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Novel signaling mechanisms in the ovary during oocyte maturation and ovulation

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Introduction

In sexually mature females, ovulation is the process whereby an egg ready to be fertilized is released from the ovary. Fully grown antral follicles are composed of mural granulosa and cumulus cells and contain a germ cell, the oocyte, which has developed the competence to mature into a fertilizable egg (Fig. 1). Ovulation is triggered by a surge of the gonadotropin luteinizing hormone (LH) secreted by the pituitary. During the periovulatory period, the oocyte completes maturation and major phenotypic changes take place in the mural granulosa and cumulus cells, culminating with rupture of the follicle and release of the matured egg. Malfunction of any step of this complex process causes impairment of fertility. Thus, a better understanding of the cellular and molecular events associated with ovulation is of paramount importance to improve assisted reproduction, as well as to develop new strategies for fertility control.

With the present review, we will summarize the current knowledge of the molecular and signaling events associated with ovulation in mammals, focusing primarily on the signaling in the follicle that controls oocyte maturation. Oocytes enter a specialized cell cycle (meiosis) during fetal life and are arrested in prophase I until reproductive maturity, after which oocytes from selected gonadotropin-responsive follicles reenter the cell cycle and complete meiosis upon fertilization. Thus, an oocyte may remain arrested in meiotic prophase for periods up to 40 years or more in women. This blockade in the meiotic cell cycle is under the control of the somatic cells of the follicle, because oocytes or cumulus-oocyte complexes that are removed from the mature follicle resume meiosis spontaneously (Pincus and Enzmann 1935; Edwards 1965). Moreover, only somatic cells of the follicle express the molecular machinery necessary to respond to LH. Therefore, somatic-germ cell communications are critical for maintaining the meiotic arrest and to induce meiotic maturation. The primary focus of this review will be the mechanisms and signals mediating these local regulations in the follicle.

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Oocyte meiotic arrest and its control

A well established concept is that meiotic arrest of the oocyte is dependent on high concentrations of the second messenger cyclic AMP (cAMP) [(Cho et al. 1974; Dekel and Beers 1978), reviewed in(Conti et al. 2002)] Given the dependence of oocyte meiotic arrest on the interaction of the oocyte with granulosa cells, a widely held view was that cAMP was provided to the oocyte by somatic cells through gap junctions (Dekel 1988). Indeed, inhibitors of gap junction permeability induced oocyte maturation (Sela-Abramovich et al. 2006), suggesting that this connection is necessary to transfer an inhibitory factor to the oocyte, cAMP being the most plausible candidate. Several studies have provided evidence that cAMP generated in somatic cells is transferred to the oocyte (Vivarelli et al. 1983; Bornslaeger and Schultz 1985; Salustri et al. 1985). A caveat of these studies is that PDE inhibitors had to be used to demonstrate this transfer. More recently and using a cAMP sensor expressed exclusively in the oocyte, Webb et al have shown that treatment of cumulus oocyte complexes with FSH which stimulates cAMP accumulation in cumulus cell causes an increase in cAMP also in oocytes; more importantly, under conditions where gap junctions are closed the oocyte transfer is abolished (Webb et al. 2002). Surprisingly, cAMP levels remain constant when gap junctions are closed, suggesting cAMP production by the oocyte. Indeed, recent data indicate that the entire molecular machinery required to produce cAMP is expressed in mammalian oocytes and that the activity of these endogenous components is sufficient to maintain cAMP at levels that prevent maturation (see below).

G-protein coupled receptors and the transduction machinery are functional in oocytes

Analysis of the oocyte transcriptome indicates that several GPCRs are expressed by rodent oocytes (Mehlmann et al. 2004; Hinckley et al. 2005). However, the function of most of these receptors remains unknown except for a cluster of receptors that includes GPR3 and GPR12. These GPCRs were initially identified as receptors with considerable constitutive activity, but that could be further activated by S1P (Kostenis 2004). As the true physiological ligand for these receptors may not be correctly identified, they should still be regarded as orphan receptors (Yin et al. 2009). Studies with genetic or oligonucleotide-mediated ablation of GPR3 in the mouse, and GPR12 in the rat, have provided evidence that these receptors are required to maintain high cAMP levels in the oocyte and meiotic arrest (Mehlmann et al. 2004; Hinckley et al. 2005; Ledent et al. 2005). GPR3 is also expressed on Xenopus and human oocytes, suggesting a conserved function across species (Deng et al. 2008; DiLuigi et al. 2008). Oocytes lacking these receptors display leaky meiotic arrest and premature reentry into the cell cycle as soon as the oocyte acquires the ability to mature (Mehlmann et al. 2004; Ledent et al. 2005). A phenotype reminiscent of premature ovarian failure was also reported for the GPR3 null mice by Ledent et al. (Ledent et al. 2005).

Downstream of these receptors, the role of a Gs protein has been demonstrated by neutralizing antibody injection (Mehlmann et al. 2002). In addition, associated adenylyl cyclases have been characterized biochemically and their function assessed genetically (Horner et al. 2003). These findings have provided a molecular explanation to earlier observations demonstrating cAMP synthesis by the oocyte (Olsiewski and Beers 1983;

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Urner et al. 1983). Thus, all the membrane machinery required for cAMP generation is expressed in oocytes and its function is required to maintain meiotic arrest.

It has long been known that inhibitors of phosphodiesterases (PDEs) were able to maintain meiotic arrest in oocytes removed from the follicle (Dekel and Beers 1978; Dekel and Beers 1980; Vivarelli et al. 1983; Bornslaeger et al. 1984). A major PDE expressed in mouse, rat, monkey and human oocytes is the product of the PDE3A gene (Richard et al. 2001; Shitsukawa et al. 2001; Jensen et al. 2002; Nogueira et al. 2006). Both pharmacological and genetic evidence indicate that the activity of this enzyme is critical for meiotic resumption (Tsafriri et al. 1996; Masciarelli et al. 2004). In the mouse and rat ovarian follicle, this enzyme is primarily expressed in the oocyte and not in granulosa cells. This property has allowed for the use of selective inhibitors to manipulate cAMP levels in the oocyte without affecting the somatic compartment (Tsafriri et al. 1996). Whether this compartmentalized expression is retained in other species is not clear, as PDE3 activity has been detected in cumulus cells of pig follicles (Sasseville et al. 2007).

