Gap-junctional communication in mouse cumulus–oocyte complexes: implications for the mechanism of meiotic maturation

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The mechanisms underlying the hormonal stimulation of meiotic maturation are not understood. The most prevalent hypothesis is that hormone-induced maturation is stimulated by an increase in the intracellular messengers, cAMP or Ca^{2+} . This study investigated whether Ca^{2+} transients in somatic cells can lead to Ca^{2+} transients in the oocyte, and whether hormones that stimulate meiotic maturation of mouse oocytes *in vitro* and *in vivo* stimulate an increase in intracellular Ca^{2+} . Of a range of potential agonists of Ca^{2+} release, ATP and UTP were the only agents that stimulated Ca^{2+} release in cumulus cells. ATP-induced Ca^{2+} release is from intracellular stores, as the response is not blocked by chelation of extracellular Ca^{2+} , but is inhibited by the Ca^{2+} -ATPase inhibitor, thapsigargin. ATP and UTP are equipotent, consistent with the receptor being of the P2Y2 type. Confocal microscopy was used to show that ATP-induced Ca^{2+} release in cumulus cells leads to a Ca^{2+} increase in the oocyte. Inhibition of gap-junctional communication using

carbenoxolone, as assayed by dye transfer, inhibited the diffusion of the Ca^{2+} signal from the cumulus cells to the oocyte. Thus, provided that a Ca^{2+} signal is generated in the somatic cells in response to maturation-inducing hormones, it is feasible that a Ca^{2+} transient is generated in the oocyte. However, FSH and EGF, both of which stimulate maturation *in vitro*, have no effect on Ca^{2+} in cumulus–oocyte complexes. Furthermore, LH, which leads to meiotic maturation *in vivo*, did not stimulate Ca^{2+} release in acutely isolated granulosa cells from pre-ovulatory mouse follicles. These studies indicate that ATP may play a role in modulating ovarian function and that diffusion of Ca^{2+} signals through gap junctions may provide a means of communication between the somatic and germ cells of the ovarian follicle. However, our data are not consistent with a role for Ca^{2+} -mediated communication in hormone-mediated induction of meiosis in mice.

Introduction

Mammalian oocytes are arrested in the first meiotic division until they are stimulated to undergo meiotic maturation just before ovulation. Meiotic maturation involves the progression from prophase of the first meiotic division to metaphase of the second meiotic division. In all mammalian species, the signal responsible for stimulating the resumption of meiosis is a surge of LH. LH receptors are present on theca and granulosa cells and, in some species, the cumulus cells but not the oocyte. How this signal in the somatic compartment of the follicle is transduced to the oocyte to stimulate meiotic maturation is not understood.

Meiotic maturation can also be stimulated by release of the oocytes from the follicle into culture medium. This 'spontaneous' maturation indicates that the follicular environment may be responsible for maintaining meiotic arrest (Pincus and Enzmann, 1935; Edwards, 1965). To override meiotic arrest, LH may lead to the suppression of the putative inhibitory follicular signals or, alternatively, may provide a positive stimulus to override the follicular

inhibition. One favoured mechanism for removing inhibitory follicular signals is the breakdown of gap junctions in the follicle (Larsen *et al.*, 1986; Downs, 1995), which would effectively restrict the flow of any inhibitory signals from the follicular cells to the oocyte. However, gap-junctional coupling persists between the cumulus cells and the oocyte until after germinal vesicle breakdown (GVBD) in sheep and mouse oocytes (Moor *et al.*, 1981; Eppig, 1982). The evidence that meiotic arrest is released by a positive stimulus is that hormonal stimuli in the form of LH (Dekel and Beers, 1978), FSH (Downs *et al.*, 1988) and epidermal growth factor (EGF) (Downs, 1989; Downs *et al.*, 1988) can override meiotic arrest *in vitro*. The action is apparently mediated by the cumulus cells and transmitted through gap junctions, as both are required for the hormonal stimulus to work (Fagbohun and Downs, 1991; Downs, 2001). Thus, with appropriate hormonal stimulation, the somatic compartment of the follicle can be induced to drive the resumption of meiosis *in vitro*. However, the nature of the signal is not known.

