Protein kinase C activity mediates LH-induced ErbB/Erk signaling in differentiated hen granulosa cells

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Abstract

While there is accumulating evidence that mitogen-activated protein kinase/Erk and protein kinase C (PKC) signaling inhibits premature differentiation of granulosa cells in hen prehierarchical follicles, it has only recently been established that these signaling pathways play an important facilitory role in promoting steroidogenesis in differentiated granulosa cells from preovulatory follicles. The present studies were conducted with differentiated granulosa cells to establish the ability of LH to initiate PKC activity, and the subsequent requirement for PKC activity in promoting the ErbB/Erk signaling cascade that ultimately facilitates LH-induced progesterone production. Incubation of differentiated granulosa cells with LH increases PKC activity within 15 min, and latently promotes Erk phosphorylation (P-Erk) by 180 min. Inhibition of PKC activity with GF109203X attenuates LH- and 8-bromo-cAMP (8-br-cAMP)-induced P-Erk, but not P-Erk promoted by an epidermal growth factor (EGF) family ligand (e.g., transforming growth factor α). Importantly, inhibition of PKC activity also blocks the LH-induced increase in the autocrine expression of mRNA encoding the EGF family ligands, such as EGF, amphiregulin, and betacellulin. Furthermore, inhibition of EGF ligand shedding at the level of the cell membrane using the matrix metalloprotease activity inhibitor, GM6001, prevents both LH- and 8-br-cAMP-induced P-Erk and progesterone production. These findings provide evidence for a facilitory role of PKC and ErbB/Erk signaling in LH-induced progesterone production, place the requirement for PKC activation upstream of ErbB/Erk activity, and demonstrate for the first time in a non-mammalian vertebrate the requirement for PKC activity in LH-induced expression of EGF family ligands in granulosa cells.

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Introduction

In both avian and mammalian species, granulosa cell differentiation and steroidogenesis during follicle maturation are mediated by the coordinately timed expression of endocrine, paracrine, and autocrine factors and the interaction of consequent signaling pathways. For instance, recent studies indicate that members of the epidermal growth factor (EGF) family of ligands play a critical role in the mammalian preovulatory follicle by mediating luteinizing hormone (LH)-induced cumulus expansion and oocyte maturation (Das *et al.* 1992, Park *et al.* 2004, Jammongjit *et al.* 2005, Shimada *et al.* 2006). Both the avian and mammalian EGF family of ligands currently consist of no <11 related proteins, including EGF, transforming growth factor- α (TGF α , amphiregulin (AR), heparin-binding EGF (HB-EGF), betacellulin (BTC), epiregulin (EPR), epigen (EPG), and neuregulins (NRG1, -2, -3, -4). All mature, biologically active forms of these polypeptides are proteolytically shed from a membrane-anchored precursor molecule by one or more matrix metalloproteinases (MMPs; Strachan *et al.* 2001,

Sweeney *et al.* 2001). The processed, soluble peptides each contain one conserved three-disulfide loop motif (the EGF-like domain; CX₆₋₇CX₄₋₅CX₁₀₋₁₃CX₁CX₈₋₁₂C), yet otherwise share relatively low homology with one another. This conserved EGF motif is considered important for tertiary structure stabilization and receptor binding (Wouters *et al.* 2005).

Data derived from mammalian species demonstrate that the EGF receptor (ErbB1/EGF-R/HER-1) is a ubiquitously expressed transmembrane receptor which contains tyrosine kinase and autophosphorylation domains (Hackel *et al.* 1999, Moghal & Sternberg 1999). Ligand binding to ErbB1 begins with the stabilization of a receptor dimer, which is capable of initiating intracellular signaling, including activation of the Erk signaling cascade. Just as the EGF family of ligands comprises structurally and functionally similar members, so do the receptors through which the mature peptides signal. In addition to ErbB1, the ErbB family includes ErbB2 (HER-2, Neu), ErbB3 (HER-3), and ErbB4 (HER-4) (Helden 1995). It is now recognized that each

ErbB receptor type can bind several different EGF family ligands and, conversely, a single EGF family ligand may interact with several ErbB receptor types.