Control of the meiotic block downstream of cAMP

High levels of cAMP in the oocyte suppress the activation of MPF via the action of cAMP-dependent protein kinase A (PKA) (Maller and Krebs 1977; Maller and Krebs 1980; Bornslaeger et al. 1986) (see Fig 1). The activity of MPF, a complex of Cdc2 and cyclin B, is negatively regulated by the phosphorylation of two highly conserved residues of Cdc2, Thr14 and Tyr15. These inhibitory phosphorylations are catalyzed by the Wee1 kinases, whereas dephosphorylation of these residues is dependent on the Cdc25 phosphatases (Lew and Kornbluth 1996). Recent studies show that PKA directly regulates the activity of both Cdc25 phosphatases and Wee1 kinases (Han et al. 2005; Zhang et al. 2008; Pirino et al. 2009; Oh et al. 2010). In GV-arrested oocytes, PKA-mediated phosphorylation of Cdc25B negatively regulates its function by sequestering it in the cytoplasm in a complex with 14-3-3 protein (Zhang et al. 2008; Pirino et al. 2009). Phosphorylation of Wee1B by PKA increases Wee1B activity and thereby inhibits the activity of MPF in the nucleus. Although MPF is mainly localized in the cytoplasm of GV-arrested oocytes, the cytoplasmic localization may be the result of rapid nuclear export and slow nuclear import (Marangos and Carroll 2004; Reis et al. 2006). Thus, Wee1B prevents the activation of MPF that is shuttling into the nucleus during meiotic arrest. On the other hand, Myt1 inhibits the activity of MPF localized in the cytoplasm, as demonstrated by morpholino oligonucleotide knockdown of Myt1 (Oh et al. 2010). When cAMP levels decrease and thereby inactivate PKA in the oocyte, Cdc25B is translocated to the nucleus (Oh et al. 2010). The accumulation of the phosphatase in the nucleus promotes the activation of MPF that is shuttling into the nucleus. In the nucleus, the activated MPF promotes the export of Wee1B to the cytoplasm. Therefore, meiotic arrest is maintained by cAMP-mediated activation of PKA via the direct regulation of the kinase and phosphatase that regulate MPF activity. These two branches of the regulatory loop likely work in a synergistic fashion to maintain an inactive MPF during meiotic arrest. A third component controlling meiotic arrest may be the equilibrium between synthesis and degradation of Cyclin B. Blockade of the APC activity responsible for cyclin B degradation causes meiotic resumption in a subset of oocytes at the GV stage (Homer et al. 2009; Holt et al. 2010; Holt et al. 2011).

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Maintenance of cAMP levels that prevent maturation in the oocyte: role of cGMP

The finding that the machinery for cAMP accumulation is expressed in its entirety in the mammalian oocyte has consolidated the idea that this second messenger is produced autonomously by the oocyte at levels sufficient to maintain meiotic arrest. However, this view needs to be reconciled with the finding that a mammalian oocyte, once removed from the follicle, is unable to maintain the meiotic arrest. To resolve these discrepancies, two hypotheses have been tested experimentally. One is that receptors involved in cAMP generation in the oocyte are regulated by signals originating in the somatic compartment. As mentioned above, GPR3 and GPR12 are the two main GPCRs regulating cAMP generation in mouse and rat oocytes, respectively. Although these receptors are thought to bind several lipid moieties, subsequent data have cast doubt on the specificity of these effects and these receptors remain orphan receptors (Yin et al. 2009). Thus, it has not been possible to directly test whether ligands are produced by granulosa cells nor to demonstrate these are necessary to maintain meiotic arrest. However, these receptors have considerable constitutive activity when expressed in a heterologous system, and some data support the idea that the cAMP signals produced by these receptors in the oocyte do not depend on ligands from somatic cells. Freudzon et al (Freudzon et al. 2005) have used the subcellular localization of Gsα as a readout of the activity of GPR3 in the oocytes (Gsα translocates to the cytoplasm upon receptor activation). By comparing the localization in follicle enclosed and follicle cell-free oocytes, they concluded that GPR3 activity does not require surrounding follicular cells. These findings are consistent with the absence of a follicular stimulus activating GPR3, with the caveat that the assay may not be sufficiently sensitive and quantitative to detect subtle changes in activity.

An additional possibility is that cAMP levels non-permissive to maturation are maintained in the oocytes via regulation of the endogenous PDE3A. Changes in PDE3 activity have been detected in oocytes during maturation (Richard et al. 2001), although the cause of this activation was unclear. More recently, a property of PDE3A has recently become the focus of further investigation (Shitsukawa et al. 2001). This enzyme hydrolyzes both cAMP and cGMP. However, the velocity of cAMP hydrolysis is one order of magnitude higher than that for cGMP hydrolysis. Given these kinetic properties, this enzyme behaves as a cGMP-inhibited cAMP-hydrolyzing PDE (Manganiello et al. 1990); work done initially in platelets has conclusively shown that cAMP levels are affected by cGMP inhibition of this PDE (Maurice and Haslam 1990). Thus, the possibility exists that cGMP, if present in the oocyte, may inhibit PDE3A and therefore contribute to maintaining cAMP levels above a certain threshold to maintain meiotic arrest.

