Epidermal growth factor activates cytosolic $[Ca^{2+}]$ elevations and subsequent membrane permeabilization in mouse cumulus–oocyte complexes

John B O'Donnell Jr, Julia L Hill and David J Gross

Department of Biochemistry and Molecular Biology, University of Massachusetts, Lederle GRC, 710 N. Pleasant Street, Amherst, Massachusetts 01003, USA

Correspondence should be addressed to D J Gross; Email: dgross@biochem.umass.edu

J B O'Donnell is now at University of Connecticut School of Dental Medicine, 263 Farmington Avenue, Farmington, Connecticut 06030, USA and J L Hill is now at Biotech Capital Ltd, Level 2, 141 St George's Terrace, Perth WA 6000, Australia

Abstract

The role of epidermal growth factor (EGF) in the maturation of mammalian oocytes is well known but not well characterized. It is known that EGF enhances oocyte maturation *in vitro* and that EGF stimulation of cumulus–oocyte complexes (COCs) induces pulsatile Ca^{2+} efflux from the cell complex. By use of quantitative Fura-2 imaging, EGF-stimulated changes in intracellular $[Ca^{2+}]$ in germinal vesicle stage murine COCs are shown to occur in a subpopulation of cumulus cells that interact cooperatively within individual COCs. Oocytes fail to respond to EGF stimulus. In many of the cumulus cells responding with a rise in intracellular $[Ca^{2+}]$, a concomitant permeabilization of the plasma membrane is found. Neither cumulus cells of control COCs nor those that show a rise in intracellular $[Ca^{2+}]$ in response to calcium ionophore treatment display a similar membrane permeabilization, although those cells responding with a prolonged $[Ca^{2+}]$ increase in response to thimerosal or thapsigargin do display plasma membrane permeabilization. Thus, EGF stimulation of mammalian COCs activates release of Ca^{2+} from intracellular stores of cumulus cells, the depletion of which activates permeabilization of the plasma membrane. This membrane permeabilization leads to loss of cell contents and presumptive cumulus cell death. This catastrophic EGF-induced plasma membrane permeabilization of individual cumulus cells within a COC leads to pulsatile Ca^{2+} efflux as previously seen, and may lead to improved cumulus cell expansion during COC maturation.

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Introduction

Epidermal growth factor (EGF) is a 6 kDa polypeptide that is a potent mitogen for various mammalian cells *in vitro* (Carpenter & Cohen 1990). A physiological role for EGF in intraovarian regulation of oocyte maturation has been suggested based on detected expression of both EGF and a related ligand, transforming growth factor- α (TGF α), in the follicular fluid from human and mouse (Toretta & DiZerenga 1989). The significance of EGF signaling *in vivo* is not clear but positive effects of EGF on both cultured follicles and isolated cumulus–oocyte complexes (COCs) from mice have been observed (Boland & Gosden 1994). It has been suggested that EGF enhances the rate of meiotic progression of oocytes of COCs isolated from small ovarian follicles (Sakaguchi *et al.* 2002).

Specific effects of EGF on the developmental competence of mouse COCs *in vitro* include promotion of meiotic maturation with increased rates of polar body

formation, and significantly improved cumulus expansion (Das *et al.* 1992). Enhanced cumulus expansion due to EGF stimulation in bovine COCs is blocked by roscovitine, an inhibitor of maturation/M-phase promoting factor (MPF) kinase activity (Vigneron *et al.* 2003) and the tyrosine kinase activity of the EGF receptor (EGFR) is required for EGF-stimulated expansion in pig COCs (Prochazka *et al.* 2003). Based on its ability to directly enhance cytoplasmic maturation in mouse and human oocytes (Das *et al.* 1991), inclusion of EGF in *in vitro* fertilization (IVF) procedures may serve to improve success. EGF has also been shown to have a positive influence on embryogenesis in both the murine and bovine systems. Following IVF in mouse oocytes, EGF stimulates both an increase in the frequency of blastocyst formation and proliferation of the blastomere (DeLafuente *et al.* 1999). In the cow, preimplantation embryo development is enhanced by addition of EGF to defined culture medium (Lonergan *et al.*, 1996).

Stimulation of these processes in both cumulus-enclosed oocytes and early embryos by EGF requires activation of its receptor, which is expressed by the COC in both the oocyte and the surrounding somatic cells, with the highest degree of expression in the preovulatory follicle (Salha *et al.* 1998). The EGFR is a 170 kDa membrane protein with a single transmembrane domain. The extracellular domain of the EGFR is responsible for ligand binding and receptor–receptor dimerization (Garrett *et al.* 2002, Ogiso *et al.* 2002). The cytoplasmic domain of the EGFR contains intrinsic tyrosine kinase activity in a domain near the membrane along with several tyrosine residues that serve as phosphorylatable substrates. Segregation of the EGFR within the plasma membrane and in intracellular organelles appears to modulate EGFR function (Carpenter 2000).

Activation of the EGFR by EGF or TGF α induces both auto- and trans-phosphorylation of EGFRs. Following this phosphorylation event cytosolic signaling proteins, containing Src homolog 2 (SH2) domains, are recruited to the activated receptor and bind to specific phosphotyrosine residues. The signaling proteins that assemble at the receptor are constituents of intracellular signaling pathways, which are initiated at the EGFR and propagate through the cytoplasm to affect various processes of cell growth and physiology.

Specific pathways coupled to EGFR signaling, considered to be putative pathways for the transduction of the EGF stimulus in COCs, include the mitogen-activated protein (MAP) kinase cascade (Nishida & Gotoh 1993) and phospholipase-C γ (PLC γ) activation (Nishibe *et al.* 1990). In both freshly isolated and established cultures of granulosa cells from pigs, tyrosine phosphorylation and activation of MAP kinase is induced by EGF in a concentration-dependent manner that requires phosphatidylinositol 3-kinase (Keel *et al.* 1995, Keel & Davis 1999, Shimada & Terada 2001). Granulosa cells in culture are stimulated by EGF to proliferate (Gospodarowicz & Bialecki 1979, Sirotkin *et al.* 2002). These results demonstrate that EGF directly activates phosphorylation cascades in granulosa cells.

In *Xenopus* oocytes that have been injected with mRNA encoding wild-type human EGFR, activation of PLC γ followed by a rapid increase in intracellular [Ca²⁺]_i is induced by EGF (Yim *et al.* 1994). EGFR expression is preferentially targeted to the animal hemisphere of the oocyte, from which a local intracellular calcium concentration ([Ca²⁺]_i) rise originates following EGF stimulation. The local [Ca²⁺]_i elevation progresses through the ooplasm, resulting in full egg activation.

