Epidermal growth factor receptor downregulation in cultured bovine cumulus cells: reconstitution of calcium signaling and stimulated membrane permeabilization

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Abstract

Cumulus cell–oocyte complexes (COCs), cultured *in vitro*, are competent for maturation and fertilization. Inclusion of epidermal growth factor (EGF) in the COC culture medium enhances *in vitro* maturation and subsequent embryonic development. It has been shown that isolated COCs exposed to EGF respond with a prolonged and pulsatile release of Ca^{2+} into the extracellular medium and that cumulus cells (CCs) of complexes exhibit both a slow rise in intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) and plasma membrane permeabilization in response to EGF. These unusual signaling responses were examined in isolated, cultured bovine CCs. Few individual CCs showed $[\text{Ca}^{2+}]_i$ increases; the lack of response was found to be due to decrease of expression of endogenous EGF receptors after dissociation. CCs transfected with a human EGF receptor–GFP fusion protein showed robust, prolonged, EGF-stimulated $[\text{Ca}^{2+}]_i$ elevations characteristic of CC responses in intact COCs. Many CCs that responded to EGF stimulation with a $[\text{Ca}^{2+}]_i$ rise also released entrapped fura-2 dye at the peak of the $[\text{Ca}^{2+}]_i$ response, suggesting that CC permeabilization and death follows activation of the EGF receptor. The $[\text{Ca}^{2+}]_i$ elevation due to EGF stimulation and subsequent membrane permeabilization was shown to be mediated by the inositol triphosphate signaling pathway.

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Introduction

Within the mammalian ovarian follicle, follicular granulosa cells surround the oocyte and provide regulatory signals to the oocyte. These somatic cells are arrayed in layers around the oocyte, with those closest to the oocyte called cumulus cells (CCs). The oocyte is coupled with its surrounding CCs via gap junctions that allow inorganic ions, second messengers and small metabolites (molar mass less than 1000 Da) to pass from cell to cell. The gap junctions transfer developmental signals and play a role in the regulation of early embryonic development.

Oocytes from primordial ovarian follicles are arrested at prophase of the first meiotic division. Progression of an oocyte through meiosis to the metaphase II stage is one aspect of the process termed maturation. *In vivo* maturation of the oocyte is stimulated by gonadotropins; it can also occur *in vitro* by removal of an immature oocyte from the follicle into culture medium under the proper

culture conditions (Edwards 1965). Oocyte maturation can be divided conceptually into nuclear and cytoplasmic processes. Nuclear maturation refers to the resumption of meiosis and progression from the first meiotic prophase (the germinal vesicle stage) to metaphase II. Cytoplasmic maturation refers to other events related to meiotic progression that prepare the oocyte for fertilization and pre-implantation development (Eppig *et al.* 1994, Eppig 1996).

Epidermal growth factor (EGF) has been shown to have a positive effect during *in vitro* maturation of oocytes within CC–oocyte complexes (COCs) in a variety of species including cattle (Kobayashi *et al.* 1994, Lorenzo *et al.* 1994, Lonergan *et al.* 1996, Rieger *et al.* 1998), humans (Das *et al.* 1991) and rodents (Das *et al.* 1991, Das *et al.* 1992). Although the mechanism whereby EGF exerts its effects on COC maturation has not been fully elucidated, evidence suggests that CCs mediate the action of EGF in the stimulation of oocyte maturation (Lorenzo

et al. 1994). EGF receptor (EGFR) expression has been detected by immunofluorescence and EGFR mRNA was detected by RT-PCR in CCs of several species (Chabot *et al.* 1986, Maruo *et al.* 1993, Singh *et al.* 1995, Goritz *et al.* 1996, Gall *et al.* 2004, Garnett *et al.* 2002). The production of other ligands for the EGFR, including amphiregulin and epiregulin, has been shown to be stimulated in COCs by luteinizing hormone, and the action of these ligands requires the presences of CCs (Park *et al.* 2004). Thus, the ability of EGF to stimulate oocyte maturation is likely due to activation of the EGFR in CCs which then signal to the oocyte.

Stimulation with EGF of COCs freshly isolated from ovarian follicles has been shown to induce pulsatile efflux of Ca^{2+} from the complexes but not from isolated oocytes (Hill *et al.* 1999). Additionally, the CCs of a COC, but not the oocyte, have been shown to respond to EGF stimulation with elevations of intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) with cell–cell co-operativity (O'Donnell *et al.* 2004). Binding of EGF to the EGFR in most cell types induces a cascade of signals transduced by activation of the tyrosine kinase activity of the EGFR (Carpenter 2000, Jorissen *et al.* 2003). Autophosphorylation of the EGFR cytoplasmic regulatory domain produces SH2 binding sites to which phospholipase $\text{C}\gamma$ ($\text{PLC}\gamma$) binds, becoming activated. Active $\text{PLC}\gamma$ hydrolyzes phosphatidyl inositol biphosphate (PIP_2) to produce diacylglycerol and IP_3 . IP_3 binds to its intracellular receptor/channel on the endoplasmic reticulum leading to release of Ca^{2+} from this store and a concomitant rise in $[\text{Ca}^{2+}]_i$. Thus, Ca^{2+} signaling mediated by EGFR activation is a likely mechanism through which EGF can mediate its enhancing effect on oocyte maturation.