As we will describe further below, recent data strongly support this view. However, it should be noted that clues that cGMP plays an important role in meiotic arrest can be traced back to reports published over the last three decades. In light of the most recent findings, all these pieces of information fit nicely in a model that may explain most of the facets of meiotic arrest in rodents. For instance, work done more than three decades ago in porcine oocytes indicated the presence of a factor in the follicular fluid that inhibited meiotic

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maturation (Tsafriri et al. 1976). This factor was partially characterized as a protein with molecular weight of about 2000. A subsequent report showed that porcine follicular fluid increased cGMP in the follicle (Kolena et al. 1993) even though the data were interpreted more in context with luteinization of granulosa cells. In hamster ovaries, cGMP levels have an opposite pattern of accumulation than cAMP during the estrous cycle, being highest in diestrus and lowest in proestrus/estrus when oocytes mature (Hubbard 1980). Also, oocyte cGMP levels were measured and shown to decrease roughly at the same time as oocyte cAMP levels (Tornell et al. 1990a). These authors further showed that atrial natriuretic peptide, which increases cGMP in the cumulus-oocyte complex, prevented rat oocyte maturation (Tornell et al. 1990b). Additional data suggest that pharmacological manipulations elevating cGMP levels in the oocyte maintain meiotic arrest (Tornell et al. 1984; Zhang et al. 2005a; Zhang et al. 2005b). In the converse experiment, inhibition of soluble guanylyl cyclase with ODQ caused oocyte maturation in follicle cultures and this effect was reversed by 8-Br-cGMP (Sela-Abramovich et al. 2008). Thus, a substantial amount of data suggested a role for cGMP for maintenance of meiotic arrest. Three recent papers have reignited interest in ovarian cGMP and have provided key observations on how meiotic arrest may be maintained in the antral follicle (Norris et al. 2009; Vaccari et al. 2009; Zhang et al. 2010).

A pool of cGMP required for maintenance of meiotic arrest is present in GV arrested oocytes. Injection of PDEs that hydrolyze only cGMP in isolated mouse oocytes or in follicle-enclosed oocytes causes oocytes to re-enter the cell cycle (Norris et al. 2009; Vaccari et al. 2009). More importantly, maturation does not occur when PDE3A knockout oocytes are injected or when PDE3 activity is blocked with an inhibitor, strongly suggesting that cGMP functions upstream of PDE3A. In addition, cGMP concentrations of about 1 μM have been measured by RIA or by FRET measurements (Norris et al. 2009; Vaccari et al. 2009). Genetic evidence for a paracrine loop that controls cGMP levels in the follicle has been recently reported by Eppig and collaborators (Zhang et al. 2010). Inactivation of either the NPPC ligand or the cognate receptor NPR2 in two genetic mouse models results in premature oocyte maturation, phenocopying the GPR3 ablation. Moreover, cGMP is decreased in these knockouts and the peptide derived from NPPC prevents spontaneous maturation (Zhang et al. 2010).

Taken together, these findings support the following model for maintenance of meiotic arrest in murine oocytes (see Fig. 2). Granulosa cells express NPPC, which is the precursor for CNP. Although direct evidence of secretion by granulosa cells is lacking, it is likely that CNP accumulates in the follicular extracellular space and activates NPR2. Activated NPR2 causes accumulation of cGMP in the granulosa cell compartment. This diffuses to the oocyte and maintains PDE3A in an inactive state. Since little cAMP accumulates in the follicle prior to LH stimulation, it is likely that cGMP, rather than cAMP, may be the diffusing molecule critical for maintaining the meiotic arrest. It remains to be determined why in earlier reports maturation could be blocked by ANP which does not bind NPR2. It is also unclear why soluble guanylyl cyclase inhibitors, which should not affect receptor guanylyl cyclases, are able to induce maturation in the rat. This may indicate species differences in the NPR expressed, or that under certain conditions ANP can bind and activate NPR2. In a recent report, ANP showed no effect on mouse oocyte maturation (Zhang et al. 2010) and

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our unpublished observations suggest that ANP does not increase cGMP in granulosa cells (Zamah et al submitted).

LH surge and associated signals

The LH surge terminates the program of FSH-dependent steroidogenesis and granulosa cell growth, while promoting differentiation of somatic cells into luteal cells. At the same time, LH induces the expression of genes required for follicle rupture and ovulation, and activates multiple signaling cascades leading to oocyte maturation.

The LHR belongs to the group of GPCRs that are dependent on interaction with Gsα and activation of adenylyl cyclase to produce cAMP (Rajagopalan-Gupta et al. 1998; Richards 2001). Since most of the LH effects are reproduced by pharmacological activation of adenylyl cyclase, for instance with forskolin, it is accepted that an increase in cAMP is the primary signal that mediates the biological effects of the LH surge. The initial cAMP signal activates a number of signaling pathways and related kinases, all having important function during ovulation. However, numerous other signals are also activated by LH in the follicle. A scheme summarizing these pathways is reported in Fig. 3. Some of these are mediated by LHR interaction with other G proteins. In rodents, the LH receptor (LHR) is expressed predominantly by theca and mural granulosa cells (Amsterdam et al. 1975; Peng et al. 1991). Therefore, paracrine signaling and intercellular communications must be essential for cumulus-oocyte complex response to the LH surge. For instance, LH-dependent local production of prostaglandins plays an essential role for ovulation (reviewed in (Robker et al. 2000).

LH activation of PLC and inositol phosphate signaling

Early studies in rat and bovine ovaries showed that LH stimulates phosphoinositide (PI) turnover in intact granulosa cells in culture (Davis et al. 1983; Davis et al. 1986; Herrlich et al. 1996). In addition, several reports have documented that LHR activation is associated with an increase in intracellular Ca^{2+} in granulosa cells (Veldhuis 1987). Using a model of recombinant receptors expression in Xenopus oocytes, it has been shown that LH causes an increase in Ca^{2+}-activated Cl^{-} current, as measured by the two-microelectrode voltage-clamp method (Gudermann et al. 1992). This indicates that Ca^{2+} mobilization from intracellular stores occurs in this reconstitution system after LHR occupancy, probably as a consequence of LHR coupling to Gq and phospholipase (PLC) endogenous to the frog oocyte. In most cases, the high concentration of LH required to elicit these signals indicated that this coupling may be important at the time of the LH surge. The physiologic role of LH-dependent Ca^{2+} signals in ovulation has not been explored in detail, but a report indicates that at least in the mouse, this pathway may not be necessary for oocyte maturation (Mehlmann et al. 2006). Conversely, LH-dependent Ca^{2+} signals may play a role in luteinization, since PKC seems to play an important role in this transition (Morris and Richards 1995).