Studies in mammals have demonstrated that EGF family members play an important role in preovulatory follicle function as downstream effectors of LH (Park *et al.* 2004, Jammongjit *et al.* 2005, Bolamba *et al.* 2006). LH-mediated G protein-coupled receptor (GPCR) signaling leads to the up-regulation of specific EGF ligands, which then activate ErbB receptors and subsequent mitogen activated protein kinase/Erk signaling, both of which are critical to oocyte nuclear maturation and cumulus expansion (Su *et al.* 2002, Park *et al.* 2004). The ErbB-mediated Erk signaling pathway works in concert with LH-induced cAMP to effectively mediate the actions of LH on follicular function. These actions include progesterone synthesis, as demonstrated by the ability of the ErbB selective inhibitor, AG1478, to inhibit progesterone production in cultured mouse preovulatory follicles (Jammongjit *et al.* 2005). The up-regulation of EGF ligands was initially proposed to selectively occur in mural granulosa cells, from which the ligands would be shed by one or more MMPs to enable paracrine signaling to the cumulus layer (Conti *et al.* 2006). However, more recent evidence suggests that induction of multiple EGF ligands occurs in both mural and cumulus cells, providing an additional autocrine mechanism for the up-regulation of EGF ligands and enhanced intracellular signaling (Shimada *et al.* 2006). In either event, the significance of EGF family ligand-initiated ErbB signaling in the periovulatory follicle is highlighted by its critical importance in ovulation. Experiments demonstrate that injection of AG1478 into the rat ovarian bursa blocks ovulation, with the mature, oocyte-containing follicles remaining intact (Ashkenazi *et al.* 2005). Taken together, these data strongly suggest that in mammals, the effects of LH on both follicular maturation and ovulation are dependent upon the ErbB-mediated Erk signaling cascade.

While a role for ErbB activation following LH stimulation has been documented in mammalian ovarian models, there is, as yet, no evidence for the conservation of this signaling cascade in non-mammalian vertebrate species. Moreover, the precise signaling sequence by which LH induces such activity has only recently been evaluated (Shimada *et al.* 2006). In addition to the well-documented role of cAMP in LH signaling, accumulating evidence from both mammalian and avian granulosa cells suggests that the actions of LH are, at least in part, dependent upon protein kinase C (PKC) activity. Studies incorporating the use of PKC inhibitors indicate that PKC is required for LH-induced progesterone synthesis in rat, hen, and quail preovulatory follicles (Jamaluddin *et al.* 1994, Morris & Richards 1995, Sasanami & Mori 1999). Moreover, in the rat, phorbol ester activation of the PKC pathway promotes luteinization in the presence of subovulatory doses of

LH, indicating a supportive role for PKC in final differentiation. Further molecular ordering places the activation of PKC prior to the tyrosine kinase activity induced by LH, although the cellular mechanisms by which this occurs were not determined (Morris & Richards 1993, 1995). While there is evidence to support a direct role for PKC in mediating the effects of LH in granulosa cells, its specific role in the activation of Erk signaling cascade has apparently not been addressed. Accordingly, the studies herein using differentiated hen granulosa cells establish an active EGF family signaling network in response to LH stimulation and directly address the role of PKC in the regulation of ErbB activation by gonadotropins.