Clues to the identity of the signalling molecules responsible for maturation may be provided by messengers in the signal transduction pathways of the LH receptor. The LH receptor is a classic G-protein-coupled seven

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Fig. 1. Monitoring of Ca^{2+} concentration in mouse oocytes and cumulus cells. Fluorescence from fura 2-loaded, partially denuded oocytes was recorded. (a) Trace showing fluorescence ratio in cumulus cells (solid lines) and oocytes (dashed lines, circled) during application of carbachol ($100 \mu\text{mol l}^{-1}$) to the bath to increase Ca^{2+} in the oocytes and application of ionomycin ($10 \mu\text{mol l}^{-1}$) to increase Ca^{2+} in both the oocytes and cumulus cells. Each trace represents a single region of interest (ROI) in either the cumulus cells or oocyte. (b) A series of representative images showing pseudocoloured fluorescence ratio (i) before application of carbachol, (ii) at the peak of the carbachol increase, (iii) after recovery of the carbachol response and (iv) after application of ionomycin. A cumulus cell and oocyte are labelled in (i).

transmembrane receptor (Segaloff and Ascoli, 1993). It activates adenylate cyclase with the result that receptor stimulation leads to a rapid increase in the concentration of cAMP (Sanchez-Yague *et al.*, 1993; Davis, 1994). Other reports indicate that LH may signal by stimulating the

hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the production of 1,4,5-triphosphate (InsP_3) and the release of intracellular Ca^{2+} (Davis *et al.*, 1986, 1991; Dimino *et al.*, 1987; Goren *et al.*, 1990; Gudermann *et al.*, 1992; Flores *et al.*, 1998). The ability of the messengers

InsP_3 , Ca^{2+} and cAMP to participate in cell signalling via gap junctions (Saez *et al.*, 1989; Sandberg *et al.*, 1992) indicates that they may be good candidates for the messengers responsible for stimulation of oocyte maturation.

There are two main models for meiotic resumption involving these messengers. In the first model, maturation is stimulated by a transient increase in cAMP. Although cAMP is normally considered to be a negative regulator of meiotic maturation (Cho *et al.*, 1974; Eppig, 1993; Downs, 1995), a number of lines of evidence indicate that it may also act as a positive regulator. Firstly, transient exposure of oocytes arrested with 200 μmol dibutyryl cAMP l^{-1} to 1 mmol dibutyryl cAMP l^{-1} stimulates the resumption of meiosis (Eppig, 1993; Downs, 1995). Secondly, a transient increase in cAMP in intact isolated follicles stimulates oocyte maturation (Dekel and Sherizly, 1983; Dekel *et al.*, 1988; Yoshimura *et al.*, 1992). Thirdly, a transient increase in cAMP has been reported in the oocyte several hours after the addition of LH to the follicle (Moor and Heslop, 1981). Thus, cAMP meets some of the requirements expected of a physiological regulator of meiotic maturation, but a causal link has yet to be established.

In the second model, Ca^{2+} transients generated in the follicular compartment diffuse to the oocyte, with a resultant increase in Ca^{2+} in the oocyte (Eppig, 1993; Homa *et al.*, 1993). In support of this model are the findings that, in intact follicles, the ability of LH to stimulate meiotic resumption is inhibited by Ca^{2+} -free medium (Goren *et al.*, 1990) and that Ca^{2+} oscillations and phosphoinositide turnover occur during resumption of meiosis (Carroll and Swann, 1992; Carroll *et al.*, 1994; Pesty *et al.*, 1994; Homa, 1995). More recently, inhibitors of phosphoinositide metabolism and intracellular Ca^{2+} buffers have been shown to inhibit hormone-induced maturation *in vitro* (Coticchio and Fleming, 1998). Perhaps the most direct evidence for a role for Ca^{2+} is the finding that in sheep cumulus–oocyte complexes (COCs), LH stimulates a Ca^{2+} increase in the cumulus cells that is transmitted to the oocyte (Mattioli *et al.*, 1998). Whether these Ca^{2+} changes occur in the granulosa cells of other species and what role they play in meiotic resumption is not known.

In this study, Ca^{2+} imaging techniques were used to test a number of hypotheses to determine whether increases in intracellular Ca^{2+} are important in LH-induced resumption of meiosis in mice.