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LH regulation of PI3-kinases and downstream pathways

Work from several laboratories has shown that gonadotropins may activate powerful signaling pathways involved in cell replication independently of PKA (Richards 2001). Granulosa cell stimulation by LH is accompanied by an increase in phosphorylation and therefore activation of the protein kinase AKT, a kinase downstream of the PI3K pathway. Class I PI3Ks (Phosphatidylinositol 3-kinases) are a family of signal transduction enzymes which catalyze the 3′ phosphorylation of the inositol ring of phosphatidyinositols to produce PIP3. PIP3 recruits AKT to the membrane, promoting its phosphorylation. An elegant study by Andric and Ascoli using immature rat granulosa cells transfected with mutant LH receptors showed that LH-mediated AKT phosphorylation is independent of cAMP and inositol phopshate production (Andric and Ascoli 2008). PI3K may also be linked to activation of other effectors such as RAS (Ramjaun and Downward 2007). LH can activate the small GTP-binding protein RAS in an MA10 mouse Leydig cell line (Shiraishi and Ascoli 2006). Similar observations on activation of RAS downstream kinases have been reported for FSH (Wayne et al. 2007). The amount of GTP bound to RAS, which is a measure of its activation, increases markedly but transiently after stimulation with an ovulatory dose of hCG (Fan et al. 2008). Moreover, granulosa cell specific knockout of RAS causes a major disruption of the ovulatory process, in addition to having a major effect on follicle growth (Fan et al. 2009a; Fan et al. 2009b). The mechanisms of LH activation of RAS are not well defined but they may be both direct and indirect through activation of tyrosine kinase receptors (see below).

LH regulation of cGMP levels in the follicle

Although some earlier observations suggested that LH regulates follicular cGMP (Davis et al. 1986), recent data using complementary approaches clearly show that LH causes a profound decrease in cGMP in both granulosa cells and the oocyte (Norris et al. 2009; Vaccari et al. 2009), thus providing a clue to how signals for maturation may develop. The decrease in oocyte cGMP levels is in a range sufficient to cause several fold activation of PDE3A. The mechanism by which activation of LHR causes a marked decrease in cGMP is at present unknown. Preliminary data from our laboratory indicate that *Nppc* mRNA is under the control of the gonadotropins FSH and LH (Zamah et al in preparation), but other mechanisms including desensitization of NPR2 are most likely involved. Some of the effects on cGMP may be indirect and dependent on activation of the epidermal growth factor (EGF) network (see below) since EGF-like growth factors produce a decrease in cGMP similar to LH (Norris et al. 2008; Vaccari et al. 2009) (Fig. 4).

LH transactivation of the EGF network

In recent years, the discovery that the LH surge is associated with activation the EGF signaling network has provided new insight and raised further questions on how the LH signal is propagated from the mural granulosa cells to the cumulus-oocyte complex for the induction of oocyte reentry into the cell cycle and ovulation. A growing body of evidence supports a critical role for LHR transactivation of the EGFR in the regulation of these processes, with recent studies aimed at defining the downstream steps involved.

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EGF has long been known to induce oocyte maturation in vitro (Dekel and Sherizly 1985), and to improve oocyte competence (De La Fuente et al. 1999), yet presence in the preovulatory follicle and follicular fluid remained inconclusive (Westergaard and Andersen 1989; Reeka et al. 1998; Hsieh et al. 2009). It is now known that the EGF-like growth factors amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC), rather than EGF, are rapidly and transiently induced in the somatic cells of the preovulatory follicle by LH (Park et al. 2004; Sekiguchi et al. 2004; Ashkenazi et al. 2005). LH/hCG induction of these EGF-like growth factors has been reported in multiple species (Fru et al. 2007; Wang et al. 2007; Chen et al. 2008; Lindbloom et al. 2008; Inoue et al. 2009; Zamah et al. 2010). The EGF-like growth factors are released from the cell surface as mature, soluble peptides by proteolytic cleavage of the ectodomain, and when added to cumulus-oocyte complexes or follicles in culture, activate the EGFR on the surfaces of the somatic cells to promote oocyte meiotic resumption and cumulus expansion. Inhibition of growth factor shedding by the matrix metalloprotease inhibitors GM6001 or TAPI-1 blocks the LH effects on maturation and cumulus expansion (Ashkenazi et al. 2005; Panigone et al. 2008).

Pharmacological inhibition of EGFR activity prevents these LH-induced events (Park et al. 2004; Ashkenazi et al. 2005), demonstrating that the EGFR is clearly involved. In addition, transcripts for the other members of the *Egfr/Erbb1* family, namely *Erbb2*, *Erbb3* and *Erbb4*, have recently been detected in granulosa cells and cumulus cells from human preovulatory follicles 36 hours after hCG stimulation, although at much lower levels than for *Egfr* mRNA (Zamah et al. 2010). ERBB2, which has no known ligand, becomes phosphorylated in mouse ovaries after hCG stimulation (Noma et al. 2010; Kim et al. 2011). Therefore, it is possible that upon ligand binding, EGFR forms not only homodimeric complexes, but perhaps also heterodimers with one or more of the other ERBB receptors in the follicle. That different ligand-receptor complexes may form likely determines the specificity of the signals produced. However, the physiological role for ERBB2, ERBB3 and/or ERBB4 in the ovary remain to be determined experimentally in vivo.

Studies using different mouse models of disruption of the EGF signaling network underscore a physiological role for this pathway in propagating the LH signal in the preovulatory follicle (Hsieh et al. 2007). Mice null for one of the EGF-like growth factors have a mild ovarian phenotype, likely due to compensation by the other EGF-like growth factors (Hsieh et al. 2007; Kim et al. 2011). A more profound effect on ovarian function was observed in mice null for amphiregulin and homozygous for the hypomorphic $Egfr^{wa2}$ allele ($Areg^{-/-}$ $Egfr^{wa2/wa2}$) (Hsieh et al. 2007). Oocyte maturation, cumulus expansion and ovulation are all impaired in these double mutant mice. In the human, AREG was found to be the most abundant EGF-like growth factor to accumulate in the follicular fluid of ovulatory follicles 36 hours after hCG stimulation (Inoue et al. 2009; Zamah et al. 2010). Human follicular fluid from hCG-stimulated follicles was capable of inducing cumulus expansion and oocyte maturation in cultured mouse cumulus-oocyte complexes (Zamah et al. 2010). However, immunodepletion of AREG, but not of other EGF-like growth factors, blocked the ability of the follicular fluid to promote both these events. In addition, AREG levels were significantly lower in follicular fluid from follicles yielding an immature oocyte or an oocyte that developed into an aberrant embryo, than in fluid from follicles producing

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healthy oocytes (Zamah et al. 2010). However, others have observed an inverse correlation between AREG and oocyte quality, raising the possibility of more complex relationship with AREG production (Inoue et al. 2009) The exact pathway(s) downstream of the LHR that control EGF-like growth factor expression and release are an area of active research, but have yet to be fully determined. Taken together, the findings in the mouse and human strongly support a critical role for the EGF-like growth factors in normal oocyte maturation and ovulation.