Materials and Methods

Animals and reagents

All studies described herein were performed with single-comb white leghorn hens 25–35 weeks of age (Creighton Bros, Warsaw, IN, USA), laying a sequence of six eggs or more. Hens were housed in individual laying batteries, with free access to feed (Purina Layena Mash; Purina Mills, St Louis, MO, USA) and water, under a controlled photoperiod of 15 h light:9 h darkness, with lights on at midnight. Hens were euthanized by cervical dislocation 16–18 h prior to a mid-sequence ovulation, and the ovary was immediately removed and placed in an ice-cold sterile 1% saline solution for dissection. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

Highly purified ovine LH was provided by the National Hormone and Pituitary Program (Torrance, CA, USA) and used at maximally effective doses (Tilly *et al.* 1991), while recombinant human TGF α (rhTGF α) was obtained from PeproTech (Rocky Hill, NJ, USA). The cell-permeable cyclic AMP analog, 8-bromo-cAMP (8-br-cAMP), was purchased from Sigma-Aldrich. The ErbB tyrosine kinase inhibitor, AG1478 (Egelblad *et al.* 2001), was purchased from Calbiochem (San Diego, CA, USA), while the broad-spectrum MMP inhibitor, GM6001, and the PKC inhibitor, GF109203X (inhibits both conventional and novel isoforms), were purchased from BioMol (Plymouth Meeting, NJ, USA). Doses for each pharmacologic inhibitor utilized have been previously described (Johnson & Bridgham 2001) or were empirically determined (e.g., for GM6001 and AG 1478) in preliminary experiments.

Granulosa cell cultures

The second (F2) and third (F3) largest stages (representing stages several days following selection into the

preovulatory hierarchy) were removed from the ovary and placed into a sterile ice-cold saline solution. Granulosa layers from preovulatory follicles were collected and cells were dispersed for culture as described previously (Tilly *et al.* 1991). The cells (5×10^5) were incubated for up to 5 h at 40 °C in 12×75 mm polypropylene tubes (Fisher Scientific, Pittsburg, PA, USA) in 2 ml Dulbecco's modified Eagle medium, which contained 2.5% FBS, 0.1 mM non-essential amino acids, and 1% antibiotic-antimycotic mixture (Invitrogen). Where appropriate, AG1478 (10 µM), GM6001 (20 µM), and GF109203X (30 µM) were preincubated with cells for 1 h prior to the addition of LH, 8-br-cAMP, or TGFα. Experiments using the MMP inhibitor, GM6001, were conducted in the absence of FBS to preclude signaling by growth factors present within the FBS.

Immunoblot analysis of phosphorylated and total Erk Proteins

Analysis of phosphorylated Erk (P-Erk; Upstate, Lake Placid, NY, USA; mouse MAB) and total Erk2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit polyclonal antibody) was conducted as described previously (Woods & Johnson 2006). Briefly, 30–60 µg protein were loaded on SDS polyacrylamide gels for electrophoresis, followed by transfer to nitrocellulose membrane. The membrane was then blocked for 1 h in 5% milk in Tris-buffered saline-0.1% Tween 20 and then incubated in primary antibody overnight at 4 °C. The membranes were washed for 5 min in Tris-buffered saline-0.1% Tween 20 and then incubated with secondary antibody for 2 h at room temperature. All primary and secondary antibodies were diluted in 5% milk in Tris-buffered saline-0.1% Tween 20. The horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were from Pierce Endogen (Rockford, IL, USA) and each used at a dilution of 1:10 000. The membranes were then incubated with ECL western blotting detection reagent (Pierce) for 1 min, and then wrapped and exposed to X-ray film for 3–10 min.

PKC activity assay

The PKC activity was assayed using the PKC activity assay kit (Upstate) according to the instructions provided

by the manufacturer. Following incubation with LH or TGFα for 15 or 60 min, granulosa cells were collected and lysed in ice-cold lysis buffer (150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM pervanadate, 1 mM EDTA, 1% Igepal, 0.25% deoxycholic acid, 1 mM NaF, and 50 mM Tris-HCl, pH 7.4). The kinase reaction mixture included 5 mM 3-morpholinopropanesulfonic acid (MOPS), 5 mM β-glyceraldehyde phosphate, 0.2 mM sodium orthovanadate, 0.2 mM dithiothreitol, 100 µM PKC substrate, 100 µg/ml 1,2-diacylglycerol protein kinase A/calmodulin kinase inhibitor mix, 15 mM MgCl₂, 100 µM ATP, and 5 µCi (3000 Ci/mmol) [γ -³²P]ATP (Perkin Elmer, Wellesley, MA, USA). The kinase reactions were prepared on ice, incubated for 10 min at 30 °C, and then spotted onto phosphocellulose paper. The paper was washed three times for 5 min each in 0.75% phosphoric acid, and finally once for 2 min in acetone. Incorporation of [γ -³²P] ATP was measured using a scintillation counter (Beckman Coulter, Fullerton, CA, USA).

