addition of transfection medium (500 µl/well) containing 1 µg total plasmid DNA and 6 µl Lipofectamine. Total DNA comprised 650 ng CYP11A/luc construct, 100 ng TK-renilla-luc (to normalize data for transfection efficiency), and 250 ng p0Luc (empty control vector) or constitutively active or inactive forms of either CamKII or CamKIV plasmids under control of Rous sarcoma virus promoter (gifts from Anthony R. Means, Duke University, Durham, NC). After transfection proceeded for 6 h at 37°C, culture medium was replaced with MEM and 3% fetal calf serum containing antibiotics for 6 h to allow cell recovery. Expression continued for an additional 4 h in serum-free MEM containing antibiotics and either vehicle [dimethylsulfoxide (DMSO)] or 10 µM forskolin. A promoterless luc expression vector was transfected in parallel cultures as a negative control.

To monitor the transcriptional response, granulosa cells were rinsed once with Dulbecco’s PBS and lysed in 100 µl lysis buffer (luc assay system, Promega). Lysates were stored at −70°C until assay. Luc activity was measured in 20 µl cellular lysate with 100 µl firefly luciferin substrate (Promega) and 100 µl renilla substrate (Promega) using a TD20e luminometer (Turner Designs, Sunnyvale, CA).

Data analysis

Each experiment was performed at least three times using cells harvested from a separate batch of 200–300 ovaries. To normalize for transfection efficiency, the luc to renilla activity ratio is expressed relative to the within-experiment (basal) CYP11A/luc control value or as a percentage of maximal luc stimulation [a dominant-negative mutant of Elk response element-binding protein (KCREB) experiments]. Means from the independent experiments were logitistically transformed and subjected to one-way ANOVA. Significant contrasts were assessed post hoc using Tukey’s multiple comparison test. P < 0.05 was considered significant.
Results

Ethidium bromide staining of agarose gel-resolved products of RT-PCR of total mRNA extracted from granulosa and theca cells and corpora lutea revealed single bands consistent with a predicted 347 bp size for CamKII and 271 bp for CamKIV in each cell/tissue type (Fig. 1). The PCR product corresponding to CamKII had 97 and 94% homology with human and rat DNA sequences, respectively, and the product associated with CamKIV, 96 and 88% homology, respectively. A hemi-cAMP response element (CRE) sequence in reverse orientation was identifiable between −57 and −45 bp in the pig CYP11A proximal promoter region.

SDS-PAGE immunoblotting of protein extracts from each of granulosa and theca cells and corpora lutea revealed bands of either approximately 52 kDa or approximately 60 kDa, consistent with CamKII and CamKIV, respectively (Fig. 2). A single band was evident for CamKII using monoclonal human antisem, whereas additional bands were observed with the polyclonal antisem to CamKIV. Immunospecific positive controls included lysates of rat brain containing CamKII and Jurkat-lymphoma cells containing CamKIV (21, 22). Neuronal CamKII consists of two major subunits of 52 and 60 kDa encoded by α-CamKII (52 kDa) and β-CamKII (60 kDa) genes, respectively (21). In many tissues, CamKIV also migrates on SDS-PAGE as a closely spaced doublet (23, 24).

To explore transcriptional responsiveness to CamK, granulosa cells were transfected with −2320/+23 CYP11A/luc and constitutively active forms of CamKIV or CamKII, corresponding inactive mutants or empty vector along with DMSO or forskolin (10 μm) (Fig. 3, top left). Constitutively active CamKIV consistently increased basal luc expression by 3.5 ± 0.9-fold (P < 0.001). None of the three control plasmids altered the activity of full-length CYP11A/luc or the promoterless luc plasmid. The adenyl cyclase-activating diterpene, forskolin, increased CYP11A/luc expression by 4.5 ± 0.9-fold (P < 0.001). The absolute effect of forskolin was not influenced by cotransfection of any vector, including CamKIV. However, the further increase achieved by forskolin combined with CamKIV was less than that induced by forskolin alone due to elevated basal −2320/+23 CYP11A/luc activity associated with CamKIV overexpression.