In a study from our laboratory, the mouse preovulatory follicle culture model was used to investigate the timing of LH transactivation of the EGFR in relation to the onset of oocyte meiotic resumption (Panigone et al. 2008). With this study, LH was found to induce the expression of *Areg* and *Ereg* mRNAs, as well as the phosphorylation of EGFR, as early as 30 minutes after stimulation, a time that precedes the onset of oocyte meiotic resumption by at least 1 hour. *Areg* and *Ereg* mRNA levels and EGFR phosphorylation increased and were maximal in follicles after 2 hours of LH stimulation. The protein synthesis inhibitor puromycin blocked LH-induced but not AREG-induced EGFR phosphorylation and oocyte GVBD, indicating that de novo protein synthesis was required. Thus, the initial activation of the EGF network by LH is rapidly amplified and maintained over time and likely serves as a mechanism to propagate and promote the LH signal throughout the follicle. Indeed, the importance for amplification, stabilization, and propagation of EGFR signals can be seen, for example, in the regulation of ERK1/2 activity in the follicle (see below).

LH regulation of MAPK

LH-dependent phosphorylation and activation of ERK1/2 has been demonstrated in granulosa cells of different species (Cameron et al. 1996; Carvalho et al. 2003; Su et al. 2003; Tajima et al. 2003; Panigone et al. 2008). Further studies showed that LH-dependent ERK 1/2 activation occurred downstream of cAMP and was dependent on PKA activation (Seger et al. 2001). We now know that multiple signaling cascades downstream of the LHR, including the EGFR and possibly PKC pathways (Woods and Johnson 2007) may also mediate ERK1/2 activation.

In the mouse, LH induces the phosphorylation of ERK1/2 in preovulatory follicles within 30 minutes, and phosphorylation levels are increased after 2 hours of stimulation (Panigone et al. 2008). This activation of ERK1/2 occurs first in the mural granulosa cells, and then, over time, also in the cumulus cells. LH-induced ERK1/2 phosphorylation was inhibited ~50–60% when preovulatory follicles were preincubated with the EGFR tyrosine kinase inhibitor AG1278, or with GM6001 or TAPI-1, matrix metalloprotease inhibitors that prevent growth factor shedding (Panigone et al. 2008; Hsieh et al. 2011). Reduced levels of phosphorylated ERK1/2 were also observed in $Areg^{-/-}$ $Egfr^{wa2/wa2}$ follicles that were stimulated with LH for 2 hours, compared to wild type. Immunostaining for phosphorylated ERK1/2 in hCG-stimulated $Areg^{-/-}$ $Egfr^{wa2/wa2}$ was decreased in the mural granulosa cells of preovulatory follicles compared to wild-type, and ERK1/2 activation in the cumulus cells was delayed and also reduced(Hsieh et al. 2011). Using a pharmacological approach, Reizel et al. (Reizel et al. 2010) found that blocking EGFR activity the last 15 minutes of incubation with LH for different time intervals resulted in reduced phosphorylation levels of ERK1/2, suggesting

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that sustained EGFR activity was required to maintain chronic ERK1/2 phosphorylation. Together, these observations show that LH transactivation of the EGFR is important for regulating ERK1/2 activation in the follicle. Because LH-induced ERK1/2 phosphorylation is not completely prevented in the *Areg*$^{-/-}$ *Egfr*$^{wa2/wa2}$ follicles, this suggests that additional pathways are involved in ERK1/2 activation.

Different experimental models have been used to evaluate the role of ERK1/2 in the ovarian follicle. In cultured mouse follicles, the MEK inhibitor U0126 caused little inhibition of LH-induced oocyte germinal vesicle breakdown (GVBD) when used at 10 μM, a concentration sufficient to prevent LH-induced phosphorylation of ERK1/2, but blocked both LH-induced GVBD and cumulus expansion when used at 100 μM, a concentration that could produce nonspecific effects (Su et al. 2003). However, when ERK1/2 was disrupted specifically in mouse granulosa cells, oocyte maturation, cumulus expansion and ovulation failed to occur in response to hCG, indicating a necessary role for these kinases in vivo (Fan et al. 2009b). Notably, activation of ERK1/2 in cumulus cells with GDF9 alone was not sufficient to stimulate oocyte maturation in cultured cumulus-oocyte complexes (Su et al. 2003). Reduced but measureable levels of phosphorylated ERK1/2 are induced by LH in *Areg*$^{-/-}$ *Egfr*$^{wa2/wa2}$ follicles, yet oocyte meiotic resumption is impaired (Hsieh et al. 2007). Together, these studies suggest that ERK1/2 is necessary but not sufficient to induce oocyte maturation. Another possible interpretation, however, is that a specific pool of ERK1/2 activated by EGFR signaling is required to promote oocyte reentry into the cell cycle, but this remains to be proven.

An additional member of the MAPK superfamily that may play an important function in LH signaling is p38MAPK. Inhibition of p38MAPK activity resulted in impaired meiotic resumption and abnormal cumulus expansion of *in vitro* maturation of porcine COCs (Yamashita et al. 2009). Three of the four p38MAPK isoforms, p38MAPKβ, p38MAPKγ, and p38MAPKδ, have been knocked out in mice, which display normal female fertility. More recently, Liu et al have generated mice with granulosa cell-specific knockout of the p38MAPKα isoform (Liu et al. 2010). In this model, female mice retained fertility, with the most notable alteration being aberrant EGF-like growth factor (specifically *Areg* and *Ereg*) gene expression patterns within the mural granulosa and cumulus cells. Interestingly, in vivo COC expansion and ovulation in these mice are normal, whereas in vitro COCs fail to expand normally. This could be overcome by addition of EGF-like growth factors to the in vitro culture media, suggesting that p38MAPK does have a role in LH-induced EGF network transactivation, but it is not essential for this function in vivo.