Two-step real-time PCR analysis of EGF family ligand expression

Forward and reverse primers for EGF, AR, BTC, and 18s rRNA were generated using MacVector software (Table 1), and were subsequently validated for use with real-time PCR by determining the optimal amplification efficiency and primer concentrations as described by the system manufacturer (Applied Biosystems, Foster City, CA, USA).

Random-primed, reverse-transcribed cDNA synthesis reactions were performed using the Promega RT System (Promega), according to the conditions described by the manufacturer. For real-time PCR, primers were added to 25 µl total reaction volume using reagents provided in the ABgene Absolute QPCR Sybr Green Mix (ABgene, Rochester, NY, USA). Final concentrations of the sense and antisense primers were determined for each primer pair based on optimal amplification efficiency. Reactions were completed on the ABI 7700 Thermocycler (Applied Biosystems). Conditions were set to the following parameters: 2 min at 94 °C followed by 40 cycles each for 15 s at 95 °C, 1 min at 60 °C, 1 min at 72 °C. The Ct

Table 1 Primer pairs designed for real-time PCR experiments in differentiated granulosa cells.

Gene	Size (bp)	Target sequence	Accession number
Amphiregulin	377	F: 5'-CAGGACTATTTTGGTGAACGCTG-3' R: 5'-GCAGTAGCCTCTGTCTTGATGAATC-3'	AY725836
Betacellulin	307	F: 5'-CGCATCCCATCCCGCTCC-3' R: 5'-CGCTGCTTCCGACAGTGGTG-3'	AY681125
Epidermal growth factor	361	F: 5'-GGTCTACTTCTGCTCCTGCTTG-3' R: 5'-TGGTTGCTTCTGTCTGTTGGC-3'	NM_001001292
18s Rrna	87	F: 5'-TTAAGTCCTGCCCTTTGTACAC-3' R: 5'-CGATCCGAGGAACCTCACTAAAC-3'	AF173612

Amplification of 18s ribosomal (r) RNA was utilized for standardization.

(defined as the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (run in triplicate) using the Sequence Detection Software (v.1.6.3), while quantification was accomplished using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001). Briefly, the target C_t was determined for each sample and then normalized to the 18s rRNA C_t from the same sample (18s rRNA C_t subtracted from the target C_t yields the ΔC_t). These values were then compared with control levels using the $2^{-\Delta\Delta C_t}$ method and expressed as fold difference compared with an appropriate control sample.

Progesterone RIA

Progesterone in media samples was quantified by RIA as described previously (Tilly & Johnson 1988). Data were expressed as a mean fold difference compared with an appropriate control for the combined replicate experiments.

Data analysis

Experiments were independently replicated for a minimum of three times unless otherwise specified. Standardized values for the combined replicate experiments were expressed as a fold difference (mean $\pm$ S.E.M.) versus cultured control cells. Data were analyzed by one-way ANOVA without including data from the control group (arbitrarily set to 1.0) and Fisher's protected least significant difference multiple range test for *post hoc* analysis. In instances where a Student's *t*-test was used for analysis, individual comparisons were made using non-transformed data.