Materials and Methods

Oocyte collection and culture

Female MF1 mice were injected with 5–10 iu equine chorionic gonadotrophin (eCG) at 48 h before oocyte collection. For collection of the COCs, mice were killed by cervical dislocation and the ovaries were removed to warm M2 (Fulton and Whittingham, 1978). The large antral follicles on the surface of the ovary were punctured with a 27-gauge needle and oocytes with an intact layer of

Fig. 2. Effects of epidermal growth factor (EGF) and FSH on resting Ca^{2+} concentration in mouse cumulus cells. Each trace represents a single region of interest (ROI) in the cumulus cells. Administration of (a) 20 ng EGF ml^{-1} and (b) 10 μg FSH ml^{-1} had no effect on Ca^{2+} concentrations.

cumulus cells were collected. The COCs were washed three times and maintained in M2 at 37°C.

Monitoring of Ca^{2+} in cumulus–oocyte complexes

For the initial imaging experiments, COCs were pipetted through a pipette smaller than the diameter of the complex but larger than the diameter of the oocyte. This technique breaks the complex open, removing some of the cumulus cells in the process. These partially denuded complexes were incubated in 2 μmol fura 2 l^{-1} in M2 containing 0.02% (w/v) pluronic acid for 20 min at 37°C. This allowed suitable loading of fura 2 into cumulus cells and the oocyte, and provided the basis of an assay to determine whether agonists could stimulate Ca^{2+} release in either the cumulus cells or the oocyte. Ca^{2+} was monitored by placing partially denuded complexes in a chamber containing 500 μl M2 without BSA. After a few minutes to allow the complexes to stick to the glass coverslip base, an additional 500 μl complete M2 was added to the chamber. The heated chamber containing the COCs was placed on the stage of a Zeiss Axiovert and imaged at $\times 20$ magnification with a 0.75 NA objective. Fluorescence was collected using a Coolview camera and processed using IonVision software (ImproVision, Coventry). Data are presented as ratio of emission intensities acquired at excitation wavelengths of 340 and 380 nm. The agonists were made up in M2 at

Fig. 3. ATP stimulated an increase in Ca^{2+} in mouse cumulus cells and this increase was inhibited by suramin. (a) Representative ratio images (i) before and (ii) after application of 1 mmol ATP l⁻¹ to mouse cumulus-oocyte complexes. Note the increase in fluorescence in the cumulus cells but not in the oocytes. (b) Each trace is the fluorescence ratio from either the oocyte or the cumulus cells, as labelled. (c) Suramin (100 $\mu\text{mol l}^{-1}$) preincubation inhibited ATP-induced Ca^{2+} release in cumulus cells.

$\times 10$ – 100 stock solutions and the appropriate volume was pipetted into the chamber during the recording to monitor the effects of agonists on Ca^{2+} in cumulus cells and oocytes. Images were acquired at 2–10 s intervals depending on the duration of the experiment.

Confocal imaging of changes in Ca^{2+} was performed using a BioRad μ Radiance confocal scanning head attached to a Zeiss Axiovert microscope. For injection of Ca^{2+} dyes into the oocyte, a holding pipette attached to a micrometer syringe was used to orient the COC while the injection pipette was inserted through the cumulus cell layers to the oocyte plasma membrane. Penetration of the plasma membrane was achieved by brief over-compensation of the negative capacitance. Fluo3 (5 mmol l^{-1} in the pipette) in injection buffer ($120 \text{ mmol KCl l}^{-1}$ and $20 \text{ mmol Hepes l}^{-1}$) was pressure-injected using a Picopump (WPI) to a final concentration of 50 – $100 \mu\text{mol l}^{-1}$. The cumulus cells were loaded by incubating intact COCs for 30 min at 37°C in $20 \mu\text{mol fluo3 AM l}^{-1}$ in M2 containing $200 \mu\text{mol dibutyl cAMP l}^{-1}$ and 0.02% (w/v) pluronic acid.

For microinjection of fluorescein to assess gap junction permeability, pipettes were backfilled with a solution of $1 \text{ mg fluorescein ml}^{-1}$ in injection buffer before microinjection as described above. The complexes were placed in heated chambers as described above and the fluo3 was excited using the 488 nm line from an argon laser. Images were acquired every 2 s for Ca^{2+} experiments or every minute for fluorescein experiments. Data were analysed off-line using Metamorph software.