More recent data collected on the direct effects of EGF on Ca²⁺ mobilization in mouse COCs have shown that EGF-stimulated Ca²⁺ efflux was detected in germinal vesicle (GV) stage COCs using a self-referencing calcium ion sensitive electrode (Hill *et al.* 1999). This response was both prolonged and oscillatory and occurred in COCs that were within 2 h of liberation from the antral follicle. Much

weaker or no efflux responses were recorded from denuded oocytes or isolated cumulus masses, suggesting that an EGF-specific response requires an intact cumulus–oocyte complex. The goal of the present work is to determine the locus within the COC responsible for the EGF-stimulated Ca²⁺ efflux, the signaling pathways responsible for the efflux, and the role of cell–cell communication in the process.

Materials and Methods

Collection of GV stage cumulus–oocyte complexes

Experiments on animals were conducted in accordance with institutional regulations approved by the United States Department of Agriculture. Female CD-1 mice (6–12 weeks old) from Charles River Laboratories (Wilmington, MA, USA) were superovulated by an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin 40 h prior to collection of ovaries. The mice were killed and ovaries were excised and placed in Tyrode-Hepes medium (TL-Hepes) (Bavister *et al.* 1983). The ovaries were dissected and the follicles mechanically separated from surrounding tissue. GV stage COCs were collected from follicles and transferred to TL-Hepes medium supplemented with 10% fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA, USA). The COCs were held in this medium up to 2 h prior to dye loading. Germinal vesicle breakdown (GVBD) was noted in some COCs during or subsequent to experiments, although GVBD was not systematically studied in all COCs.

Preparation of COCs for fluorescence imaging

Intracellular Ca²⁺ concentration ([Ca²⁺]_i) was measured by ratiometric analysis of images of cells loaded with the Ca²⁺-sensitive dye, Fura-2. For dye loading, COCs were incubated for 90 min at 37°C in TL-Hepes + 10% FCS containing 2 μ M of the acetoxymethylester (AM) form of the dye (Molecular Probes, Eugene, OR, USA). Fura-2 AM was dispersed in the medium by addition of 0.02% pluronic acid (Sigma Chemical Co., St Louis, MO, USA). After dye loading, COCs were washed in fresh TL-Hepes and then transferred to a polylysine-coated coverslip mounted in an 800 μ l microscope cell chamber held at 37°C. The Fura-2 fluorescence appeared to be uniformly distributed throughout the cytosol of COC cumulus cells and oocytes.

Cell viability was examined by the use of fluorescein diacetate (FDA), a nonfluorescent membrane-permeable vital stain that is converted by live cells to highly fluorescent fluorescein. COCs were incubated for 90 min at 37°C in TL-Hepes + 10% FCS containing 2 μ g/ml FDA (Sigma Chemical Co.). After dye loading, COCs were washed in fresh TL-Hepes and then transferred to a polylysine-coated coverslip mounted in an 800 μ l microscope cell chamber held at 37°C. The dye loading appeared uniform in the cell cytosol.

Preparation of cumulus cell explants for fluorescence imaging

In some experiments, the cumulus cell layer of a COC was mechanically teased away from the oocyte and placed on a polylysine-coated coverslip in TL-Hepes + 10% FCS for 2–6 h at 37 °C. These cumulus cell explants were labeled with Fura-2 and treated with EGF exactly as for the COCs. Fura-2 loading was uniform in the cells.

Fluorescence imaging experiments

Experiments were conducted on a Zeiss IM-35 inverted fluorescence microscope equipped with a Hg-Xe arc lamp/interference filter illumination system using electronic shutters and excitation wavelength under computer control. Fura-2 fluorescence was excited at two different wavelengths selected by bandpass interference filters, one of 334 nm center wavelength with 10 nm bandpass and the other of 365 nm center wavelength with 10 nm bandpass (Omega Optical, Brattleboro, VT, USA); sample illumination was alternated between 334 nm and 365 nm with exposure times usually of 1250 ms and 25 ms respectively, which produced image pairs of roughly equal signal intensity considerably above the camera noise level. The dichroic mirror used was a 395 nm long-pass (FT395, Carl Zeiss, Thornwood, NY, USA) and the barrier filter a 399 nm long-pass glass (Schott Glass Technologies, Duryea, PA, USA). Sequences of Fura-2 fluorescence image pairs were collected at ~10-s intervals with a Photometrics CCD camera employing a Thomson CFS TH 7882 CCD (Photometrics, Ltd, Tucson, AZ, USA). Image collection, display and analysis were accomplished through home-written routines based in IDL (Research Systems, Boulder, CO, USA).

Individual cells in the COC or in explanted cumulus cells were identified by user creation of a set of regions of interest in the first image of a series. After appropriate background fluorescence subtraction, the 334 nm/365 nm ratio image was formed and the mean ratio and 365 nm fluorescence values were extracted from each region of interest in all images of the series. Reported herein are the ratio values for individual cells and the average fluorescence for the 365 nm excitation image. The Fura-2 334 nm/365 nm ratio has previously been shown to be linear with pCa (Linderman *et al.* 1990), i.e. a twofold change in ratio represents a tenfold change in $[Ca^{2+}]$. The 365 nm excitation image is relatively insensitive to changes in $[Ca^{2+}]$, thus it dominantly reports Fura-2 dye concentration. In some experiments movement of the COC occurred over time. For these, a drift-correcting algorithm was applied, based on image correlation software (<http://idlastro.gsfc.nasa.gov/contents.html>) that compared the location of the initial and subsequent 365 nm images. Fura-2 334 nm/365 nm ratio values reported here are normalized for the different exposure times used for the image pairs, and thus represent the actual ratio signal

for the arc lamp, filter set and dye combination used in the experiments.

Fluorescein fluorescence for FDA-loaded COCs was excited via a 436 nm bandpass filter and detected through a 546 nm bandpass barrier filter (Omega Optical); both had 10 nm full width at half maximum bandpass. Exposure time of 2000 ms produced images of signal intensity considerably above the camera noise level. Time lapse image sequence collection in the presence or absence of EGF stimulation was carried out at 37 °C with identical protocols as used for the Fura-2 experiments.