Initial promoter analysis was carried out using successive 5′-deletional constructs of −2320/+23 CYP11A/luc comprising −1100, −500, −100, −73, and −50 bp upstream to +23 bp downstream of the transcriptional start site. None showed modulation by CamKII. Figure 3 summarizes responses of −2320 (above), −100, −73, and −50/+23 CYP11A/luc to overexpression of CamKIV. The two intermediate-sized sequences evinced transcriptional responsiveness equivalent to that of the full-length −2320 fragment to CamKIV; viz., 4.5 ± 0.4 and 4.5 ± 0.6-fold that of empty expression vector (P < 0.001 vs. unstimulated). In four additional experiments, basal expression of −50/+23 CYP11A/luc was significantly reduced to 13% that of −100/+23 CYP11A but remained responsive to overexpression of constitutively activated CamKIV and stimulation with forskolin (10 μm); viz, respectively by 2.3 ± 0.2-fold (P < 0.01) and 2.6 ± 0.3-fold (P < 0.01). Combined CamKIV and for-
skolin were not additive in effect (2.9 ± 0.4-fold) [n = 4 experiments].

Inspection of the proximal (−100 bp) 5′-upstream regulatory region revealed a putative hemi-CRE sequence in reverse orientation (−57/−45 bp). To test possible involvement of the major CRE-binding protein (CREB) in CamKIV-dependent stimulation, we cotransfected GL cells with −100/+23 bp CYP11A/luc, constitutively active CamKIV or pSG5 (empty vector), and KCREB (a gift from Richard Goodman, Vollum Institute, Oregon Health Sciences University, Portland, OR). The KCREB vector contains a full-length CREB cDNA harboring a single base pair substitution that introduces an Arg (287) to Leu (287) transversion in the corresponding protein-DNA binding domain. Thereby, KCREB dimerizes with and inactivates nuclear CREB (25). Cotransfection of KCREB (10, 100, and 250 ng DNA) and Renilla/luc (100 ng) inhibited CamKIV (250 ng)-stimulated −100/+23 bp CYP11A/luc (400 ng) expression in a concentration-dependent manner; viz., by 17, 47, and 48% (P < 0.01; pooled SEM ± 2%; Fig. 4A). Under the same conditions, KCREB: 1) did not alter basal −100/+23 bp CYP11A/luc expression; 2) suppressed the −100/+23 bp reporter response to forskolin by 12, 38, and 52% (P < 0.01 concentration response); and 3) inhibited stimulation of −100/+23 bp CYP11A/luc by combined forskolin and CamKIV by 9.5, 40, and 43% (P < 0.01). In corollary, we tested the impact of KCREB (250 ng) on basal (DMSO) and CamKIV vs. forskolin (10 μM) stimulation of −50/+23 CYP11A/luc. Basal (unstimulated) −50/+23 CYP11A/luc activity was only 13% that of −100/+23 CYP11A/luc (Fig. 4B). KCREB did not change basal or CamKIV-induced −50/+23 CYP11A/luc expression but inhibited that...
driven by forskolin (by 51 ± 8.3%) and in lesser measure that induced by forskolin combined with CamKIV (by 21 ± 4.7%) \((P < 0.01\) contrast, \(n = 4\) experiments). Thus, the actions of CamKIV and forskolin are distinguishable on the −100/+23 but not the −100/+23 CYP11A/luc fragment.

Discussion

The activated Ca\(^{2+}\)/calmodulin complex is a potent stimulus of CamKs, phosphatases, and isotypes of adenylyl cyclase (26). Among Ca\(^{2+}\)/calmodulin-dependent protein kinases, CamKII is expressed predominantly in neural tissues, whereas CamKIV is represented in brain, thymus, T lymphocytes, testis, and ovary (27, 28). The nuclear localization of CamKIV peptide facilitates its phosphorylation of relevant transcription factors, such as CREB, CCAAT/enhancer-binding protein, and serum response factor (29, 30). Although little is known about the role of specific CamK effector pathways in gonadal cells, transgenic silencing of the CamKIV gene impairs granulosa-cell luteinization and ovulation in mice (30). The present experiments extend the latter insight by demonstrating expression of CamKIV and CamKII gene transcripts and immunoreactive proteins in (porcine) granulosa and theca cells and corpora lutea and documenting that exogenous CamKIV but not CamKII stimulates \textit{in vitro} transcriptional activity of CYP11A in primary cultures of GL cells.