Results

Monitoring of Ca^{2+} in cumulus cells and oocytes

Agonists known to increase intracellular Ca^{2+} were applied to establish that changes in cumulus cells and oocytes could be monitored using partially denuded COCs. Carbachol ($100 \mu\text{mol l}^{-1}$) increases Ca^{2+} concentration in oocytes via activation of muscarinic acetylcholine receptors (Carroll and Swann, 1992). An immediate increase in Ca^{2+} concentration in the oocyte was observed after addition of carbachol to fura 2-loaded COCs (Fig. 1). No Ca^{2+} increase was observed in the cumulus cells; however, a large Ca^{2+} increase was reported after application of $10 \mu\text{mol ionomycin l}^{-1}$ ($n = 8$). These findings demonstrate that the experimental conditions allowed measurement of Ca^{2+} concentrations in oocytes and their attached cumulus cells.

Agonist-induced Ca^{2+} release in cumulus cells

Several candidate agonists were screened to examine the hypothesis that a Ca^{2+} increase in the somatic compartment of the follicle is passed to the oocyte via gap junctions. EGF and FSH stimulate mucification of the cumulus cells (Downs *et al.*, 1988; Downs, 1989) and increase Ca^{2+} in a variety of cell types (Flores *et al.*, 1990, 1992). Addition of EGF (20 ng ml^{-1} ; $n = 9$) or FSH ($10 \mu\text{g ml}^{-1}$; $n = 10$) to COCs failed to cause an increase in Ca^{2+} in either the cumulus

Fig. 4. ATP-induced release of Ca^{2+} from intracellular Ca^{2+} stores. (a) Addition of EGTA (2 mmol l^{-1} final concentration) to the bath before application of ATP ($500 \mu\text{mol l}^{-1}$) had no effect on the ability of ATP to trigger Ca^{2+} release in mouse cumulus cells. (b) Depletion of intracellular Ca^{2+} with thapsigargin ($10 \mu\text{mol l}^{-1}$) inhibited the ability of ATP to stimulate Ca^{2+} release in mouse cumulus cells.

cells or oocyte (Fig. 2), despite induction of cumulus expansion in parallel experiments (data not shown). Angiotensin II ($n = 5$) and GnRH ($n = 5$) increased Ca^{2+} in cultured granulosa cells (Currie *et al.*, 1992); however, in the present study, no effect of either agent on Ca^{2+} concentrations in cumulus cells was observed. In addition, no increase in Ca^{2+} in response to dibutyl cAMP (1 mmol l^{-1} ; $n = 6$) or forskolin ($200 \mu\text{mol l}^{-1}$; $n = 6$) was observed (data not shown), although both have been reported to modify Ca^{2+} in ovarian cells of some species (Flores *et al.*, 1992). LH was not tested because mouse cumulus cells do not express LH receptors (Eppig *et al.*, 1997).

Effect of ATP on Ca^{2+} release in mouse cumulus cells via activation of P_2 receptors

The final agonist of Ca^{2+} release tested in the present study was ATP, as it stimulates Ca^{2+} release in ovarian cells from many species (chicken: Morley *et al.*, 1994; human:

Fig. 5. ATP and UTP stimulated Ca^{2+} release in mouse cumulus cells with similar potencies. A dose-response curve for Ca^{2+} release in response to 10, 20, 100 and 500 $\mu\text{mol l}^{-1}$ (a) ATP and (b) UTP is shown. Time of addition of ATP or UTP is indicated by the arrow.

Lee *et al.*, 1996; Squires *et al.*, 1997; pig: Aguirre *et al.*, 2000). ATP (between 250 $\mu\text{mol l}^{-1}$ and 1 mmol l^{-1}) stimulated a significant increase in intracellular Ca^{2+} concentration in cumulus cells ($n = 33$) (Fig. 3a,b). There was no evidence of any increase in Ca^{2+} in the oocyte (Fig. 3a,b), indicating that gap-junctional coupling may be incapable of propagating the Ca^{2+} increase to the oocyte or that the gap junctions were not patent in the conditions of the experiment (see below).

Suramin is an effective inhibitor of P2 receptors and acts by blocking the ATP binding site. Application of 100 $\mu\text{mol l}^{-1}$ suramin before ATP inhibited the ability of ATP to increase Ca^{2+} concentration ($n = 9$) (Fig. 3c). P1 receptors linked to phosphoinositide turnover do not appear to be present on cumulus cells, as adenosine and 2-chloroadenosine did not increase intracellular Ca^{2+} concentration (data not shown).