Results

LH-induced PKC and P-Erk in differentiated granulosa cells

Treatment of differentiated granulosa cells from preovulatory follicles with LH (100 ng/ml) demonstrated a modest but significant increase in the PKC activity following a 15-min incubation, and the activity increased further after 60 min. By comparison, TGF α (25 ng/ml) increased PKC activity following a 15-min challenge, but the activity returned to control levels by 60 min (Fig. 1). In contrast to the rapid increase in the PKC activity following LH treatment, levels of P-Erk did not significantly increase until 180 min, with levels remaining elevated at 240 min (Fig. 2A). Preincubation with the ErbB tyrosine kinase inhibitor, AG1478 (10 μ M), for 1 h prior to a 240-min challenge with LH or 8-br-cAMP (1 mM) completely blocked agonist-induced levels of P-Erk (Fig. 2B). This latter finding establishes the requirement of active ErbB signaling for LH-induced Erk phosphorylation.

Figure 1 PKC activity in differentiated hen granulosa cells from preovulatory follicles incubated for 15 or 60 min with LH (100 ng/ml); A: or TGF α (25 ng/ml); B: Data are expressed as fold difference versus control (Con) cells cultured for 60 min. * $P < 0.05$, † $P < 0.01$ versus Con by *t*-test; $n = 3$ (A) or 4 (B) replicate experiments.

LH-induced P-Erk is dependent upon PKC activation

To determine whether active PKC signaling is required for P-Erk induced by LH, differentiated granulosa cells were preincubated for 1 h in the presence of GF109203X (30 μ M) prior to a 180-min challenge with LH. GF109203X significantly attenuated LH- and 8-br-cAMP-induced levels of P-Erk (Fig. 3A). To rule out the possibility of a requirement for PKC signaling in EGF family ligand-induced P-Erk, cultures of differentiated granulosa were pretreated for 1 h in the presence or absence of GF109203X, then challenged for 20 min with TGF α . Levels of P-Erk were induced equally in cells cultured with and without the selective PKC inhibitor (Fig. 3B). Not unexpectedly, the ErbB inhibitor, AG1478, completely prevented TGF α -induced P-Erk (Fig. 3B). Taken together, these data indicate that the requirement for PKC in gonadotropin-induced Erk activation lies upstream of ErbB receptor activation.

LH-induces expression of EGF family ligands through a PKC-dependent mechanism

To investigate whether gonadotropin-induced Erk activation is mediated through a PKC-dependent up-regulation of one or more EGF family ligands, differentiated granulosa cells were preincubated with or without GF109203X for 1 h prior to an additional 180-min treatment with or without LH. Treatment

Figure 2 A) Western blot analysis of phosphorylated Erk (P-Erk) and total Erk2 in differentiated granulosa cells. Dispersed cells were treated for 30, 60, 180, or 240 min with LH (100 ng/ml). Con, cultured (240 min) control cells. ^{A,B} $P < 0.05$, by ANOVA. B) Cells were pretreated with AG1478 (10 μM) or without (Con) for 1 h prior to a 240-min incubation with or without LH (100 ng/ml) or 8-br-cAMP (8br; 1 mM). a, b, c $P < 0.01$. Note that elevated levels of P-Erk occur without a change in levels of Erk2 protein.

with LH induced a robust increase in levels of EGF, AR, and BTC mRNA, whereas GF109203X blocked this response (Fig. 4).

LH-induced P-Erk is dependent upon MMP activity

To evaluate the requirement for ligand shedding following the up-regulation of EGF family ligands, MMP activity was prevented using the inhibitor GM6001. While differentiated granulosa cells treated with LH or 8-br-cAMP for 180 min revealed significantly elevated levels of P-Erk compared with the untreated control, preincubation with the MMP activity inhibitor, GM6001 (20 μM), for 1 h reduced LH- and 8-br-cAMP-induced P-Erk to control levels (Fig. 5A). Furthermore, preincubation of granulosa cells with GM6001 significantly attenuated progesterone production following a 3-h challenge with LH or 8-br-cAMP (Fig. 5B). Combined with the requirement for active ErbB signaling (Fig. 2B), these data collectively indicate