Cells were maintained at 37 °C throughout the experiments by a temperature-controlled stage and objective lens. Imaging was done through coverslips mounted in the cell chamber where the COCs were bathed in buffer solutions. The experiments were carried out in either of two solutions: Hepes-buffered saline (HBS) with 2 mM $[Ca^{2+}]$ or holding medium (130 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 10 mM Hepes, pH 7.4), a solution of low calcium ion concentration.

Stimulation of COCs or cumulus cell explants was accomplished by addition of reagents directly to the cell chamber, approximately 1 mm distant from the cells. For growth factor treatment, EGF was added at a final concentration of 16 nM in media that were supplemented with 1 mg/ml BSA (Sigma Chemical Co.). For some experiments additions of 100 μ M thimerosal (Sigma Chemical Co.), 10 μ M probenecid (Sigma Chemical Co.), 1 μ M Br-A23187 (Molecular Probes) or 20 μ M thapsigargin (Sigma Chemical Co.) were made.

Statistical analysis

Statistical significance of differences in responses of cells for different treatments was analyzed using a two-tailed *t*-test (Weiss 1989). In all cases the total numbers of cells examined were from those distinguishable as individuals in the fluorescence images which were less than the actual number of cumulus cells within a given COC.

The randomness of the response of cumulus cells in a COC was tested by determining the deviation of the number of responding cumulus cells compared with the number predicted for a random binomial distribution, which would be expected for a cell population that exhibited two states (responding and not responding). The predicted number for each COC was computed by multiplying the probability *P* of response of a cumulus cell for the whole population of cumulus cells for each treatment (total number of responsive cells n_{Tot} divided by total number of cells N_{Tot}) with the number *N* of cumulus cells in each COC. This value was subtracted from the actual number *n* of responding cells in each COC and the absolute value of this difference taken. This difference was normalized to the expected standard deviation of the binomial distribution of responsive cells for each COC ($s.d. = \sqrt{NP(1 - P)}$). A random response of the cumulus cells should thus lead to a difference equal to 0 with a

normalized standard deviation of 1. The normalization for each COC by the expected standard deviation of number of responsive cells allows experiments with different numbers of cells and probabilities of response to be compared more easily. This analysis was done for cumulus cells in all COCs and separately for cumulus cells only in COCs that responded to each treatment.

Results

Effects of EGF on intracellular $[Ca^{2+}]_i$ levels in COCs

COCs from antral follicles were mounted in a 37°C microscope chamber containing HBS or holding medium and analyzed for increases in intracellular $[Ca^{2+}]_i$ following stimulation with 16 nM EGF using a Fura-2 fluorescence imaging system. Experiments were conducted only on those COCs that exhibited appropriate Fura-2 fluorescence and appeared to be healthy. All experiments on COCs were completed within 4 h of excision from the ovary.

Figure 1 shows the response of a COC to 16 nM EGF added to the HBS bathing medium. Of the 73 cumulus cells that were analyzed in this COC, 22 responded to EGF stimulus with an increase in $[Ca^{2+}]_i$. Of these, two abruptly lost their content of Fura-2 dye subsequent to the $[Ca^{2+}]_i$ rise. Figure 1A shows the $[Ca^{2+}]_i$ response of three representative cumulus cells, one of which does not respond to EGF and two of which have increases in $[Ca^{2+}]_i$, one initiating at $t \approx 400$ s and the other at $t \approx 1000$ s. As shown in Fig. 1B, the 365 nm-excited Fura-2 signal drops rapidly at $t \approx 1070$ s in one cumulus cell, indicating rapid loss of the dye. One difficulty in the measurement of COC cumulus cell $[Ca^{2+}]_i$ signals is illustrated by this cell. Although the cell lost all of its Fura-2, Fura-2 fluorescence from out-of-focus cumulus cells and the oocyte contributes to the image signal. This limits the data collection to those cumulus cells that are distinctly imaged, i.e. ones at the surface or along the edge of the COC, and also demonstrates that calibration of the Fura-2 ratio for quantitation is not feasible. The remainder of the single cell $[Ca^{2+}]_i$ data here is analyzed as a response/no response binary system.

An additional four cumulus cells in this COC did not display a $[Ca^{2+}]_i$ increase but did lose Fura-2 abruptly (not shown). Thus, cumulus cells in COCs have three distinct responses to EGF: a $[Ca^{2+}]_i$ increase with subsequent dye loss; a $[Ca^{2+}]_i$ increase with no dye loss, and dye loss with no apparent change in $[Ca^{2+}]_i$. In all, 47% of cumulus cells that responded to EGF stimulus displayed dye loss. Individual experiments were carried out over a range of 50–80 min.

A cumulus cell $[Ca^{2+}]_i$ increase coupled with a loss of dye was the most common response to EGF stimulation in 12 COCs that responded to EGF. A common motif in these responses was a spatial clustering of responsive cumulus cells. Figure 2 shows a second COC stimulated

with 16 nM EGF in HBS. The cumulus cells in this COC were arranged in three clusters. All of the cumulus cells in one cluster responded to EGF stimulus with a prolonged rise in $[Ca^{2+}]_i$ (Fig. 2A) that was terminated by a rapid loss of Fura-2 (Fig. 2B). No cumulus cells in the other clusters responded. Three representative cumulus cells from the responding clump are shown along with a cumulus cell from a nonresponding clump and the oocyte. The responding cumulus cells all initiated their respective $[Ca^{2+}]_i$ increases from 1 to 3 min subsequent to EGF stimulus, and all had a prolonged period of elevated $[Ca^{2+}]_i$. The loss of dye occurred at different times in different cells, and was precipitous. The initiation of the rapid dye loss phase is indicated by dashed lines in panels A and B. The cells disappeared from the fluorescence image within ~ 30 s after rapid dye loss began, indicating that the Fura-2 loss was catastrophic. Figure 2C shows pseudocolor images of the COC at six time points. The pseudocoloring represents ratio by color and 365 nm-excited fluorescence by intensity. In these images, cells that respond to EGF first show a ratio increase which is followed with a rapid decrease in image intensity as the Fura-2 dye leaks out. Note in Fig. 2 that three factors lead to a decline in Fura-2 fluorescence: dye bleaching plus passive efflux, elevation of $[Ca^{2+}]_i$ (the 365 nm excitation fluorescence decreases modestly with increasing $[Ca^{2+}]_i$) and the rapid dye loss phenomenon. The latter can be distinguished by a simultaneous decline in the 365 nm-excited fluorescence and the 334 nm/365 nm ratio. The drop in the 334 nm/365 nm ratio upon loss of Fura-2 is due to the increasing dominance of cell autofluorescence, which has a different fluorescence excitation spectrum compared with that of Fura-2. Thus, once the Fura-2 signals drop to very low levels, the 334 nm/365 nm ratio no longer represents $[Ca^{2+}]_i$ within the cell. For these cells with this imaging system, a loss of Fura-2 produces a drop in the 334 nm/365 nm ratio to levels below starting values. This signal coupled with the drop in 365 nm-excited fluorescence is diagnostic for Fura-2 dye loss.