Partial characterization of the P2 receptor subtype

P2 receptors occur in two major forms: ligand-gated ion channels (P2X receptors) and G-protein-coupled receptors (P2Y) (Burnstock and King, 1996). The roles of extracellular and intracellular Ca^{2+} in the response to ATP were examined to determine whether the cumulus cells expressed the P2X or P2Y receptors. Removal of

extracellular Ca^{2+} by addition of 2 mmol l^{-1} EGTA l^{-1} to the chamber before addition of ATP had little effect on ATP-induced Ca^{2+} release ($n = 21$) (Fig. 4a). This finding indicates that intracellular stores are probably the source of Ca^{2+} in response to ATP. The Ca^{2+} -ATPase inhibitor, thapsigargin, was applied before ATP to deplete intracellular Ca^{2+} . Application of ATP after depletion of intracellular Ca^{2+} stores failed to elicit an increase in Ca^{2+} ($n = 15$), further implicating P2Y receptors in the ATP response (Fig. 4b).

Effects of other di- and tri-nucleotides on Ca^{2+} in cumulus cells

The effects of other tri- and di-nucleotides were examined to characterize further the P2Y receptor present on cumulus cells. UTP stimulated Ca^{2+} release in cumulus cells with approximately equal potency to ATP. Dose-response curves for ATP and UTP are shown (Fig. 5). No response was observed at 10 $\mu\text{mol l}^{-1}$, whereas a small increase was observed at 20 $\mu\text{mol l}^{-1}$, which increased from 100 $\mu\text{mol l}^{-1}$ to 500 $\mu\text{mol l}^{-1}$. This finding indicates that the receptor stimulated by these agents is equally sensitive to both agonists and, therefore, would be placed tentatively in the P2Y2 category (Burnstock and King, 1996).

No change in Ca^{2+} concentration was detected in

response to 1–100 $\mu\text{mol l}^{-1}$ (data not shown); however, higher concentrations (between 500 $\mu\text{mol l}^{-1}$ and 1 mmol l^{-1}) caused a small Ca^{2+} increase in the cumulus cells that appeared to be followed by an increase in Ca^{2+} concentration in the oocytes ($n = 9$) (Fig. 6a). In one experiment ($n = 3$), a further addition of 500 $\mu\text{mol l}^{-1}$ ADP caused an additional Ca^{2+} increase in the oocytes but not in the cumulus cells (Fig. 6a). This finding indicates that the ADP-induced Ca^{2+} increase in the oocytes is independent of the Ca^{2+} change in the cumulus cells, consistent with previous data for ATP and UTP. Further support for this is provided by the observation that ADP also increases Ca^{2+} concentration in oocytes denuded completely of cumulus cells ($n = 3$) (Fig. 6b). These experiments indicate that both cumulus cells and oocytes may have an ADP-sensitive receptor. One feature of the ADP response is that in all the COCs tested ($n = 5$), the oocyte remained capable of responding to an additional application of ADP, whereas cumulus cells did not (Fig. 6a,b).

Effect of ATP on Ca^{2+} in the oocyte of intact cumulus–oocyte complexes

As described above, ATP-induced Ca^{2+} release in partially denuded COCs did not lead to any increases in intracellular Ca^{2+} concentration in the attached oocytes. This finding indicates that Ca^{2+} and InsP_3 are unable to traverse the processes and gap junctions between the cumulus cells and oocyte. To determine whether this is also true in intact COCs, fluo3 was microinjected into the oocyte and the cumulus cells were loaded with fluo3 AM; Ca^{2+} changes in the oocyte and cumulus cells were monitored using confocal microscopy. In intact COCs, ATP caused an increase in Ca^{2+} in the cumulus cells, which was followed 46.8 ± 8.1 s ($n = 17$) later by an increase in Ca^{2+} in the oocyte (Fig. 7a,b). This finding indicates that increases in Ca^{2+} in the cumulus cells lead to increases in Ca^{2+} in the oocyte provided that the cumulus cell layer is intact.