Figure 3 A) Western blot analyses of P-Erk and total Erk2 in differentiated granulosa cells pretreated with or without (Con) the selective PKC inhibitor, GF109203X (GF; 30 μM) for 1 h prior to a 240-min incubation with or without LH or 8-br-cAMP (8br). ^{A,B} $P < 0.05$. B) Cells were pretreated with or without (Con) GF109203X or AG1478 (AG; 10 μM) for 1 h prior to an additional 15-min incubation with or without TGFα (25 ng/ml). ^{A,B} $P < 0.01$; $n = 3$ (B) or 4 (A) replicate experiments.

that the bioavailability of one or more soluble EGF ligands is a requisite for LH-induced activation of Erk signaling and the facilitation of progesterone production in differentiated hen granulosa cells.

Discussion

The novel findings presented herein provide evidence that in differentiated hen granulosa cells collected from preovulatory follicles, LH-induced ErbB tyrosine kinase activity is induced by *de novo* synthesis and the subsequent membrane shedding of one or more EGF family ligands, and that the upstream actions of LH are mediated through PKC signaling (Fig. 6). Among the proposed roles for LH-induced Erk signaling at this stage of granulosa cell development are the facilitation of progesterone production and the final maturation of the follicle in preparation for ovulation. By direct contrast, evidence has previously been provided that constitutively active ErbB/Erk signaling serves to tonically inhibit premature granulosa cell differentiation within prehierarchal follicles (e.g., prior to follicle selection into the

Figure 4 Quantitative PCR for A: amphiregulin, B: betacellulin, and C: epidermal growth factor (EGF) in differentiated granulosa cells. Cells were incubated with or without (Con) GF109203X (GF; 30 μ M) for 1 h prior to a 180-min incubation with or without LH. Data are standardized to 18s rRNA and expressed as fold difference versus Con. A, B, C, D, E, F $P < 0.02$.

preovulatory hierarchy; Johnson & Bridgham 2001, Johnson *et al.* 2004, Woods & Johnson 2005, 2006, Woods *et al.* 2005). This plasticity in both the regulation and the function of Erk signaling demonstrated between these two distinct stages of follicle development highlight the dynamic changes in intracellular signaling that occur in the granulosa cell layer during follicle maturation.

There is accruing evidence from various mammalian ovarian models that the actions of LH are, in part, dependent upon the ErbB/Erk signaling cascade. It has been shown in the mouse ovary that the effects of LH on ErbB activation are latent, as maximal phosphorylation of ErbB1 is observed 4 h following hCG injection (Park *et al.* 2004). Similarly, in hen granulosa cells, levels of P-Erk are increased by LH following 180 min of treatment *in vitro* (Fig. 2A). While differentiated hen

Figure 5 A) Western blot analysis of P-Erk and total Erk2 in differentiated granulosa cells pretreated with or without (Con) the MMP inhibitor, GM6001 (20 μ M), for 1 h prior to a 240-min incubation with or without LH (100 ng/ml) or 8-br-cAMP (8br; 1 mM). A, B $P < 0.001$; $n = 4$. B) Progesterone production in differentiated granulosa cells following preincubation with GM6001 (20 μ M) or without (Con) for 1 h prior to a 240-min treatment with or without LH or 8br. a, b $P < 0.05$; $n = 7$.

granulosa cells are considered LH-R dominant, they also express limited levels of follicle-stimulating hormone (FSH)-R capable of inducing cAMP accumulation (Tilly *et al.* 1991), and a maximally effective dose of recombinant human FSH (100 ng/ml) induces Erk activation with a time course similar to LH (Woods, unpublished observations). The latent induction of P-Erk is also consistent with the previous finding in mouse cumulus-oocyte complexes collected from preovulatory follicles that FSH-induced P-Erk is sustained for at least 4 h. Such results support the hypothesis that up-regulation of EGF ligands occurs prior to ErbB receptor tyrosine kinase activation (Park *et al.* 2004). Furthermore, the activation of Erk following treatment with 8-br-cAMP (Figs 3A and 5A) places gonadotropin-induced P-Erk downstream of cAMP accumulation. While the exact mechanisms by which Erk activation modulates steroidogenesis have not been determined in granulosa cells, it has recently been shown in Leydig cells that activation of Erk is critical for maintaining mitochondrial membrane potential (Renlund *et al.* 2006). Thus, it is possible that the requirement for Erk signaling in steroidogenesis occurs independently from the regulation of steroidogenic enzymes and StAR protein expression and phosphorylation. The proposed