LH regulation of gap junction permeability

Gap junctions play an important role in signaling between somatic cells of the follicle, as well as between the cumulus cells and the developing oocyte. The gap junctions between different cells are distinguishable based on the dominant connexin protein present in the junction. Connexin-43 (Cx43) is the predominant connexin present in gap junctions connecting granulosa cells to granulosa cells, whereas Connexin-37 (Cx37) is the major connexin present in junctions between the cumulus cells and oocyte (Beyer et al. 1989; Simon et al. 1997). In the preovulatory follicle, LH induces decreased permeability of Cx43

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gap junctions but not of Cx37 gap junctions (Norris et al. 2008). LH induces the phosphorylation of Cx43 on specific serine residues, and this phosphorylation is MAPK-dependent (Norris et al. 2008). Phosphorylation of Cx43 is associated with gap junction closure, which under experimental conditions appears sufficient to induce oocyte meiotic resumption, presumably by blocking passage of an inhibitory signal to the COC. In mutant mouse models with inactivating mutations of EGFR, LH-induced Cx43 phosphorylation is impaired (Andric et al. 2010)(Hsieh et al. 2011). Subsequent work further demonstrated that LH-induced EGFR transactivation is required for gap junction closure (Norris et al. 2010). However, other as yet undefined signals appear able to promote maturation in the absence of gap junction closure, since a MEK inhibitor (UO126) which prevents gap junction closure does not prevent LH-mediated meiotic resumption (Norris et al. 2008). It is possible that this additional signal may be the decrease in cGMP.

Conclusions

The available information summarized above clearly shows that a complex array of signaling cascades is activated by LH at the time of oocyte maturation and ovulation. These pathways act in parallel or sequentially and undoubtedly produce profound and often irreversible changes in the cells of the follicle. Some of the pathways we have described are intracellular, whereas others require extracellular events including the release of factors that act in an autocrine or paracrine fashion. All these events are necessary to propagate the LH signal from the periphery of the follicle to the center where the oocyte resides. The spatial dimension of signal propagation within the follicle has received little attention, but there is no doubt that exploring how different cellular domains function in time and space will provide a better understanding of the ovulatory process.

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Highlights

- cAMP signaling controls MPF and prevents cell cycle reentry in the oocyte
- cGMP signaling intersects with cAMP signaling to control meiotic arrest
- the ovulatory LH signal activates multiple signaling pathways in the follicle
- LH signals are propagated through the ovulatory follicle via release of paracrine factors

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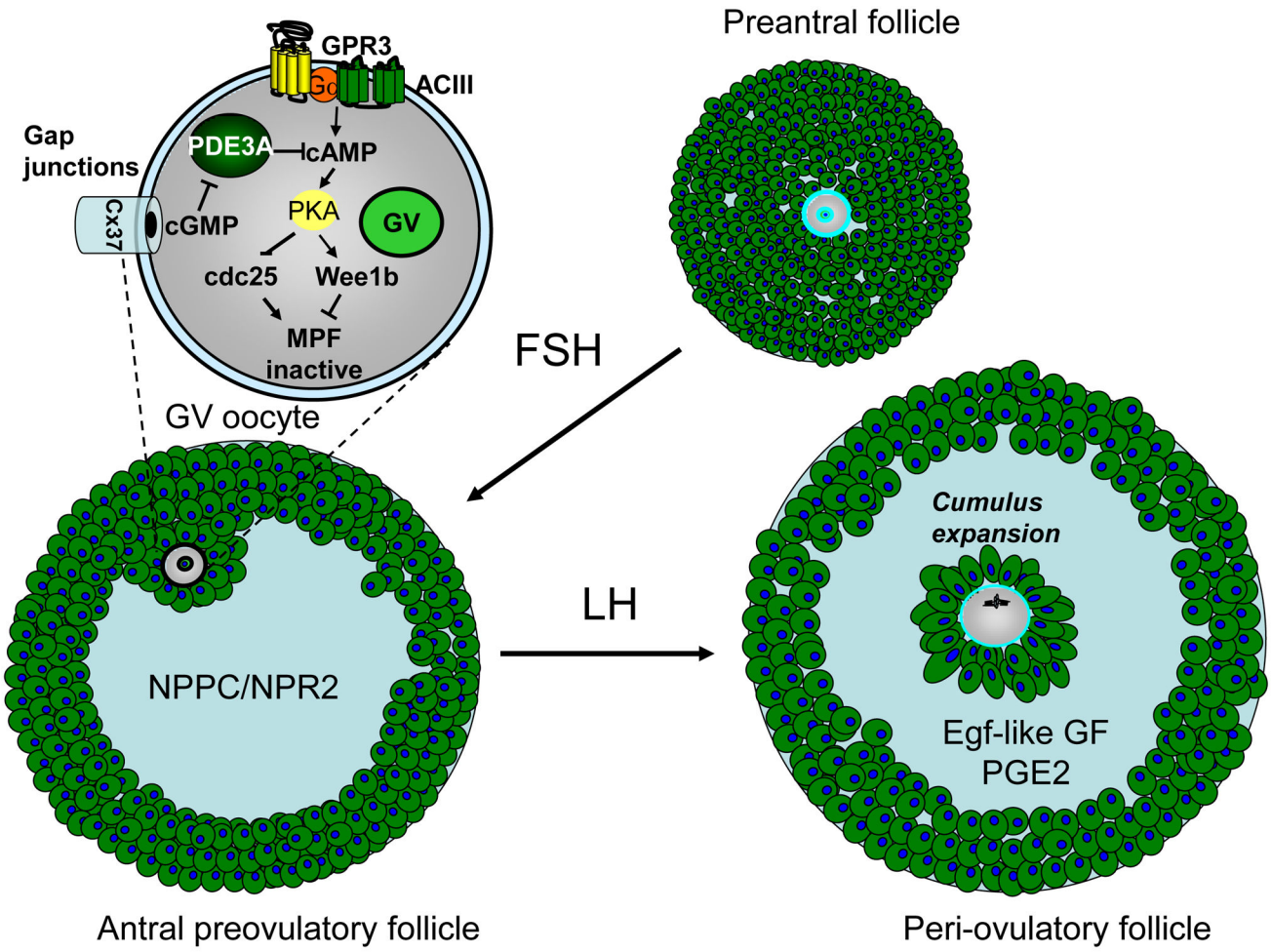