The increase in Ca^{2+} concentration in the oocyte may be brought about by two mechanisms. Firstly, the Ca^{2+} or InsP_3 generated in the cumulus cells may diffuse through gap junctions and, secondly, ATP may induce the release of a messenger from cumulus cells that stimulates a Ca^{2+} -mobilizing receptor on the oocyte plasma membrane. The gap junction inhibitor, carbenoxolone, was applied (500 $\mu\text{mol l}^{-1}$ for 20 min) before application of ATP to examine the role of gap junctions. Preincubation with carbenoxolone completely abolished the transfer of FITC–dextran from the oocyte to the cumulus cells, indicating that gap-junctional coupling was probably inhibited ($n = 5$) (Fig. 8a,b). In carbenoxolone-treated COCs, ATP-induced Ca^{2+} transients did not propagate from the cumulus cells to the oocyte ($n = 6$) (Fig. 9a,b). In two experiments, the ability of the oocyte to generate a Ca^{2+} increase was verified by application of carbachol (not shown). Thus, increases in Ca^{2+} in the cumulus cells can be transmitted to the oocyte via gap-junctional coupling.

Fig. 6. Mouse oocytes and cumulus cells have an ADP receptor that stimulates Ca^{2+} release. (a) ADP was added to the bath as indicated by the arrows. A small increase in Ca^{2+} is visible in the cumulus cells (solid lines) and a larger change is apparent in the oocytes (dashed lines). Only the oocytes responded at the second addition of ADP. (b) Addition of ADP to a denuded oocyte. The denuded oocyte responded in a similar way to oocytes with some cumulus cells attached. Arrows indicate times of addition of 500 $\mu\text{mol l}^{-1}$.

Effect of LH on Ca^{2+} in granulosa cells

The ability of LH to increase Ca^{2+} in the somatic compartment of the follicle would provide support for a role for Ca^{2+} in meiotic resumption *in vivo*. However, to date, the physiological agonists FSH and EGF have not been found to influence Ca^{2+} in cumulus cells. Granulosa cells were recovered from individual follicles from PMSC-primed ovaries to determine whether LH may stimulate a Ca^{2+} increase in mouse follicular cells. The application of LH (1–10 $\mu\text{g ml}^{-1}$) did not cause an increase in Ca^{2+} in granulosa cells in three separate experiments. ATP was applied to confirm that the granulosa cells were responding to agonists. Similar to its effects on cumulus cells, ATP caused an increase in Ca^{2+} concentration in granulosa cells (Fig. 10).

Fig. 7. ATP-induced Ca^{2+} waves travelled from mouse cumulus cells to the oocyte. (a) Fluorescence changes in different cumulus cells (blue traces) and the oocyte (red trace) in a representative mouse cumulus–oocyte complex. Note that the Ca^{2+} increase in the cumulus cells is followed by an increase in the oocyte. (b) A series of confocal images showing the cumulus cells and the oocyte. Images were acquired at the times indicated.

Discussion

The cell signalling pathway underlying hormone-induced meiotic maturation is essentially a problem of cell–cell signalling. How does the oocyte detect the binding of LH to receptors on the theca and granulosa cells? In the present study, conventional and confocal imaging techniques were used to monitor Ca^{2+} concentration in oocytes, cumulus cells and granulosa cells. It was found that ATP and UTP, but not FSH or EGF, cause Ca^{2+} transients in mouse follicular cells, that ATP-induced Ca^{2+} changes in cumulus cells can be passed to the oocyte via gap junctions and, finally, that LH has no effect on Ca^{2+} concentration in granulosa cells. These data provide evidence against a role for phosphoinositide turnover and Ca^{2+} transients in hormone-induced maturation in mice.

Experiments in the present study were designed to identify agonists of Ca^{2+} release in cumulus cells with the aim of determining whether changes in Ca^{2+} in cumulus cells could diffuse to the oocyte. Despite the fact that there are many agents that release Ca^{2+} in populations of ovarian cells from a number of species (Flores *et al.*, 1990, 1992; Currie *et al.*, 1992), the results of the present study indicate that mouse cumulus cells respond to few agonists of Ca^{2+} release. Of the agonists tested, only ATP and UTP consistently resulted in an increase in Ca^{2+} in cumulus cells. The P2 receptor subtype is apparently the G-protein-coupled P2Y family, as the ability of ATP to increase Ca^{2+} concentration was not dependent on extracellular Ca^{2+} and required intracellular Ca^{2+} stores. Furthermore, the similar sensitivity of the response to both ATP and UTP indicates that the receptor may be a P2Y2 receptor (Fredholm *et al.*,