Figure 6 Proposed model depicting the molecular order of intracellular signaling events leading to LH-induced P-Erk and the facilitation of progesterone production in differentiated granulosa cells from hen preovulatory follicles. LH receptor (LH-R) signaling promotes the rapid activation of PKC activity, which in turn induces expression of multiple EGF family ligands (EGFL, including amphiregulin, betacellulin, and epidermal growth factor). Subsequent EGFL-induced ErbB receptor activation is dependent upon the proteolytic shedding of mature EGFL. While the potential action(s) of ErbB-induced PKC activity has not been studied, the activation of P-Erk signaling is proposed to facilitate progesterone production at the level of the mitochondria (D Woods, unpublished observations). MMPs, matrix metalloproteinases.

action for Erk at the level of the mitochondrial membrane is depicted in Fig. 6 (dashed line).

It has been reported that ErbB-associated receptor tyrosine kinase activation is required for LH-induced oocyte maturation and progesterone production in the mouse (Su *et al.* 2002, Park *et al.* 2004, Jammongjit *et al.* 2005) and ovulation in the rat (Ashkenazi *et al.* 2005). These effects are attributed to the stimulatory actions of LH, acting at least in part through cAMP, on the up-regulation of expression and subsequent protease-induced ectodomain shedding of one or more EGF family ligands (Conti *et al.* 2006). In light of data presented herein that the ErbB tyrosine kinase inhibitor, AG1478 (Fig. 2), and the MMP inhibitor GM6001 (Fig. 5), each prevent both LH- and 8br-cAMP-induced accumulation of P-Erk, it is apparent that the signaling cascade leading to Erk activation is conserved in the hen. Collectively, these data confirm that LH-induced Erk signaling is dependent upon ErbB receptor activation, and that these effects also lie downstream of cAMP accumulation.

Studies from mouse embryonic cells lacking candidate sheddases have demonstrated a role for MMPs in the shedding of EGF ligands. In particular, MMPs from the ADAM (a disintegrin and metalloprotease) family of

proteases are responsible for the release of multiple EGF family ligands from the membrane surface. ADAM10 is considered the major sheddase for both EGF and BTC, while EPR, TGF α , AR, and HB-EGF are proteolytically processed by ADAM17 (Sahin *et al.* 2004). While there is extensive evidence implicating MMP activity in the degradation of extracellular membrane components and tissue remodeling within ovarian follicles (Jo & Curry 2004, Curry & Smith 2006), there is additional evidence to substantiate their importance in EGF family ligand signaling in the ovary as well. Studies from mouse preovulatory follicles have implicated MMPs in ovarian steroidogenesis (Jammongjit *et al.* 2005), and in the present studies with hen granulosa cells, inhibition of sheddase activity significantly reduced LH- and cAMP-induced progesterone synthesis (Fig. 5B). The inhibition of LH- and cAMP-induced progesterone synthesis is associated with the absence of P-Erk detected under the same conditions (Fig. 5A). While the events by which Erk signaling facilitates progesterone synthesis in granulosa cells from hen preovulatory follicles are currently under investigation, this evidence is consistent with the current hypothesis that active Erk signaling following treatment with LH is critical for steroid biosynthesis. Moreover, these data suggest that MMP activity assumes a physiological role as an upstream component of the steroidogenic pathway in differentiated granulosa cells. Although mechanisms involved in the regulation of ADAM family members with regard to LH signaling remain to be determined, there is evidence that MMP activity can be mediated both through GPCR signaling and the Erk signaling cascade (Gechtman *et al.* 1999, Prenzel *et al.* 1999). Moreover, PKC signaling may play a role in regulating ADAM activity. It has been shown in human glioma cells that the induction of ADAM17 by phorbol ester is dependent upon PKC, as GF109203X inhibits the stimulatory actions of phorbol myristate acetate (PMA; Nagano *et al.* 2004). Additionally, there is evidence that PMA treatment can induce the expression of one or more ADAM family members (Gechtman *et al.* 1999). Further experiments in the ovary are warranted to determine what, if any, role PKC signaling may have in the regulation of sheddase activity following LH stimulation.