It has recently been shown that activation of the EGFR in CCs, both in COCs and in cumulus mass explants, leads to catastrophic plasma membrane permeabilization and cell death (O'Donnell *et al.* 2004). Both this event and the EGF-stimulated elevation of $[\text{Ca}^{2+}]_i$ were shown to occur over a 10- to 20-min time-course, which is much slower than calcium signals in other EGF-stimulated somatic cells. The EGF-activated CC death was suggested to be regulated by the $\text{PLC}\gamma$ signaling pathway. This earlier work also showed that CCs act co-operatively in their response to EGF. Thus, questions arise as to the role of cell–cell interactions and a possible role of the oocyte in the regulation of EGF signaling in CCs within COCs. Luciano *et al.* (2000) have shown that CCs dissociated from bovine COCs and cultured for 18 h have a significantly reduced response to EGF compared with COCs as measured by stimulation of DNA synthesis and inhibition of CC apoptosis. To characterize the intrinsic EGFR signal transduction system in CCs, the mechanisms of EGF signaling in individual, cultured CCs dissociated from bovine COCs were investigated here by observation of $[\text{Ca}^{2+}]_i$ responses and cell death in response to EGF presentation to the cells.

Materials and Methods

Bovine COCs were purchased from OvaGenix (San Angelo, TX, USA), transported in a shipper incubator at 38°C and received within 24 h of slaughter. Mouse EGF (receptor grade) was purchased from Harlan Bioproducts for Science (Indianapolis, IN, USA), Effectene transfection reagent from Qiagen (Valencia, CA, USA), Medium 199 (phenol red-free) and Pluronic F-127 from Gibco/BRL (Grand Island, NY, USA), fetal calf serum (FCS) from Atlanta Biologicals (Norcross, GA, USA), xestospongins C (XeC) from Calbiochem (San Diego, CA, USA), hyaluronidase, thimerosal (Thi), polyclonal anti-EGFR and bovine serum albumin (BSA) from Sigma Chemical Co., fura-2/AM and Alexa Fluor 546-conjugated donkey anti-sheep antibody from Molecular Probes (Eugene, OR, USA) and paraformaldehyde from Aldrich Chemical Company Inc. (Milwaukee, WI, USA).

CC dissociation and culture

Bovine COCs matured for 20–24 h *in vitro* were treated with 10 mg/ml hyaluronidase in Medium 199 at 37°C for 15–30 min to completely dissociate CCs from the oocyte. The dissociated CCs were then cultured on 22 × 22 mm glass slides in 30 mm Petri dishes with 2 ml Medium 199 supplemented with 10% FCS at 37°C in an atmosphere of 5% CO_2 /95% air with saturated humidity.

Cell culture

Human epidermoid carcinoma A431 cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagles' medium (Gibco/BRL) plus 10% FCS in a 37°C humidified incubator with a 5% CO_2 /95% air atmosphere. Chinese hamster ovary (CHO) cells of the K1 subline (American Type Culture Collection) were grown in Ham's F12 medium supplemented with 5% FCS in a humidified 37°C, 5% CO_2 /95% air atmosphere. CHO cells expressing a GFP–EGFR fusion protein (see below) were grown under the same conditions.

GFP–EGFR construction and transfection

The wild-type human EGFR gene was a generous gift from Roger Davis, University of Massachusetts Medical School (Countaway *et al.* 1989, 1992). The XbaI site of the XbaI–HindIII fragment of pXHER which contains the human EGFR coding sequence was converted to a SalI site and the SalI–HindIII fragment was cloned into pBK-CMV (Stratagene, La Jolla, CA, USA). This plasmid was designated pCMV-EGFR. A histidine tag sequence (MRGSHHHHHH; $(\text{His})_6$) was inserted to the pCMV-EGFR at the C terminus of the EGFR (just prior to the HindIII site) using standard PCR techniques and was designated pCMV-EGFR ($(\text{His})_6$). An AgeI site was created at the C terminus of the EGFR ($(\text{His})_6$) using PCR techniques. The pCMV-EGFR ($(\text{His})_6$) was cut with SalI and AgeI and cloned into the pEGFP vector

containing the S65T GFP gene (Clontech, Palo Alto, CA, USA).

CCs were transfected with the GFP-EGFR gene construct following the protocol included with the Effectene transfection reagent. In short, after dissociation, the CCs were cultured for 12–24 h to 20–60% confluence, then treated with a DNA-Effectene solution (0.8 µg DNA, 100 µl buffer EC, 6.4 µl enhancer and 4.0 µl Effectene in 1.6 ml Medium 199 with 10% FCS for each dish) in an atmosphere of 5% CO₂ in air with saturated humidity at 37°C. After treatment for 16 h, the transfection reagent was washed away and replaced with fresh Medium 199 with 10% FCS.

[Ca²⁺]_i imaging

Between day 1 and day 8 (the day on which the CCs were dissociated from COCs was designated as day 0), transfected or untransfected CCs were loaded with fura-2 by adding 2 mM fura-2/AM plus 1 mM Pluronic F-127 for 30 min in HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 10 mM α-D-glucose, pH = 7.4) plus 1 mg/ml BSA at 37°C. Loaded CCs were washed with HEPES-buffered saline plus 1 mg/ml BSA, then observed and stimulated in the same buffer at 37°C. [Ca²⁺]_i changes were monitored as the ratio of the fura-2 fluorescence excited sequentially at 334 and 365 nm in a quantitative imaging microscope system comprised of a Zeiss IM-35 inverted fluorescence microscope equipped with a Hg-Xe arc lamp/interference filter illumination system under computer control. The excitation interference bandpass filters both had full width at half maximum (FWHM) of 10 nm bandpass; the 365 nm center wavelength filter was from Omega Optical (Brattleboro, VT, USA). The 334 nm Hg emission line was selected with a 340 nm center wavelength UVxtreme bandpass filter from Thermo Corion (Franklin, MA, USA). The dichroic mirror used was a 395 nm long-pass mirror (Carl Zeiss, Thornwood, NY, USA) and the barrier filter a 546 nm center wavelength, 10 nm bandpass interference filter (Omega Optical). Sequences of fura-2 fluorescence image pairs were collected at ~10 s intervals with a Photometrics CCD camera (Photometrics Ltd, Tucson, AZ, USA). Image collection, display, and analysis were accomplished through home-written routines based in IDL (Research Systems, Boulder, CO, USA). CCs in the microscope cell chamber were stimulated by infusion of EGF or other agents into the cell culture chamber. The concentration of EGF used was 16 nM and that for Thi was 200 µM. For CCs treated with XeC, 20 µM of the reagent was applied during fura-2 loading.