1994; Burnstock and King, 1996). In addition to the putative P2Y2 receptor, there is also evidence from the present study for an ADP receptor on both the cumulus cells and the oocyte. In most tissues, ecto-ATPases are responsible for metabolizing ATP, producing ADP in the process (Dubyak and El-Moatassim, 1993). This process provides a source of extracellular ATP, perhaps from hypoxic (Forrester, 1990) or dying cumulus cells, to initiate Ca^{2+} transients in the oocyte. ATP increases Ca^{2+} concentration in chicken, pig and human granulosa cells (chicken: Morley *et al.*, 1994; human: Lee *et al.*, 1996; Squires *et al.*, 1997; pig: Aguirre *et al.*, 2000), which indicates that P2 receptor-mediated regulation of follicular function is widely conserved. P2 signalling pathways regulate many physiological processes (Gordon, 1986; Dubyak and El-Moatassim, 1993), but their role in ovarian function is not known. One possibility is that ATP from sympathetic nerve terminals or dying cells (Stefenson *et al.*, 1981; Gordon, 1986) leads to the release of Ca^{2+} in the somatic compartment of the follicle and that this Ca^{2+} modulates the sensitivity of follicular cells to steroid and peptide hormones (Sadighian *et al.*, 1989; Bodis *et al.*, 1993; Davis, 1994). Thus, ATP may act as a paracrine mediator of follicular development. Recent evidence showing that ATP activates MAP kinase in human granulosa-lutein cells supports this idea (Tai *et al.*, 2001).

The finding of an agonist, ATP, that stimulates Ca^{2+} increases specifically in the cumulus cells provides the opportunity to determine whether a Ca^{2+} increase in the somatic compartment of the follicle is followed by a Ca^{2+} transient in the oocyte. Our data using confocal microscopy demonstrate clearly that ATP-induced Ca^{2+} increases in cumulus cells are followed by Ca^{2+} increases in the oocyte. Early experiments using conventional imaging of partially denuded COCs indicated that no cell-cell communication was taking place. The difference between intact and partially denuded COCs indicates that a full complement of gap junctions is required for sufficient diffusion of the messengers, or that the partial denuding process, perhaps by stimulating an increase in intracellular Ca^{2+} (Arellano *et al.*, 1990), inhibits gap-junctional communication in the remaining cells. Whatever the mechanism, these findings demonstrate that, during *in vitro* maturation, an intact COC is required for normal gap-junctional communication between the oocyte and cumulus cells.

The increase in Ca^{2+} concentration in oocytes after stimulation of intact COCs with ATP appeared to be stimulated via diffusion through gap junctions. Gap junction inhibitors that prevent dye transfer between oocyte and cumulus cells also abolished the ability of ATP-induced Ca^{2+} transients in the cumulus cells to reach the oocyte. The messenger diffusing through gap junctions may be InsP_3 or Ca^{2+} . In the case of the COC, where the gap junction forms at the end of a narrow process that winds through the zona pellucida (Anderson and Albertini, 1976), the distance required for diffusion is at least 7 μm . As Ca^{2+} is heavily buffered by cytosol (Allbritton *et al.*, 1992), it is likely that InsP_3 is the diffusible messenger. Irrespective of which

Fig. 8. Carbenoxolone inhibits dye transfer between mouse oocyte and cumulus cells. (a) Fluorescence in arbitrary units in the cumulus cells in control (black) and carbenoxolone-treated (red) mouse cumulus-oocyte complexes. (b) Representative images at the start and end of the 30 min recording. Oocytes were injected with fluorescein and fluorescence was recorded in the cumulus cells and oocyte for 30 min in the presence or absence of carbenoxolone. Note that the fluorescence did not diffuse into associated cumulus cells. The decrease in fluorescence in the oocyte is a result of photobleaching of the fluorescein.

messenger molecule is diffusing between cumulus cells and oocytes, these studies demonstrate that if the hormone responsible for meiotic maturation leads to an increase in Ca^{2+} in the somatic compartment of the follicle, it may be transmitted to the oocyte.