Fig. 1. Schematic representation of follicles at different stages of maturation and primary regulatory signals

During preantral follicle growth, oocytes are not competent to reenter meiosis. With the formation of the antrum, the NPPC/NPR2 paracrine regulation becomes active and maintains oocytes arrested in meiotic prophase via regulation of intrafollicular and oocyte cGMP. With the LH surge, a switch in paracrine regulation takes place with inactivation of the NPPC/NPR2 module and activation of EGF-like growth factors and the PGE2 paracrine regulations. The enlargement of a GV oocyte included in the figure summarized the components of the cAMP signaling pathway involved in meiotic arrest and functional in an antral preovulatory follicle.

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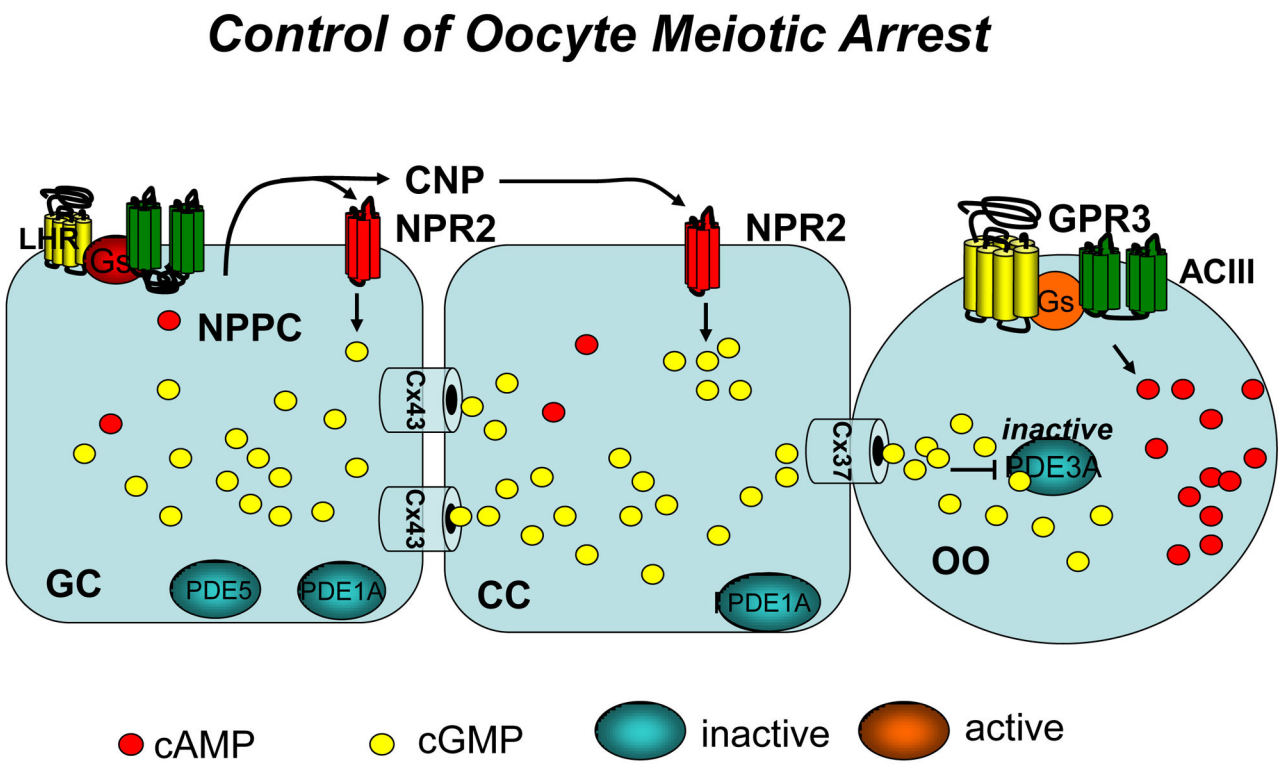

Fig. 2. Model summarizing the cyclic nucleotide signaling pathways involved in maintenance of meiotic arrest in the mouse follicle

The presence of cGMP PDEs in granulosa cells is inferred from biochemical and inhibitor data. Although NPR2 expression in cumulus cells may be higher than in mural cells, mRNA for this receptor is detectable also in mural granulosa cells. GC, granulosa cells; CC, cumulus cells; OO, oocyte. The major connexin subunit expressed in the oocyte is connexin 37 whereas connexin 43 is expressed in the somatic compartment. Heterodimers of connexin 37/43 mediate some communication between the oocyte and cumulus cells.