GFP-EGFR imaging

Fluorescence of the GFP-EGFR construct expressed in CCs was examined using the same microscope system as above, substituting a 436 nm center wavelength, 10 nm

bandpass excitation filter (Omega Optical) and a 580 nm long-pass dichroic mirror (Carl Zeiss).

Quantitation of EGFR expression on CCs by immunofluorescence

COCs or dissociated CCs were fixed with 4% paraformaldehyde (Aldrich Chemical Company Inc., Milwaukee, WI, USA) in phosphate-buffered saline (PBS) at room temperature (RT) for 10 min, then washed three times with PBS buffer. They were then treated with anti-EGFR sheep polyclonal antibody (Sigma) for 1 h in PBS plus 1 mg/ml BSA at RT and washed three times with PBS. This was followed by treatment with Alexa Fluor 546-conjugated donkey anti-sheep antibody from Molecular Probes for 1 h in PBS plus 1 mg/ml BSA, followed by washing three times with PBS and mounting in 1:1 PBS:glycerol. EGFR expression was observed by confocal microscopy using a Bio-Rad MRC-1000 on a Nikon Diaphot 200 inverted microscope. Fluorescence was excited at 568 nm and imaged through a GR2 filter cube. Average cell fluorescence values were analyzed using Scion Image (Scion Corp., Frederick, MD, USA).

Quantitation of EGFR mRNA and protein levels in CCs

EGFR mRNA levels in CCs were analyzed using PCR. An RNeasy mini kit and Hot Star Taq were from Qiagen, Superscript II RT was from Invitrogen (Carlsbad, CA, USA), and primers 1 (5–3: TGT GAG GTG GTC CTT GGG AAT TTG G) and 2 (5–3: TGC TGA CTA TGT CCC GCC ACT GGA) were obtained from IDT (Coralville, CA, USA).

PCR was accomplished following the protocol of Raynor *et al.* (2002). In brief, CCs were collected after hyaluronidase treatment of bovine COCs and a fraction of the cells were lysed and stored at –80°C. The remaining CCs were cultured as above. The cultured CCs were collected and lysed on day 4. Total mRNA from the day 0 and day 4 lysed cells was purified according to the manufacturer's instructions with an RNeasy mini kit (DNase free). Purified mRNA was then amplified using Superscript II RT. In brief, 1 µl 0.5 mM oligo(dT), 1 µl 10 mM dNTP mix, 2 µl purified mRNA and 8 µl sterile water were heated at 65°C for 2 min. To this was added 4 µl 5 × buffer, 2 µl 0.1 M dithiothreitol and 1 µl RNaseOUT which was then incubated at 42°C for 2 min. Then 1 µl Superscript II RT was added and incubated at 42°C for 50 min. Two microliters of the synthesized cDNA was added to 50 µl PCR reaction and heated at 95°C for 15 min, then followed by 30–50 cycles of 94°C for 1 min each, 70°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min.

Western blot analysis was used to detect the level of EGFR expression in CCs. CCs were collected from bovine COCs with hyaluronidase treatment; some were boiled in 1 × SDS buffer for 5 min, others were cultured in medium for 4 days. The cultured CCs were scraped from the culture plate and pelleted by centrifugation and were then

boiled in $1 \times$ SDS buffer for 5 min. Cell lysates from 5×10^4 cells were separated by SDS-PAGE on a 7.5% gel. Proteins were transferred to nitrocellulose membrane overnight in a transfer buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol at pH 8.3. The membrane was blocked with 5% dry milk powder in TBS-Tween (20 mM Tris, 150 mM NaCl and 0.05% Tween 20) for 60 min at RT. The membrane was incubated with monoclonal anti-EGFR antibody F4 (1:1000 dilution; Sigma Chemical Co.) in 1% milk powder in TBS-Tween at RT for 2 h. The membrane was then washed with 1% milk powder in TBS-Tween and then incubated in horseradish peroxidase-linked polyclonal anti-mouse IgG (dilution of 1:1000; Amersham Biosciences Biotech, Amersham, Bucks, UK) in 1% milk powder in TBS-Tween at RT for 1 h. After washing in 1% milk powder in TBS-Tween the membrane was developed with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Population average responses of cells, treated and control, were compared using a two-sided *t*-test for samples with unequal variance. Statistical significance of differences in EGFR expression on the surfaces of untransfected CCs was determined by using a two-sided *t*-test for samples with unequal variance.

Results

EGFR signaling in dissociated CCs

Previous work has shown that CCs of COCs transduce EGF activation of the EGFR with an elevation in $[Ca^{2+}]_i$ that initiates approximately 15 min after EGF stimulation, and continues for many minutes, often being terminated with plasma membrane permeabilization (O'Donnell *et al.* 2004). It was shown that the activation of a CC is more probable if neighboring CCs also activate, suggesting that CCs respond to EGF in a co-operative fashion. At least two mechanisms can account for this co-operativity: gap junctional communication between cells and paracrine signaling between neighboring cells. To better study the signaling pathways activated by the EGFR in individual cells that do not directly couple via gap junctions, CCs dissociated from bovine COCs and grown in culture were loaded with fura-2 and examined for EGF-stimulated $[Ca^{2+}]_i$ signals and plasma membrane permeabilization. Table 1 shows the distribution of individual CCs responding to 16 nM EGF stimulation with a $[Ca^{2+}]_i$ rise, where a positive response was defined as an increase in the fura-2 334 nm/365 nm ratio of more than 10% that continued more than 30 s. The probability of response of CCs to EGF was very low, with only a few cells responding. The only cells that responded were those in culture 2 days after dissociation from the COC. This response rate was in marked contrast to CC responses in intact COCs where 13–19%

Table 1 Probability of response of CCs to 16 nM EGF.