Control experiments, conducted in the absence of EGF, did not show such responses. Only two cumulus cells in one COC (of 263 cells examined) displayed a rise in the 334 nm/365 nm ratio; no control cells showed a rapid loss of Fura-2. Table 1 summarizes the data collected from a total of 67 COCs in HBS under various conditions. Cell responses were characterized as response/no response, with the criterion for a response being a change in the 334 nm/365 nm ratio of at least 10% lasting more than 1 min and/or a rapid loss of Fura-2.

A COC was classified as responsive if one or more cells in the COC responded. With this classification, COCs were three times more responsive subsequent to EGF treatment compared with controls. Within this class of responsive COCs, the probability of response of individual cumulus cells was greater than that for all cumulus cells from all COCs (Table 1). This indicates that cumulus cells within individual COCs act cooperatively when

Figure 1 EGF-stimulated COC cumulus cells respond with $[Ca^{2+}]_i$ elevations and Fura-2 dye loss. (A) The 334 nm/365 nm Fura-2 fluorescence ratio for three cells in a mouse COC stimulated with 16 nM EGF at $t = 230$ s. The initiation of response in two cells occurs about 2 min after EGF addition (red and violet traces); one cell does not respond (blue trace). (B) Fura-2 fluorescence excited at 365 nm shows a modest, slow decline in two cells (blue and red), and a sharp drop in the third cell (violet) that is simultaneous with a sharp drop in the 334 nm/365 nm ratio shown in panel A. Inset: the 365 nm excitation Fura-2 image of the COC with representative cumulus cells indicated by the arrows. Out of focus cumulus cells are the source of the fluorescence halo at the left and below the in-focus portion of the COC.

responding to EGF stimulation. Considering cumulus cells alone, the response rate was more than tenfold enhanced in the presence of EGF, and this result was highly statistically significant ($P < 0.01$).

The loss of Fura-2 dye in cells responding to EGF appears to be rapid, with near complete dye loss occurring in less than 1 min. The speed with which this loss occurs seems inconsistent with activation of anion pumps in the plasma membrane that could remove the Fura-2

anion from the cytoplasm. To test further this possibility, COCs were incubated with 10 μ M probenecid, an inhibitor of anion pumps (Di Virgilio *et al.* 1990), to determine if the rapid loss of Fura-2 was via pumps. In the presence of probenecid, cells lost Fura-2 dye in response to EGF stimulation just as in the absence of probenecid, demonstrating that the dye loss was not via this process (Fig. 3). As shown in Table 1, the responses of oocytes and cumulus cells in the presence of probenecid were statistically

Table 1 Responses of oocytes and cumulus cells in HBS. The results show the fraction of responding complexes or cells given various treatments. The number in parentheses are the number of COCs or cells examined.

Treatment ¹	COCs responding ^a	Oocytes responding ^b	Cumulus responding ^c	Active COC oocytes responding ^d	Active COC cumulus responding ^e
None	0.17 (6)	0.00 (5)	0.01 (263)	0.00 (1)	0.04 (54)
EGF	0.50 ^f (24)	0.10 ^f (21)	0.13 ^g (780)	0.20 ^f (10)	0.19 ^g (530)
Probenecid	0.67 ^l (3)	0.00 ^f (3)	0.04 ^f (92)	0.00 ^f (2)	0.07 ^f (61)
Probenecid/EGF	1.00 ^{g,h} (12)	0.14 ^f (7)	0.24 ^{g,h} (815)	0.14 ^f (7)	0.24 ^g (815)
Ionophore	1.00 ^g (6)	1.00 ^g (6)	1.00 ^g (196)	1.00 ^g (6)	1.00 ^g (196)
Thimerosal	0.92 ^g (13)	0.92 ^g (13)	0.86 ^g (326)	1.00 ^g (12)	0.95 ^g (295)
EGF/thimerosal	1.00 ^{g,h} (3)	1.00 ^{g,h} (3)	1.00 ^{g,h,i} (63)	1.00 ^{g,h} (3)	1.00 ^{g,h,i} (63)

¹ Concentrations of agents are EGF, 16 nM; probenecid, 10 μ M; Ca^{2+} ionophore Br-A23187, 1 μ M; thimerosal, 100 μ M.

^a Fraction of COCs in which at least one cell responded; ^b the fraction of total oocytes that responded for all COCs; ^c the fraction of total cumulus cells that responded for all COCs; ^d the fraction of responding oocytes in COCs in which at least one cell responded; ^e the fraction of responding cumulus cells in COCs in which at least one cell responded; ^f Not significantly different from control at the 90% confidence level; ^g significantly different from control at the 99% confidence level; ^h significantly different from EGF stimulus at the 99% confidence level; ⁱ significantly different from thimerosal stimulus at the 99% confidence level; ^j significantly different from control at the 95% confidence level.

similar to controls ($P < 0.10$). For EGF stimulation in the presence of probenecid, the fraction of responding COCs and total population of cumulus cells responding was significantly different from those for EGF stimulation alone ($P < 0.01$), although this level of significance was not found for active COC cumulus cells.

Cooperative responses of cumulus cells within COCs

As shown in Fig. 2, often a localized group of cumulus cells in a COC responded to EGF. This suggests that the cumulus cell response might be cooperative. If the response of an individual cell is independent of other cells, then the distribution of responsive cells within a COC should follow a binomial distribution. The individual cumulus cell responses for the COCs listed in Table 1 were compared with the predicted random response for all COCs and for only COCs that included one or more responsive cells. The deviation of the actual and predicted cell responses are given in Table 2. Control COC responses fell within the expected range of average deviation, suggesting that cumulus cell activity for unstimulated COCs is random. For EGF-stimulated COCs, the deviation from random activity was well beyond the expected maximum of one.