Evidence from both mammalian and avian models suggests that the effects of LH are partially mediated by active PKC signaling (Morris & Richards 1993, 1995, Jamaluddin *et al.* 1994, Sasanami & Mori 1999). Results from the present studies indicate that PKC signaling is required for LH-induced Erk activation (Fig. 3A), as inhibition of PKC using the non-selective PKC isoform antagonist, GF109203X, inhibits the effects of both LH and cAMP on the activation of Erk. Further evidence suggests that the requirement for PKC in LH-induced Erk signaling lies upstream of EGF ligand-mediated activation of ErbB receptor signaling, since GF109203X blocks LH-induced P-Erk, but does not

block EGF ligand-induced P-Erk (Fig. 3). Moreover, the PKC activity is rapidly induced (within 15 min) by LH, and such activity is sustained after 60 min (Fig. 1A). This is in contrast to the activation of PKC by TGF α which rapidly activates, but does not sustain, PKC activity (Fig. 5B). The early enhancement of PKC activity by LH, combined with the requirement for active PKC signaling in LH-induced P-Erk, indicates that activation of PKC signaling precedes LH-mediated Erk signaling. While the present studies assess the molecular ordering of PKC signaling in LH-induced Erk activation, they clearly do not selectively evaluate the role of specific PKC isoforms. Thus, future studies are warranted to determine a role for LH-induced activation of specific PKC isoforms in the Erk signaling pathway.

In light of evidence that active PKC signaling is required for LH-induced P-Erk upstream of EGF ligand-mediated ErbB receptor signaling, we postulated that PKC activity may be required for LH-induced up-regulation of one or more EGF ligands. Accordingly, we mined the BBSRC EST database (<http://www.chick.umist.ac.uk/>) for EGF family ligands expressed within the ovary (Boardman *et al.* 2002). cDNAs encoding three ligands (AR, BTC, and EGF) were subsequently amplified specifically from granulosa cells and verified by nucleic acid sequencing. Our studies also documented the expression of EPR, EPG, and TGF α mRNA within hen ovarian theca and stromal tissues, but not within granulosa cells at any stage of follicle development investigated (Woods *et al.* 2005; D Woods, unpublished observations). Previous reports in mammals have demonstrated rapid (within 4 h) agonist-induced transcription of AR, EPR, and BTC both *in vivo* and *in vitro* (Park *et al.* 2004, Shimada *et al.* 2006). In hen, AR, BTC, and EGF mRNA are rapidly up-regulated following a 180-min challenge with LH. This induction was blocked by the inhibition of PKC with GF109203X, indicating that PKC activity is required for LH-induced EGF ligand mRNA expression (Fig. 4). Collectively, these data place LH-induced PKC activity downstream of cAMP but upstream of EGF ligand transcription in the LH-induced Erk signaling cascade.

In summary, the data presented represent the first of its kind in a non-mammalian species to demonstrate the active role of LH-induced Erk signaling through an EGF family ligand network. Moreover, the molecular ordering of this action supports a role for PKC in the up-regulation of such ligands (Fig. 6). While active Erk signaling in differentiated granulosa cells from preovulatory follicles is proposed to facilitate LH-induced progesterone synthesis, a physiological role for ErbB-promoted PKC activity is yet to be established in hen granulosa cells.

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