Day ^a	Untransfected (<i>n</i> ^b)	Transfected (<i>n</i> ^b)
1	0.00 (7)	—
2	0.25 (32)	0.00 ^c (27)
3	0.00 (32)	0.20 ^d (15)
4	0.00 (31)	0.17 ^c (110)
5	0.00 (7)	0.37 ^c (128)
6	0.00 (17)	0.41 ^c (63)
7	0.00 (17)	0.39 ^c (61)
8	0.00 (6)	0.37 ^c (19)
9	—	0.20 (20)
All	0.05 (149)	0.29 ^c (443)

^a The number of days subsequent to CC dissociation.

^b The number of CCs examined.

^c Statistically significantly different from untransfected at a >99% confidence level.

^d Statistically significantly different from untransfected at a >90% confidence level.

of CCs responded to EGF stimulus, and demonstrated that the EGFR signaling system is rapidly and nearly completely attenuated for CCs in culture for 24 or more hours.

Detection of EGFR protein levels in CCs

The above data showed that EGFR signaling was attenuated in CCs dissociated from COCs for several days in culture. One possible cause of this attenuation is the decrease of endogenous EGFR expression in cultured CCs. To test this hypothesis, EGFR expression in CCs on different days after dissociation was examined by Western blot analysis and immunofluorescence imaging. Western analysis of EGFR expression showed no detectable EGFR in freshly dissociated CCs or CCs cultured for 4 days (Fig. 1A). EGFR from A431 cells and CHO cells permanently transfected with a GFP–EGFR construct was detected as a control. Thus, total CC EGFR expression levels were considerably below the levels in A431 cells that are known to overexpress the EGFR and CHO cells expressing a GFP–EGFR chimera.

Dissociated CCs were examined by double immunofluorescence confocal microscopy to determine the extent of cell surface expression of the EGFR. Figure 1B (inset) shows that EGFR expression on CCs was detected via this method for up to 4 days in culture and that it decreased over time after CC dissociation from COCs. The level of expression of cell surface EGFR on CCs was quantitated and normalized to the level on A431 cells (Fig. 1B). The data showed that EGFR expression on CCs declined to a statistically significant extent (at a 94 to over 99% confidence level). Results were similar in two other experiments.

EGFR mRNA levels in CCs

EGFR gene expression was examined by determining the levels of EGFR mRNA in CCs either freshly dissociated from bovine COCs or in CCs that were cultured for 4

Figure 1 (A) Western blot of 5×10^4 CCs (lanes 1 and 2), A431 cells (lane 4), CHO cells (lane 5) or CHO cells expressing the GFP-EGFR construct (lane 6) and 10 ng EGFR from Sigma Chemical Co. (lane 3). The primary antibody (F4) used detects the intracellular domain of the EGFR. (B) The average EGFR expression level in untransfected CCs on different days after dissociation. Fluorescence values represent the average fluorescence signal in units of image gray level for cells double immunolabeled for the EGFR, with fluorescence levels expressed as a fraction of the level on A431 cells. The primary antibody is polyclonal and detects multiple EGFR epitopes. All imaging measurements were done with laser power, photomultiplier gain and microscope parameters held fixed. Error bars represent S.E.M. The numbers (N) beside the bars indicate the number of cells imaged. Hatched bars indicate that the mean is statistically significantly different from the day 1 mean at >99% confidence level. The day 3 cells are statistically different from day 1 at a 94% confidence level. Two other experiments gave similar results. (Inset) Representative immunofluorescence images for day 1-5 CCs. All images are shown with identical gain and contrast.

days. Figure 2 shows the PCR-amplified mRNA from cell lysates (inset) along with the quantified values for the detected mRNA. In this experiment, 5×10^4 cells were used for preparation of mRNA for each sample and the PCR amplification was for 40 cycles.

Figure 2 EGFR mRNA levels in CCs. EGFR mRNA was measured using RT-PCR. The CHO-K1 parental line and CHO-K1 cells expressing the GFP-EGFR construct are shown along with untransfected CCs immediately after dissociation from COCs (day 0) and after 4 days in culture. (Inset) Gel of EGFR mRNA expression. Lane 1, 1 kb DNA marker ladder (Promega), 0.2 µg DNA; lane 2, 15 µl RT-PCR product of day 0 CCs; lane 3, 15 µl RT-PCR product of day 4 CCs; lane 4, 15 µl RT-PCR product of CHO cells expressing the GFP-EGFR construct; lane 5, 15 µl RT-PCR product of the parental CHO-K1 cell line.

It could be seen that the EGFR mRNA level was higher in freshly dissociated (day 0) CCs than in the cultured (day 4) CCs, and that levels for both of them were much less than those for the positive control CHO cell line expressing the GFP-EGFR construct (Fig. 2). For a negative control, the parental CHO-K1 cell line that expresses little or no EGFR (Fig. 1A) showed nearly undetectable EGFR mRNA levels. In other experiments starting with different numbers of cells, the detected mRNA levels were proportional to cell number, indicating that this method is a reliable indicator of mRNA expression (not shown). Thus, EGFR mRNA levels declined in CCs subsequent to dissociation from the COC by 60% over 4 days of culture. Again, these results were consistent with both the decline in EGFR protein expression and in EGFR-mediated signaling in the untransfected, cultured CCs.