Dye loss versus calcium elevation and inositol trisphosphate (IP_3) receptor activation

The role of elevated $[\text{Ca}^{2+}]_i$ in the loss of dye was investigated by the use of the calcium ionophore Br-A23187 (Fig. 3; in this example 16 nM EGF was added prior to 1 μ M Br-A23187 and $[\text{Ca}^{2+}]_i$ rose rapidly following the ionophore despite the lack of response to EGF in this cell). All oocytes and cumulus cells in all COCs treated with Br-A23187 responded with a rapid increase in $[\text{Ca}^{2+}]_i$ (Table 1). In all cases the $[\text{Ca}^{2+}]_i$ response was transient and in no case was loss of Fura-2 dye found. Thus, elevation of $[\text{Ca}^{2+}]_i$ alone is insufficient to induce dye loss.

Activation of the EGFR leads to activation of multiple downstream signaling pathways including that of $\text{PLC}\gamma$, which in turn leads to EGF-stimulated IP_3 production. The role of endoplasmic reticulum-resident IP_3 receptors in the stimulated loss of Fura-2 was examined by treating COCs with 100 μ M thimerosal, a reagent that enhances the sensitivity of the IP_3 receptor for IP_3 (Elferink 1999). Similar to other cell types, cumulus cells and oocytes of COCs responded to the application of thimerosal with $[\text{Ca}^{2+}]_i$ elevations (Fig. 3, Table 1), presumably due to the enhanced sensitivity of the IP_3 receptor to endogenous levels of cytoplasmic IP_3 . In some cases, the oocyte

Figure 2 EGF-stimulated COC cumulus cells responses are localized and cooperative. (A) The 334 nm/365 nm Fura-2 fluorescence ratio for four cumulus cells and the oocyte in a mouse COC stimulated with 16 nM EGF at $t = 310$ s. The initiation of response in the three cumulus cells (L, C, R; violet, green and blue traces respectively) in the lower clump of cumulus cells occurs about 1 min after EGF addition and does so at slightly offset times. No increase in the 334 nm/365 nm Fura-2 fluorescence ratio is seen for the terminal cumulus cell (T, red trace) in another clump or for the oocyte (black trace). A large drop in the Fura-2 ratio is seen for the three cells in the lower clump, and the ratio changes are slightly offset in time. Inset: four 365 nm excitation Fura-2 images of the COC near the peak of the $[\text{Ca}^{2+}]_i$ elevations and during the drop in the ratio values. The time of image collection is indicated (in seconds). (B) Fura-2 fluorescence excited at 365 nm shows little change in the nonresponding cumulus cell (red trace) and a slight increase in the oocyte (black trace) that corresponds with a shape change of the oocyte. Sharp drops in the three responding cumulus cells (L, C and R) are simultaneous with the sharp drops in the 334 nm/365 nm ratio (dashed lines). The Fura-2 dye loss occurs within 1 min in the three cells, but at slightly offset time points. The more gradual decline in the 365 nm excitation signal for the three responding cells is due to the increase in $[\text{Ca}^{2+}]_i$ in the cells; the sharp drop is due to dye loss. (C) Six time points (in seconds) in the image sequence of A and B. The color in each image encodes the 334 nm/365 nm ratio value (blue is low, green is higher, red is highest) while the brightness in the image is proportional to the 365 nm fluorescence signal. Note that the cells labeled L, C and R and their near neighbors in panel A show increasing ratio and eventually disappear, while the cell clump near cell T shows very little ratio change and no dramatic dye loss. The very high ratio value for cells in the upper left corner is due to a 365 nm excitation filter edge effect for this data series.

Figure 3 Representative individual cell responses to different stimuli. Normalized Fura-2 ratio values for individual cumulus cells that responded to 16 nM EGF in the presence of 10 μ M probenecid (green trace), 16 nM EGF followed by 1 μ M Br-A23187 (red trace), 100 μ M thimerosal (blue trace), and 20 μ M thapsigargin followed by 16 nM EGF (violet trace) are shown. All experiments were in HBS except the thapsigargin/EGF treatment, which was in holding medium. The times of addition of the various agents are indicated by arrows. Each 334 nm/365 nm ratio trace was normalized to its baseline value (average of 10–20 points) to ensure that all data were on the same scale. Each trace is offset for clarity. Scale markers indicate 25% ratio change with respect to baseline and 350 seconds. A 25% ratio increase corresponds to $\sim$ 75% increase in $[Ca^{2+}]_i$ (Linderman *et al.*, 1990).

$[Ca^{2+}]_i$ response was oscillatory; this behavior was not observed in cumulus cells (not shown). The thimerosal response deviated even more from randomness than did the EGF-activated response of cumulus cells (Table 2) and

Table 2 Deviation of cumulus cell response from predicted random behavior. The normalized deviation of numbers of responding cumulus cells in individual COCs from the number predicted for a random binomial response is calculated by dividing the difference in these two values by the expected standard deviation for a randomly responding cell population. See Materials and Methods for more details.

Treatment ¹	P_{all}^a	P_{resp}^b	Mean deviation of actual response from predicted response ^c	
			All COC cumulus	Active COC cumulus ^d
None	0.01	0.04	0.85	0.00
EGF	0.13	0.19	1.80	1.71
Probenecid	0.04	0.07	1.04	0.80
Probenecid/EGF	0.24	0.24	4.07	4.07
Ionophore	1.00	1.00	—	—
Thimerosal	0.86	0.95	2.84	1.81
EGF/thimerosal	1.00	1.00	—	—

¹ Concentrations of agents are EGF, 16 nM; probenecid, 10 μ M; Ca^{2+} -ionophore Br-A23187, 1 μ M; thimerosal, 100 μ M. ^a Probability of response of a cumulus cell from all COCs; ^b probability of response of a cumulus cell from COCs in which at least one cell responded; ^c a value between 0 and 1 is consistent with random cell response; ^d an active COC, had at least one responsive cell.

the response of oocytes to thimerosal was much more probable to thimerosal than EGF. Cumulus cell $[Ca^{2+}]_i$ responses subsequent to thimerosal treatment were highly temporally correlated (not shown). Also significant was the loss of Fura-2 in cells responding to thimerosal; 35% of all cumulus cells lost dye subsequent to the $[Ca^{2+}]_i$ rise.