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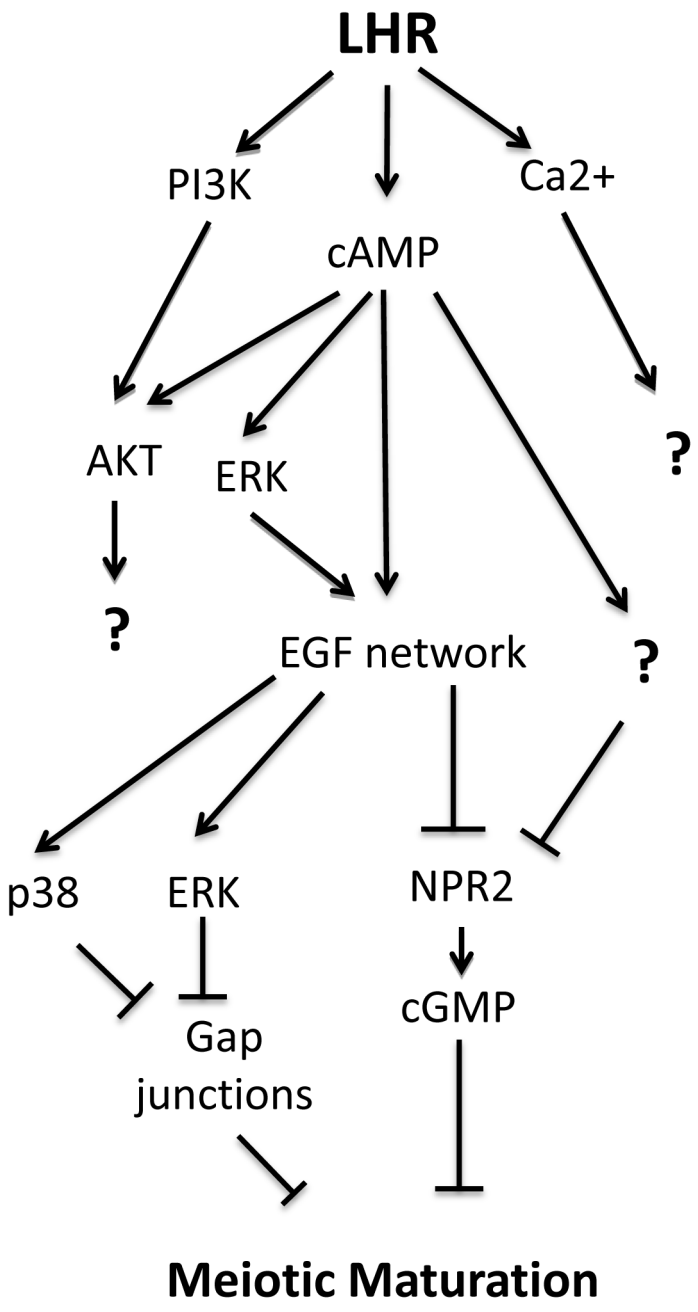

Fig. 3. Scheme of LH Receptor-activated signaling pathways regulating oocyte meiotic maturation

The scheme summarizes our current knowledge on how the signal emanating from the LHR receptor branches to regulation of different signaling pathways. Question marks indicate that the intracellular mechanism linking a signal to downstream responses has not been established. The figure is an updated version of a figure published by Hsieh et al (Hsieh et al. 2011).

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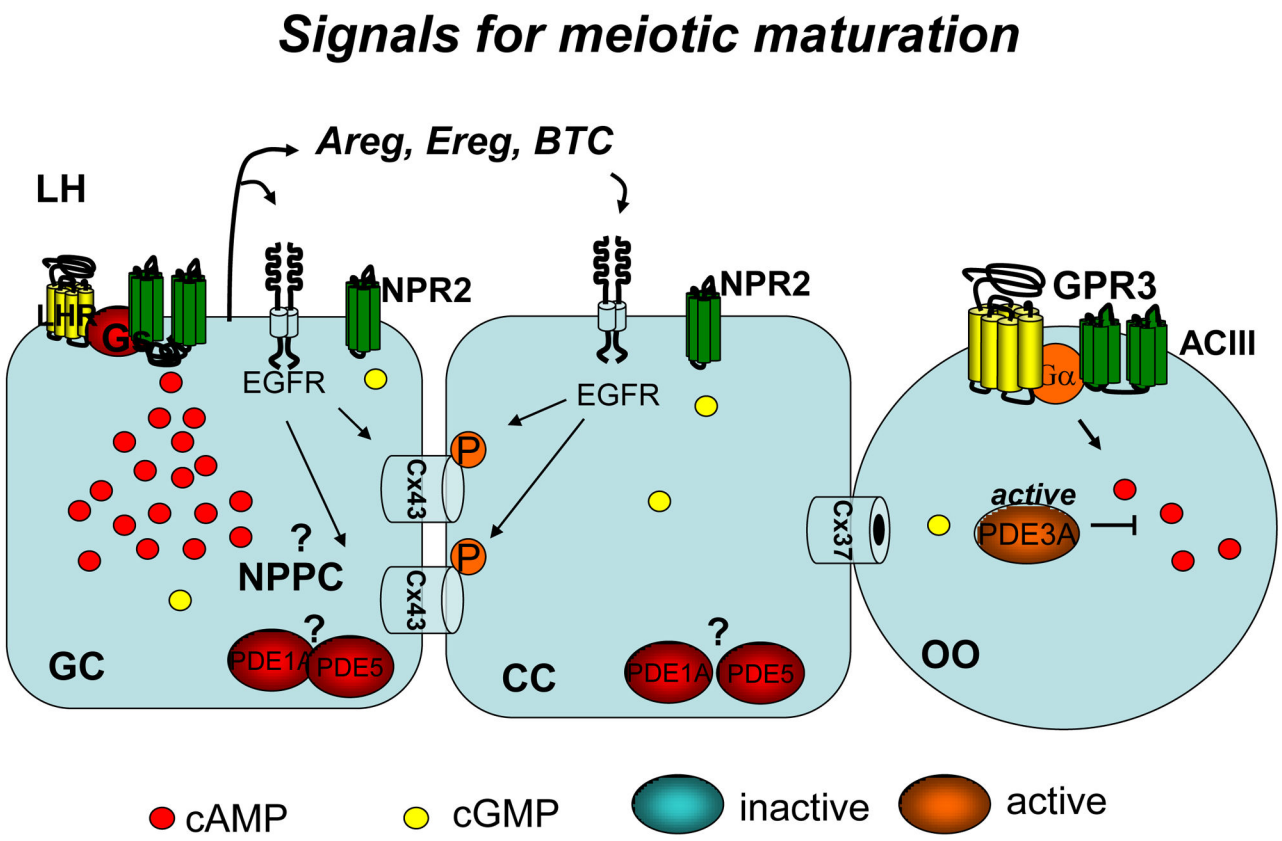

Fig. 4. Model summarizing the different signaling pathways in the ovarian follicle mediating the LH-induced oocyte maturation

Question marks indicate putative changes not yet substantiated by experimental data. P in a red circle indicates phosphorylation. The inactivation of NPR2 may be consequent to decrease in ligand production by granulosa cells or desensitization of the receptor through unknown mechanisms. Activation of a cGMP-specific phosphodiesterase may also contribute to the decrease in cGMP in the follicle.