EGFR transfection into CCs restores the EGF response

It appears that EGFR expression in cultured CCs is considerably reduced compared with other cells, and that EGFR expression is attenuated with time in culture. This was consistent with the hypothesis that lowered EGFR levels are responsible for attenuated signaling as described earlier (Table 1). To test this hypothesis, CCs were transfected with a plasmid containing the gene for the EGFR-GFP fusion protein that is stably expressed in the CHO

Figure 3 GFP–EGFR expression in representative CCs. All cells were transfected on day 1 in culture, images are for cells in culture from day 2 through 6 as indicated.

cell line of Figs 1 and 2. Transfection efficiency was nearly 100% as judged by observation of GFP fluorescence in cultured CCs and the level of expression of the GFP–EGFR construct was very similar from cell to cell (Fig. 3). GFP–EGFR expression could be detected by visualizing GFP fluorescence 1 day after transfection (i.e. on day 2 of culture), and it persisted for more than 10 days (not shown). GFP–EGFR expression was distributed nearly uniformly in the plasma membrane, consistent with results for a similar construct expressed in COS cells (Carter & Sorkin 1998).

Some cells showed elevated GFP–EGFR concentration near the nucleus (see day 3 of Fig. 3), perhaps due to localization of endoplasmic reticulum pools of freshly synthesized GFP–EGFR.

Dissociated, cultured CCs expressing the GFP–EGFR fusion protein responded to 16 nM EGF stimulation with a $[Ca^{2+}]_i$ rise in a higher fraction of cells as compared with untransfected CCs. Table 1 shows the fraction of responsive cells at different days subsequent to initial CC dissociation. The fractional response of the transfected CC population to EGF stimulus was statistically significantly different from that of untransfected cells for all days for which a comparison was possible. Additionally, the fractional response of the CC population on average, 0.29, was similar to the fractional response of CCs in mouse COCs stimulated with 16 nM EGF, 0.13–0.19 (O'Donnell *et al.* 2004).

Also similar to the findings of O'Donnell *et al.* (2004) for CCs in COCs was the phenomenon of sudden fura-2 dye loss subsequent to an EGF-stimulated $[Ca^{2+}]_i$ rise (Fig. 4). Only those CCs that showed an EGF-stimulated increase in $[Ca^{2+}]_i$ also displayed this sudden dye loss. Unresponsive CCs showed only a gradual, monotonic decrease in fura-2 fluorescence attributed to photobleaching. As with CCs in intact COCs, the loss of fura-2 led to a concomitant decrease in the 334 nm/365 nm ratio image as cell autofluorescence began to dominate the fluorescence image. Thus, a sharp drop in the 334 nm/365 nm

ratio, which paralleled the sharp decrease in fura-2 fluorescence excited at 334 nm and at 365 nm, was diagnostic for rapid fura-2 dye loss.

$[Ca^{2+}]_i$ responses to EGF stimulation were mediated by production of IP_3

To further explore the mechanism of CC $[Ca^{2+}]_i$ response to EGF stimulation, the IP_3 pathway blocker XeC was employed. XeC antagonizes the Ca^{2+} -releasing activity of IP_3 , thus blocking Ca^{2+} release at the last step in the IP_3 – Ca^{2+} release pathway (Gafni *et al.* 1997). XeC-treated transfected CCs failed to show any $[Ca^{2+}]_i$ response to EGF stimulation (Table 2), demonstrating that IP_3 -mediated Ca^{2+} release from intracellular stores is necessary for the EGF-stimulated $[Ca^{2+}]_i$ response. No membrane permeabilization in response to EGF stimulation was found for XeC-treated transfected CCs (not shown), demonstrating that this phenomenon also requires IP_3 metabolism. The concentration of XeC used was 20 μ M.

To confirm the role of IP_3 signaling in the transfected CC response to EGF stimulation, the IP_3 receptor sensitizer Thi was used. Thi acts on the IP_3 receptor both to increase mean channel open time and to enhance its single-channel conductance (Thrower *et al.* 1996). Pretreatment of transfected CCs treated with 200 μ M Thi was followed by stimulation with 16 nM EGF. All CCs (of 60 tested) showed a robust $[Ca^{2+}]_i$ response to EGF (Table 2), a statistically significant increase in the fraction of responsive cells.

Treatment with 200 μ M Thi alone also produced a $[Ca^{2+}]_i$ response and membrane permeabilization that was similar in time-course to EGF stimulation in all treated cells (Table 2). Thus, sensitization of the IP_3 receptor with Thi is sufficient to amplify a low-level basal production of IP_3 to release calcium from intracellular stores. This suggests that IP_3 is being produced continuously at low levels in the transfected CCs in the absence of EGF stimulation.

Figure 4 A group of six GFP–EGFR-transfected CCs responding to 16 nM EGF added at $t = 0$. (A) Eight images at key time-points subsequent to EGF addition. Each cell is numbered in the top left panel. The time in min subsequent to EGF stimulation is shown at the lower left in each panel. The color in each image corresponds to the fura-2 334 nm/365 nm ratio, with the scale running from blue through green to red as ratio increases. The brightness in each image is proportional to fura-2 fluorescence intensity. (B) Plot of the fura-2 334 nm/365 nm ratio for each of the cells (cell numbering as in A). Each trace is offset for clarity. The solid bar at the top left indicates 0.5 ratio units. The dashed vertical lines indicate the times for each of the illustrative images in (A). (C) Plot of the 365 nm fluorescence signal for each of the cells (numbering as in A). The dashed vertical lines indicate the times of collection of images in (A).

Table 2 Response of CCs to XeC or Thi in the presence or absence of EGF.