For COCs first treated with 16 nM EGF and then followed 2–3 min later by application of 100 μ M thimerosal, the probability of response of both cumulus cells and oocytes rose to 100% (Table 1), demonstrating that EGF stimulation of COCs leads to IP₃ production. Aside from the enhanced overall responsiveness of COC cells to EGF plus thimerosal, the time course of action of thimerosal was enhanced when EGF preceded it (Fig. 4A). This figure shows the initiation time for individual cell $[Ca^{2+}]_i$ responses to various treatments, examining only cumulus cells that responded to a treatment. Both EGF (filled blue triangles) and thimerosal (open green squares) alone showed similar initiation kinetics, with most cells activating within about 25 min. For cells first stimulated with EGF and then treated with thimerosal (filled red diamonds), the cell response to thimerosal was much more rapid, being essentially complete within 5 min. As expected, the response to calcium ionophore (black line) was very rapid, with nearly all cells responding within

Figure 4 The initiation of $[Ca^{2+}]_i$ elevations (A) or Fura-2 dye loss (B) in responding cumulus cells at times subsequent to stimulation by 16 nM EGF (filled blue triangles), 100 μ M thimerosal (open green squares), 1 μ M Br-A23187 (solid black line) and 100 μ M thimerosal subsequent to 16 nM EGF (solid red diamonds). The time of initiation was determined by eye as the first data point at which a clear trend in the cell response had begun. Note the time scale difference in panels A and B.

1 min of application. This time was likely limited by diffusion of the ionophore to the cells.

In this figure the cooperative response of cumulus cells within individual COCs can be seen. The distinct vertical jumps in numbers of responding cells at nearly the same time result from synchronized responses of cumulus cells in individual COCs. This effect is most pronounced for cells that respond earliest to any stimulus.

Panel B of Fig. 4 shows the time course of Fura-2 dye loss in cumulus cells stimulated with EGF (filled blue triangles), thimerosal (open green squares) or EGF followed by thimerosal (filled red diamonds). In contrast to the time course of initiation of the $[Ca^{2+}]_i$ response in panel A, the loss of dye from thimerosal-stimulated cumulus cells was noticeably delayed compared with the loss due to EGF stimulation. Similar to the results in panel A,

dye loss in cumulus cells first treated with EGF and then with thimerosal occurred more rapidly.

Dye loss and [Ca²⁺]_i elevation in Ca²⁺-free medium

It has been shown that Ca²⁺ efflux from EGF-stimulated COCs occurs with a time delay of ~30 min and that it has both prolonged and pulsatile components (Hill *et al.* 1999). These results are consistent with the idea that a similar mechanism might underlie the Ca²⁺ efflux and the [Ca²⁺]_i response/dye loss described above. For technical reasons the Ca²⁺ efflux experiments of Hill *et al.* (1999) were carried out in nominally Ca²⁺-free holding medium, thus they are not directly comparable to the present work. Therefore, Fura-2 imaging experiments were carried out in holding medium to help establish the relationship between Ca²⁺ efflux and [Ca²⁺]_i elevation.

Similar responses of cumulus cells and oocytes in holding medium were found compared with those for cells in HBS. Control COCs responded at a very low level, and cell responses to EGF shared the same characteristics for both media: [Ca²⁺]_i elevations and dye loss. Table 3 summarizes these results. As expected, Br-A23187 induced rapid and high-probability [Ca²⁺]_i elevations similar to cells treated in HBS. Thus, COCs in holding medium respond similarly to those in HBS.

Hill *et al.* (1999) also showed that treatment of COCs with thapsigargin, an inhibitor of intracellular Ca²⁺-store Ca²⁺-ATPase activity (Thastrup *et al.* 1994), induced Ca²⁺ efflux. Thapsigargin treatment (20 µM) of COCs in holding medium induced both [Ca²⁺]_i elevation and Fura-2 dye loss in 70% of the cumulus cells and all oocytes (Table 3). The dye loss kinetics were similar in nature to those for EGF and thimerosal stimulus in HBS medium (not shown). Stimulation with 16 nM EGF subsequent to 20 µM thapsigargin (Fig. 3) produced responses similar to thapsigargin treatment alone in that the subsequent EGF stimulus did not increase the fraction of responding cells (Table 3).

Fluorescein release from stimulated cumulus cells

Although unstimulated, control COCs showed no Fura-2 dye release during imaging, further experiments were

carried out to confirm that this EGF-stimulated phenomenon was not due to ultraviolet light illumination or other nonspecific effects. To this end, COCs were loaded with fluorescein by application of 2 µg/ml FDA for 90 min. COCs were mounted in the cell chamber in HBS as for the Fura-2 experiments. COCs stimulated with 16 nM EGF showed sudden fluorescein dye loss in 68% of the 184 cumulus cells examined while no sudden loss was found for unstimulated cells. Figure 5 shows the single-cell fluorescein dye signal from representative individual cumulus cells in 16 nM EGF-stimulated COCs (data from three separate experiments). The fluorescein dye loss, when it occurred, did so sooner in stimulated COCs as compared with the Fura-2 dye loss, with the mean time between stimulus and dye loss being 310 ± 120 s for the former and 1260 ± 560 s for the latter (Figs 4B and 5B). Because the fluorescein images involve only single-wave-length dye excitation, they are more difficult to quantitate than those for the dual excitation Fura-2 data.

FDA hydrolysis and subsequent fluorescein entrapment is routinely employed to evaluate the viability of cells. Thus, these data support the view that the EGF-stimulated loss of Fura-2 is due to plasma membrane permeabilization of a cumulus cell that indicates the death of the cell.

Response of excised cumulus cells to EGF stimulus

The above data suggest that cumulus cells in COCs respond to EGF stimulation with a rise in [Ca²⁺]_i and plasma membrane permeabilization that results in cell death. Because the cumulus cells are in communication with each other and with the oocyte via gap junctions, it is possible that the oocyte could influence this signaling activity of the cumulus cells. To test this, cumulus cell clumps were excised from COCs, placed in culture for 2–6 h, loaded with Fura-2 AM and stimulated with 16 nM EGF in an identical protocol as used for the COCs. Figure 6 shows a representative experiment in which a cumulus explant was mounted in HBS medium and exposed to 16 nM EGF after 30 prestimulus images were taken. The time at which EGF was added to the medium was designated as t = 0.

Table 3 Responses of oocytes and cumulus cells in holding medium. The results are the fraction of responding complexes or cells given various treatments. The numbers in parentheses are the numbers of COCs or cells examined.