Primary cultures of GL cells were used to assess putative mechanisms of CamKIV-specific enhancement of transcriptional activity of CYP11A. Transient overexpression of a constitutively active CamKIV minigene stimulated transcriptional activity of a −2300/+23 bp 5′-upstream fragment of swine CYP11A by 3.5-fold. In contrast, analogous expression vectors driving active CamKII or inactive CamIV were ineffective. More than 2.5-fold transcriptional responsiveness to CamKIV was retained by putative proximal promoter regions −100/+23 (2.9-fold) and −50/+23 CYP11A/luc (2.6-fold), albeit at reduced basal expression levels. The −100/+23 bp CYP11A fragment contains an apparent hemi-CRE sequence (−57/−45), which in principle could be responsive to endogenous CREB. Accordingly, we tested the possible relevance of CREB by cotransfection with a dominant-negative mutant CREB minigene (KCREB). KCREB expression repressed tran-
scriptional drive by CamKIV in a CREB-concentration-dependent fashion. In the case of $-100/+23$ CYP11A/luc, maximal inhibition by KCREB was approximately 50% of the CamKIV-stimulated effect. Incomplete repression by KCREB suggests that CamKIV may augment $-100/+23$ CYP11A/luc transcriptional activity in part via non-CREB-dependent mechanisms. KCREB analogously inhibited forskolin's stimulation of $-100/+23$ and $-50/+23$ CYP11A/luc activity by about 50%. In contrast, KCREB failed to reduce the 2.3-fold stimulatory effect of CamKIV on $-50/+23$ CYP11A/luc, wherein the putative hepatic CRE region is truncated. The last observation points to distinguishable mechanisms of CamKIV and forskolin action on the most proximal region of the putative porcine CYP11A promoter studied here. The nature of such inferred mechanisms is not known.

The individual stimulatory effects of CamKIV and forskolin on transcriptional activity of deletional (−2320 to −50/+23 bp) fragments of CYP11A/luc were concordant. However, responses to combined agonists were not additive. Thus, collective data are consistent with partially convergent actions of cAMP/PKA and CamKIV. For example, intracellular Ca$^{2+}$ availability in granulosa cells is required for maximal forskolin-stimulated accumulation of CYP11A mRNA (12). In addition, the present work shows that, except for $-50/+23$ CYP11A/luc, KCREB antagonizes transcriptional stimulation of CYP11A by CamKIV and cAMP/PKA comparably. Because putative inhibition of CREB blocks up-regulation by CamKIV and forskolin by approximately 50%, our findings allow for roles of other (non-CREB) Ca$^{2+}$-modulated transcriptional factors in stimulating CYP11A expression in ovarian cells.

In summary, the present studies document the expression of transcriptional and translational products of the CamKII and CamKIV gene in porcine corpora lutea and granulosa and theca cells. Transient overexpression of a constitutively active CamKIV but not CamKII minigene in primary cultures of GL cells induces transcriptional activity of −2320/+23 and −100/+23 bp 5′-upstream fragments of homologous CYP11A by 3.5-fold. Transcriptional stimulation by CamKIV is nonadditive with that of forskolin and repressed significantly but incompletely by an exogenous CREB antagonist. Based on available data, we postulate that endogenous CamKIV contributes to Ca$^{2+}$- and forskolin-driven expression of CYP11A gene expression in GL cells.

Acknowledgments

The authors thank Drs. Anthony Means (Duke University, Durham, NC) for donating the CamK plasmids, Richard Day (University of Virginia, Charlottesville) for providing the luc vector, and Richard Goodman (Oregon Health Sciences University, Portland, OR) for supplying KCREB.

Received November 11, 2003. Accepted July 28, 2004.

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This work was supported in part by National Institutes of Health (NIH) Training Grant T32 HD07382 and NIH R01 HD16393-19 (to J.D.V.) and NIH Specialized Cooperative Center for Studies in Women's Health (to the University of Virginia, Charlottesville, VA).

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Endocrinology, December 2004, 145(12):5616–5622 5621

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*Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.