Hormone-induced *in vitro* maturation is stimulated by FSH and EGF (Downs *et al.*, 1988; Eppig, 1991), and the signal responsible for stimulation of maturation apparently requires intact gap-junctional communication (Fagbohun and Downs, 1991). Thus, Ca^{2+} is a good candidate as the mediator of hormone-induced meiotic maturation.

Fig. 9. Inhibition of gap-junctional communication inhibited ATP-induced Ca^{2+} transients in mouse oocytes. (a) Trace of fluo3 fluorescence from cumulus cells (blue) and oocyte (red) and (b) images of the same cell at selected times during recording. Data are from a representative mouse cumulus–oocyte complex preincubated for 20 min in carbenoxolone.

Fig. 10. LH did not stimulate Ca^{2+} release in freshly isolated mouse granulosa cells. Addition of LH ($10 \mu\text{g ml}^{-1}$) at the times indicated (arrows) did not modify intracellular Ca^{2+} concentration. Subsequent addition of 1 mmol l^{-1} ATP showed that granulosa cells also respond to ATP.

However, in this system there was no evidence to implicate Ca^{2+} in FSH- or EGF-induced maturation. The finding that

neither agonist promoted an increase in Ca^{2+} indicates that their mode of action is via the stimulation of other signal transduction pathways, probably involving cAMP and tyrosine kinases, respectively. The lack of evidence for Ca^{2+} in hormone-induced maturation is in contrast to a recent study in which inhibition of phosphoinositide signalling and Ca^{2+} transients using lithium chloride, neomycin and BAPTA all inhibited hormone-induced but not spontaneous maturation (Cottichio and Fleming, 1988). As there was no evidence for an increase in Ca^{2+} in response to FSH and EGF in the present study, it remains to be shown that the inhibitors used to block phosphoinositide metabolism and Ca^{2+} transients were operating in the intended manner. In contrast to Ca^{2+} , there is compelling evidence for a role for cAMP in mediating FSH-induced maturation. Firstly, it is well established that FSH stimulates cAMP production in granulosa cells (Richards, 1980) and, secondly, analogues of cAMP can mimic the ability of FSH to induce meiotic

maturation *in vitro* (for reviews, see Eppig, 1993; Downs, 1995). Thus, the stimulus (FSH) increases cAMP and an increase in cAMP is sufficient to stimulate maturation. A lack of a specific method for inhibiting the generation of cAMP means that further studies must be undertaken to determine whether it is a necessity for hormone-induced maturation. On the basis of these data, an increase in cAMP, rather than Ca^{2+} , remains the best candidate for mediating hormone-induced maturation *in vitro* in mice.

In vivo maturation is stimulated by the action of LH on the granulosa cells. It is not clear how LH leads to the stimulation of maturation. The studies described above do not discount a role for Ca^{2+} in the induction of maturation in intact follicles. If LH stimulates an increase in Ca^{2+} in the granulosa cells, it remains possible that this Ca^{2+} may reach the oocyte via gap-junctional communication (Eppig, 1993; Homa *et al.*, 1993). This hypothesis was tested in the present study but it was found that LH had no effect on Ca^{2+} in granulosa cells from mouse follicles. Thus, there is little evidence to support a role for Ca^{2+} in stimulating maturation in intact follicles in mice. Species differences are apparent. LH has been shown to increase InsP_3 production in granulosa cells (Davis *et al.*, 1986; Dimino *et al.*, 1987), although there is a delay of several minutes between LH application and detection of InsP_3 . The differentiation status of granulosa cells in culture may explain some of these differences. The most recent data indicating that Ca^{2+} transients may be involved in hormone-induced maturation is the finding that in sheep COCs, LH triggers a Ca^{2+} increase that is observed in the oocyte within 5 min. Like ATP-induced Ca^{2+} changes in mice, the Ca^{2+} transient in the sheep oocyte is apparently dependent on intact gap junctions.

In conclusion, the results of the present study demonstrate that Ca^{2+} waves generated in the somatic compartment of mouse follicles can travel between heterologous gap junctions and stimulate Ca^{2+} transients in the oocyte. This finding demonstrates that Ca^{2+} may be an important mediator of cell-cell communication between the oocyte and the somatic cells of the ovary. However, the inability of FSH, EGF and LH to stimulate Ca^{2+} release in mouse follicular cells indicates that Ca^{2+} is probably not central to the mechanism of hormone-induced oocyte maturation in mice.

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