Treatment ¹	COCs responding ^a	Oocytes responding ^b	Cumulus responding ^c	Active COC oocytes responding ^d	Active COC cumulus responding ^e
None	0.29 (7)	0.00 (4)	0.004 (468)	0.00 (2)	0.03 (73)
EGF	0.33 ^f (12)	0.00 ^f (9)	0.05 ^g (625)	0.00 ^f (5)	0.08 ⁱ (417)
Ionophore	1.00 ^g (4)	1.00 ^g (4)	0.97 ^g (131)	1.00 ^g (4)	0.97 ^g (131)
Thapsigargin	1.00 ^g (4)	1.00 ^g (2)	0.70 ^g (120)	1.00 ^g (2)	0.70 ^g (120)
Thapsigargin/EGF	1.00 ^{g,h} (3)	0.50 ^f (2)	0.79 ^{g,h} (157)	0.50 ^f (2)	0.79 ^{g,h} (157)

¹ Concentrations of agents are EGF, 16 nM, Ca²⁺ ionophore Br-A23187, 1 µM; thapsigargin, 20 µM.
^a Fraction of COCs in which at least one cell responded; ^b the fraction of total oocytes that responded for all COCs; ^c the fraction of total cumulus cells that responded for all COCs; ^d the fraction of responding oocytes in COCs in which atleast one cell responded; ^e the fraction of responding cumulus cells in COCs in which atleast one cell responded.
^f Not significantly different from control at the 90% confidence level; ^g significantly different from control at the 99% confidence level; ^h significantly different from EGF stimulus at the 99% confidence level; ⁱ significantly different from control at the 95% confidence level.

Figure 5 Fluorescein dye loss in COC cumulus cells exposed to 16 nM EGF. (A) Individual representative cumulus cell responses from three experiments. Some cumulus cells retained dye at close to initial, prestimulus concentration for the duration of the experiments (green traces). A small number of cells released fluorescein slowly (purple trace) with no distinguishable release initiation time. More typical dye release occurred more suddenly, with a clear inflection point in the plot of fluorescein fluorescence versus time. The times between EGF stimulus (dotted line) and inflection point are indicated. (B) For all cumulus cells with distinct fluorescein dye loss initiation times, the timing of the initiation is shown. Note the time scale differences in panels A and B.

As can be seen in the top left panel ($t = 0$), individual cells prior to stimulus show high levels of Fura-2 accumulation and the $[Ca^{2+}]_i$ of individual cells varies somewhat from cell to cell. At times subsequent to EGF stimulation, an increasing number of cumulus cells show elevated $[Ca^{2+}]_i$ and, eventually, massive leakage of Fura-2. Selected cells are highlighted with a red arrow to note that $[Ca^{2+}]_i$ has increased since the previous image and with a yellow arrow to note that Fura-2 has begun to leak rapidly since the previous image. The overall brightness of cells that did not rapidly leak dye declined from start to finish in the experiment due to a combination of

photobleaching and possible nonspecific dye leakage. These processes are easily distinguished from the EGF-stimulated dye leakage by their much slower kinetic signature.

Statistical analysis indicated that explanted cumulus cells responded to EGF stimulus in a significant manner compared with nonstimulated controls (Table 4). The fractions of excised cells responding to EGF stimulus in COC cumulus cells and cumulus cell explants were similar (compare Tables 1, 3 and 4).

Discussion

The role of EGF in the enhancement of *in vitro* maturation of oocytes has become clearer in recent years. The work described here demonstrates that EGF stimulation of COCs activates $[Ca^{2+}]_i$ elevations in cumulus cells in a statistically significant manner ($P < 0.01$), while it does not do so for oocytes. A second, unexpected cellular response to EGF stimulation is the catastrophic release of the Fura-2 Ca^{2+} indicator employed in these experiments. Sensitization of the IP_3 receptor produces similar effects as EGF stimulus, suggesting that IP_3 production is constitutively active in cumulus cells and that such basal activity in the presence of thimerosal, a reagent that sensitizes the IP_3 receptor for IP_3 , is sufficient to produce both prolonged $[Ca^{2+}]_i$ elevation and dye loss in a subset of cumulus cells. Additionally, sensitization of the IP_3 receptor subsequent to EGF stimulus increases the rate and fraction of both oocyte and cumulus cell responses. For the latter, the time course of responses, both $[Ca^{2+}]_i$ and dye loss, was dramatically reduced, thus demonstrating that IP_3 production, at least in part, plays a role in these cellular responses to EGF. $[Ca^{2+}]_i$ elevations stimulated by EGF in nominally Ca^{2+} -free holding medium also support the idea that release of Ca^{2+} from intracellular stores underlies the EGF signal transduction system in cumulus cells within COCs.

Other experiments with a vital dye, fluorescein diacetate, also found dye loss subsequent to EGF stimulation of cumulus cells of COCs with more rapid kinetics as compared with Fura-2 experiments. This dye loss demonstrates two points. First, it confirms that EGF stimulation induces the loss of cumulus cell viability that is consistent with a permeabilization of the cumulus plasma membrane. Secondly, it suggests that the membrane permeabilization depends, in part, on intracellular calcium mobilization. Examining Figs 4B and 5B, it can be seen that fluorescein dye loss occurs on average about twice as fast as Fura-2 dye loss. Since fluorescein is of lower molecular weight than Fura-2 (332 vs 637 g/mol) and both are anions, but Fura-2 is a Ca^{2+} buffer while fluorescein is not, the slower dye loss when Fura-2 is in the cumulus cell cytosol may be related to damping of calcium ion movements in the cytosol. Such effects could be

Figure 6 Excised cumulus cells responding to 16 nM EGF stimulation. Six illustrative time points are shown: just prior to EGF addition ($t = 0$) and at various times (in seconds) subsequent to EGF addition. The color in each panel encodes the 334 nm/365 nm ratio value (blue is low, green is higher, red is highest) while the brightness in the image is proportional to the 365 nm fluorescence signal. As a cell responds with a rise in $[Ca^{2+}]_i$, its color changes towards green. As a cell loses its entrapped Fura-2, it becomes very dim. All intensities are shown at the same scale for all images. The numbers in the upper right corner of each panel are the time in seconds from EGF addition. Red arrows indicate cells that have initiated a rise in $[Ca^{2+}]_i$ since the previous image. Yellow arrows indicate cells that have initiated a marked dye loss since the previous image. Not all responding cells are indicated for sake of clarity.

due both to reduction in the peak $[Ca^{2+}]_i$ in the cells or in accelerated transport of Ca^{2+} in the cytosol.