Day ^a	Fraction of cells responding (<i>n</i> ^b)				
	Transfected			Untransfected	
	EGF + XeC	Thi	EGF + Thi	Thi	EGF + Thi
1	—	—	—	0.94 (63)	0.94 (51)
2	0.00 (49)	1.00 ^c (34)	1.00 ^c (6)	0.86 ^f (36)	1.0 ^h (23)
3	0.00 ^d (61)	1.00 ^c (14)	1.00 ^c (13)	0.88 ^f (24)	1.0 ^h (35)
4	0.00 ^c (56)	1.00 ^c (7)	1.00 ^c (9)	0.83 ^e (52)	1.0 ^h (44)
5	0.00 ^c (65)	—	1.00 ^c (11)	1.0 (28)	1.0 (59)
6	0.00 ^c (62)	—	1.00 ^c (12)	1.0 (9)	1.0 (19)
7	—	1.00 ^c (5)	1.00 ^c (12)	1.0 (7)	1.0 (5)
All	0.00 ^c (293)	1.00 ^c (60)	1.00 ^c (63)	0.90 ^e (219)	0.99 ^{f,g} (228)

^a The number of days subsequent to CC dissociation.^b The number of CCs examined.^c Statistically significantly different from EGF alone (Table 1) at a >99% confidence level.^d Statistically significantly different from EGF alone (Table 1) at a >90% confidence level.^e Statistically significantly different from comparably treated transfected CCs at a >99% confidence level.^f Statistically significantly different from comparably treated transfected CCs at a >90% confidence level.^g Statistically significantly different from Thi treatment alone (untransfected cells) at a >99% confidence level.^h Statistically significantly different from Thi treatment alone (untransfected cells) at a >90% confidence level.

Untransfected CCs treated with Thi displayed weak EGFR activity

The above results suggested that, for transfected CCs, EGF stimulation activates IP₃ production that leads to release of intracellular calcium and that low-level basal production of IP₃ is found in these cells. To assess whether this is the case for untransfected CCs, and also to determine if signaling through remnant endogenous EGFRs is detectable in these cells, 200 µM Thi was employed in the presence or absence of EGF to sensitize the IP₃ receptor. Thi alone induced a [Ca²⁺]_i response in the untransfected CCs of significantly higher probability than for untreated cells, but produced a response of lower probability than for transfected CCs, with day 2–4 cells and the population average being statistically significantly different from transfected cells (Table 2). The fraction of untransfected CCs that responded to 16 nM EGF following Thi pretreatment was higher than the fraction responding to Thi alone with day 2–4 cells and the population average being statistically significantly different. Although the probability of cells responding to EGF after Thi was very high in the untransfected cells, the average response for all days after dissociation was weakly statistically significantly different (at a 90% confidence level) compared with that for transfected cells. The general trends were for a lesser response to Thi in the untransfected cells and the average response over all days to Thi or Thi plus EGF was reduced between transfected and untransfected cells. These results taken together were consistent with the idea that untransfected CCs express EGFRs at reduced levels, that unstimulated

EGFRs activate a small but non-zero production of IP₃ and that basal EGFR activity is present at diminished levels in CCs cultured for up to 4 days.

Kinetics of the [Ca²⁺]_i and dye loss responses in untransfected and EGFR-transfected CCs

The time over which individual CCs respond to EGF, both in the time to initiation of a [Ca²⁺]_i rise and the duration of the calcium elevation, was much longer than that of [Ca²⁺]_i responses to EGF stimulation of other somatic cells. For CCs transfected with the EGFR–GFP, all cells showed a lag of 10 min or longer between EGF stimulation and [Ca²⁺]_i rise (Fig. 5B) and a lag of over 25 min between EGF stimulation and fura-2 dye loss (Fig. 5D). Similar results were found for the small number of untransfected CCs that responded to EGF (Fig. 5A and C). The mean times to EGF-stimulated [Ca²⁺]_i rise and dye loss are given in Table 3, as are mean values for cells stimulated with Thi or with EGF following Thi. When transfected CCs were incubated first with Thi and then 3 min later with EGF, the time for individual cells to respond with a rise in [Ca²⁺]_i was noticeably reduced compared with those times for Thi only or EGF only treatment (Fig. 5B and Table 3). The response times for dye loss did not change under these conditions (Fig. 5D and Table 3).

In no case did dye loss in an individual cell precede the rise in [Ca²⁺]_i in the cell for cells treated with EGF, Thi or Thi followed by EGF (Fig. 6). For EGF-treated CCs, the time to dye loss was roughly proportional to the time to a calcium response in an individual cell, independent of the number of days post-transfection (as shown by increasing size of the solid triangle symbols). This same trend was also found for Thi-treated CCs. For Thi plus EGF-treated CCs, the time to dye loss was notably longer compared with the time to a calcium response for individual cells. Additionally, for those cells that responded rapidly to EGF stimulation after Thi pretreatment, there appeared to be a minimum time to initiation of dye loss of about 25 min (red boxes in Fig. 6).

Discussion

EGF has been shown to enhance maturation of oocytes *in vitro* and *in vivo*. Data from several laboratories suggest that CCs surrounding the oocyte are the primary site of action of EGF. Because CCs in COCs communicate with each other and with the oocyte via gap junctions, the signal transduction properties of the EGFR in individual CCs may be altered by these cell–cell interactions. To characterize EGF action in individual cells, CCs dissociated from COCs were isolated and cultured for up to 2 weeks. Quantitative microscopic imaging employing the indicator dye fura-2 demonstrated that EGF stimulation induced [Ca²⁺]_i elevations in CCs isolated for up to 24 h, but not for longer times. The drop in CC response to EGF was

shown to be due to a concomitant decline in EGFR expression levels. Transfection of dissociated CCs with a GFP-EGFR fusion protein restored EGF-stimulated $[Ca^{2+}]_i$ signals which remained activatable for over a week after transfection. In the transfected CCs, the $[Ca^{2+}]_i$ response to EGF was similar to, but slightly slower than, that in CCs in intact COCs (O'Donnell *et al.* 2004): the $[Ca^{2+}]_i$ rise subsequent to EGF stimulus initiated ~ 30 min after stimulus showed a slow time-course of development, and often culminated in catastrophic loss of the fura-2 indicator dye. This suggests that the slow kinetic response and plasma membrane permeabilization in CCs are intrinsic to individual cells, and are not dramatically altered by gap junctional cell-cell communication found in CCs of intact COCs.