The fact that some EGF-stimulated COC cumulus cells showed dye loss without a precursor increase in $[Ca^{2+}]_i$ demonstrates that calcium elevation sufficient for detection by Fura-2 is not necessary for dye loss. Additionally, elevation of $[Ca^{2+}]_i$ by application of the calcium ionophore Br-A23187 did not induce the dye loss phenomenon, again demonstrating that the loss of dye is not solely dependent on elevation of intracellular calcium ion concentration. These data cannot rule out the possibility that two independent EGF-activated pathways can lead to dye loss, however they do not suggest that multiple pathways are necessary. What is clear from these data is that EGF receptor activation in COCs is dominantly transduced in the cumulus cells, requires IP_3 production and release of Ca^{2+} from intracellular stores, and can lead to major modifications of the cumulus cell plasma membrane. These data alone do not address the mechanism or implications of this dye loss.

The fact that isolated cumulus cell clumps respond to EGF stimulus similarly to cumulus cells in COCs in the

presence or absence of extracellular calcium ion clearly demonstrates that the cumulus cell responses of $[Ca^{2+}]_i$ elevation and membrane permeabilization do not require the presence of the oocyte. Thus, the locus of EGF signal transduction in the COC lies in the cumulus cell layer, as has been suggested previously.

The mechanism by which stimulated, rapid Fura-2 dye loss occurs was shown not to be due to activation of anion pumps in the plasma membrane because probenecid, an inhibitor of anion pumps, did not alter cumulus or oocyte response patterns. This suggests that the dye loss is via a mechanism involving direct permeabilization of the cell membrane, with the permeabilization pores being of sufficient size to allow release of a molecule the size of Fura-2 (molecular weight = 636.5 g/mol). Such drastic and rapid permeabilization would be a characteristic of cell death, although the precise nature of this phenomenon remains to be determined. However, the speed with which the permeabilization occurs suggests that this phenomenon is due to necrotic rather than apoptotic cell death, although spontaneous apoptotic cell death has been demonstrated in cumulus cells of bovine COCs (Ikeda *et al.* 2003). Stimulated permeabilization of the plasma membrane would lead to loss of cytosolic contents at least as large as Fura-2, including intracellular Ca^{2+} . In holding medium in which the $[Ca^{2+}]$ is low, such rapid loss of cellular calcium would produce a local and transient flux of Ca^{2+} from the COC. Such a localized pulse of calcium release would appear as Ca^{2+} efflux from the COC, and could account for the Ca^{2+} efflux from EGF- and thapsigargin-stimulated COCs measured by a Ca^{2+} -selective extracellular probe (Hill *et al.* 1999).

Table 4 Responses of explanted cumulus cells. The results are the fraction of cumulus cells responding to 16 nM EGF. The numbers in parentheses are the number of cells examined.

Treatment	Cumulus responding HBS medium	Cumulus responding holding medium
None	0.006 (165)	0.02 (185)
EGF	0.23 ^a (340)	0.08 ^b (274)

^a Significantly different from control at the 99% confidence level;
^b significantly different from control at the 97% confidence level.

The time course of EGF-induced $[Ca^{2+}]_i$ elevations in COCs is different from that observed in A431 and other cells treated with EGF (Cheyette & Gross 1991). Two primary differences between cumulus cell responses and other cell responses is the long time delay and prolonged time of $[Ca^{2+}]_i$ elevation found in the cumulus cells. The mechanism by which an IP_3 -mediated $[Ca^{2+}]_i$ response can be so slow relative to other cells is not clear. This difference in response time may reflect the unique physiology of cumulus cells that are associated with the cumulus-oocyte complex, i.e. in that they are connected by gap junctions, and may contribute to the membrane permeabilization that is an endpoint of EGF stimulus in many responsive cumulus cells. The slow time course of EGF-stimulated $[Ca^{2+}]_i$ responses in COC cumulus cells explains previous findings that EGF does not induce $[Ca^{2+}]_i$ elevations in these cells as earlier measurements were carried out over considerably shorter times than those of the present work (Webb *et al.* 2002).

COC cumulus cells were found to respond to EGF, thimerosal and thapsigargin in a cooperative fashion. The cell responses were often spatially and temporally clustered to small groups of adjacent cells. This observation suggests that cell-cell communication, possibly via gap junctions, of a signal between cumulus cells is an important factor in the overall response of the COC. Two likely signal species are IP_3 and Ca^{2+} . Because not all cumulus cells in a COC respond to EGF, the calcium mobilizing elevation of IP_3 concentration must be regulated such that a signal strong enough to activate a cumulus cell can spread to its neighbors, but not globally through the whole COC. One possible mechanism to regulate locally gap junctional signal propagation would involve local phosphorylation of gap junction connexins, a process that involves protein kinase C and phosphatidylinositol 3-kinase (Shimada *et al.* 2001). Spatially localized inactivation of gap junctions in response to EGF would limit the local activation of cumulus cells. One or more cumulus cells within the local clusters might produce more IP_3 than the average cell. By diffusive propagation, this elevated $[IP_3]$ would be sampled as an elevated background of IP_3 in nearby cells, thus enhancing the local cluster response probability. In some cells at the cell cluster boundary, inactivation of gap junctions would proceed more rapidly than transmission of the IP_3 signal, leading to definition of the edge of responsive cluster. Thus, cumulus cells would activate cooperatively with each other in responsive clusters, which is consistent with the analysis presented in Table 2. In those COCs that fail to respond to EGF, the gap junction inactivation may occur before localized sites of IP_3 production can dominate. Such localized responses may be important for *in vivo* processes that employ the EGF receptor in select COCs.

The data presented here demonstrate that COCs respond to EGF stimulation of the EGF receptor by a standard signal transduction pathway that leads to a commonly found endpoint - elevation of $[Ca^{2+}]_i$. The kinetics

of this response are dramatically slower than in other somatic cells and, further, the EGF signaling system appears to activate cell membrane permeabilization and cell death over a similar time course. Although EGF stimulation of A431 cells is known to lead to apoptotic cell death for high levels of EGF stimulation (Kawamoto *et al.* 1983), this response is slower than that seen in cumulus cells of the COC and is not typical of most somatic cell responses to EGF. A speculative reason to explain why a fraction of cumulus cells exposed to EGF might proceed through a receptor-regulated cell death program is to diminish the physical barriers to sperm access to the oocyte through the cumulus layers. Whether or not IP_3 -mediated signals play such a role in COCs *in vitro* or *in vivo* remains to be determined.

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