The CCs in this study were isolated from bovine COCs matured for 24 h prior to CC dissociation. These CCs were further cultured for up to 2 weeks. Although the CCs cultured in this fashion were clearly different from the CCs in freshly isolated, intact COCs, it nevertheless remains that the two main features of EGFR signaling that were studied here, $[Ca^{2+}]_i$ elevation and plasma membrane permeabilization, remained intact in these cultured CCs when the expression level of the EGF receptor was restored by transfection of the cells with exogenous EGF receptor. This cell culture system thereby permits examination of these signaling events in the absence of direct cell-cell communication when the cells are sparsely plated for experiments. CCs dissociated from freshly excised bovine COCs and cultured for 18 h also showed reduced efficacy of EGF signaling as compared with COC CCs (Luciano *et al.* 2000), suggesting that reduction of EGFR signaling capacity in dissociated CCs occurs whether or not oocyte maturation has proceeded.

Both the EGF-stimulated $[Ca^{2+}]_i$ rise and dye loss were shown to be mediated by activation of IP_3 production since an IP_3 pathway blocker, XeC, totally blocked both effects and an IP_3 receptor sensitizer, Thi, amplified both. Although an IP_3 -mediated signaling pathway was

Figure 5 Time-course of the $[Ca^{2+}]_i$ and dye loss responses of CCs. (A) Untransfected cells treated at $t = 0$ with 16 nM EGF (solid lines), 200 μ M Thi (dashed lines) or 16 nM EGF (dotted lines) following 200 μ M Thi addition at $t = -3$ min that displayed a rise in $[Ca^{2+}]_i$. The total number of cells that responded to each treatment is shown. The time of response is defined as the first time at which an elevation of $[Ca^{2+}]_i$ lasting at least three time-points is detected and, for EGF following Thi, the time of EGF addition is defined as zero. (B) CCs transfected with the GFP-EGFR construct and treated at $t = 0$ with 16 nM EGF (solid lines), 200 μ M Thi (dashed lines) or 16 nM EGF (dotted lines) following 200 μ M Thi added at $t = -3$ min that displayed a rise in $[Ca^{2+}]_i$. (C) Untransfected cells treated at $t = 0$ with 16 nM EGF (solid lines), 200 μ M Thi (dashed lines) or 16 nM EGF (dotted lines) following 200 μ M Thi added at $t = -3$ min that displayed a loss of fura-2. (D) CCs transfected with the GFP-EGFR construct and treated at $t = 0$ with 16 nM EGF (solid lines), 200 μ M Thi (dashed lines) or 16 nM EGF (dotted lines) following 200 μ M Thi added at $t = -3$ min that displayed a loss of fura-2.

Table 3 Mean times from EGF stimulus to $[Ca^{2+}]_i$ or dye loss responses.

Treatment	Mean time to $[Ca^{2+}]_i$ response (min) ^a		Mean time to dye loss (min) ^a	
	Untransfected	Transfected	Untransfected	Transfected
EGF	33 ± 9	50 ± 26 ^c	60 ± 12	66 ± 24
Thi	44 ± 24	49 ± 22	69 ± 21	77 ± 22
Thi/EGF ^b	38 ± 24 ^d	23 ± 14 ^{c,d}	67 ± 26	62 ± 22 ^d

^a Mean ± S.D. are indicated.
^b Thi added 3 min prior to EGF. Time indicated is from EGF addition to individual cell response.
^c Statistically significantly different at a 99% confidence level compared with untransfected cells with the same treatment.
^d Statistically significantly different at a 99% confidence level compared with Thi treatment alone.

responsible for these responses, the time-course over which transfected CCs responded to EGF was much slower than IP₃-mediated signals found in other cell types. In human epidermoid carcinoma A431 cells, initiation of the $[Ca^{2+}]_i$ response is within seconds at similar stimulus levels and its time-course is in the order of a minute (Cheyette & Gross 1991), whereas in CCs these times were in the range of tens of minutes (Figs 5 and 6 and Table 3). This difference in $[Ca^{2+}]_i$ signaling time-course, which was also found in CCs of intact COCs (O'Donnell *et al.* 2004), is thus an intrinsic aspect of the IP₃ signal transduction pathway in CCs and appears not to be due to cell–cell gap junctional signal modulation.

The catastrophic loss of fura-2 subsequent to EGF stimulation that was seen in CCs of COCs and attributed to plasma membrane permeabilization (O'Donnell *et al.* 2004) was found in dissociated, cultured CCs. The permeabilization of the CC plasma membrane was sufficient to permit fura-2, of 636.5 g/mol molar mass, to pass

through the membrane, suggesting that many components of the CC cytosol are also released. In intact COCs, fura-2 and fluorescein leakage was shown not to involve plasma membrane anion pumps (O'Donnell *et al.* 2004) while in the present work the stimulated production of IP₃ was found to be necessary. In intact COCs, plasma membrane permeabilization due to EGFR activation was found to occur in some individual CCs in the absence of a detectable rise in $[Ca^{2+}]_i$ (O'Donnell *et al.* 2004), while all cultured CCs studied here that responded to EGF showed a membrane permeabilization that was preceded by a rise in intracellular calcium ion concentration. Because both thapsigargin, an inhibitor of calcium ATPase activity in intracellular Ca^{2+} stores, and Thi can induce dye leakage in the absence of other stimuli, it appears that release of Ca^{2+} from IP₃-sensitive Ca^{2+} stores precedes the permeabilization of the plasma membrane.

Individual CC responses (Fig. 6) showed that plasma membrane permeabilization occurred subsequent to $[Ca^{2+}]_i$ elevation, and that these two events were roughly correlated temporally. However, under treatment conditions in which the initial $[Ca^{2+}]_i$ elevation occurred within a few minutes of stimulus, the plasma membrane permeabilization required about 20 min to develop. Although prolonged elevation of $[Ca^{2+}]_i$ can lead to membrane permeabilization and cell death, the fact that no detectable rise in $[Ca^{2+}]_i$ was found in some CCs that showed EGF-stimulated membrane permeabilization (O'Donnell *et al.* 2004) suggests that other signals may underlie the phenomenon of plasma membrane permeabilization. One possible mechanism consistent with our findings is suggested by the thapsigargin, XeC and Thi results: the response was initiated by production of IP₃, leading to Ca^{2+} from IP₃-sensitive stores. This calcium release often, but not always, elevates cytosolic $[Ca^{2+}]_i$ sufficient to detect a rise. Following calcium release,

Figure 6 Individual GFP–EGFR-transfected CC $[Ca^{2+}]_i$ and dye loss response kinetics. CCs were treated at $t = 0$ with 16 nM EGF (solid black triangles), 200 μ M Thi (blue X symbols) or 16 nM EGF following 200 μ M Thi added at $t = -3$ min (red open boxes). Only cells that responded to stimulus are shown. Data from 2 to 9 days after transfection with the GFP–EGFR construct are indicated by increasing plot symbol size with increasing time after transfection. The solid diagonal line has a slope of 1, indicating the points above which the time to dye loss is greater than the time of initial calcium response for individual cells. The series of data points along the top of the plot are for cells that showed a rise in $[Ca^{2+}]_i$ but which had no dye loss over the course of the experiment.

depletion of IP₃-sensitive Ca²⁺ stores produces a signal that leads to CC plasma membrane permeabilization. The nature of this signal would be reminiscent of the so-called capacitative Ca²⁺ entry pathway that replenishes intracellular Ca²⁺ stores (Putney & Bird 1993, Clapham 1995).

The nature of the plasma membrane permeabilization initiated by EGF treatment of CCs could be either apoptotic or necrotic. The development of permeability over roughly a 1-h time-course suggests necrosis, although it could indicate the initial stage of apoptosis that is apparent in a subpopulation of CCs. Ikeda *et al.* (2003) have shown that bovine CCs in COCs that are matured *in vitro* undergo apoptosis that is maximal in 24 h but that is weakly detectable at 6 h. The role of activation of the EGFR in this process requires further study.

It is clear that mammalian CCs display unusual IP₃-mediated Ca²⁺ signaling properties. First, the kinetics of these responses is much slower than in other somatic cells. Additionally, the kinetics of the rise in [Ca²⁺]_i subsequent to EGF stimulation in individual CCs is much slower than the activation of the EGFR tyrosine kinase activity in COCs (Prochazka *et al.* 2003), a step that precedes the activation of PLCγ and subsequent production of IP₃. Secondly, a well-known but not well-characterized signaling system, that of stimulated Ca²⁺ entry in response to intracellular calcium store depletion, may be subverted to other functions that lead to catastrophic permeabilization of the CC plasma membrane. Further examination of the modulators of the IP₃-signaling pathway in these cells is necessary to clarify these unusual CC signaling characteristics. Ding & Foxcroft (1994) have described EGF-stimulated cumulus expansion in COCs in which little mucification was found, but rather in which CC disaggregation from the COC was accompanied by optical changes in the CCs. Whether this observation is related to our finding of CC plasma membrane permeabilization in response to EGF requires further study.

The fact that GFP-EGFR expression levels in transfected CCs are relatively constant both over time and from cell to cell (Fig. 3) indicates that the fractional response of a population of CCs (Table 1) is not due to lack of expression of the upstream activator of the signaling cascade, the EGFR. Rather, the results reported here suggest that negative feedback loops in the IP₃ pathway that attenuate the IP₃-mediated [Ca²⁺]_i and permeabilization signals act to dampen these signals before they develop into full strength, observable events. This idea is consistent with the slowed kinetics of these responses in that such attenuation would diminish a nascent response, extending the time necessary for a signal to develop. Thus, the relatively low fractional responsiveness of CCs to EGF may be directly related to the abnormally slow kinetic response times to EGF stimulation.

EGF has been shown to provide an anti-apoptotic effect in many cell types including granulosa cells (Quirk *et al.* 2000, Danielson & Maihle 2002). This protective effect is mediated by the Ras-to-Erk pathway in some cells

(Caragila *et al.* 2003). In contrast, it has been found that EGF can induce apoptosis in some cells that express the EGFR at high copy number (Barnes 1982, Armstrong *et al.* 1994). This cell death signal occurs when such cells are exposed to high concentrations of EGF, and some work has suggested that EGF-mediated cell detachment, or anoikis, may mediate the effect in these circumstances (Schaerli & Jaggi 1998, Kottke *et al.* 1999). This appears not to be the case for all cell types, however (Anto *et al.* 2003). It remains to be seen if EGFR activation of CCs induces apoptotic or necrotic cell death in a subpopulation of the cells, and if this death modulates the effects of the CCs on the oocyte.

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