Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the Gs G protein

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The arrest of meiotic prophase in mouse oocytes within antral follicles requires the G protein Gs and an orphan member of the G protein–coupled receptor family, GPR3. To determine whether GPR3 activates Gs, the localization of Gs in follicle-enclosed oocytes from Gpr3+/+ and Gpr3−/− mice was compared by using immunofluorescence and GαsGFP. GPR3 decreased the ratio of Gαs in the oocyte plasma membrane versus the cytoplasm and also decreased the amount of Gαs in the oocyte. Both of these properties indicate that GPR3 activates Gs. The follicle cells around the oocyte are also necessary to keep the oocyte in prophase, suggesting that they might activate GPR3. However, GPR3-dependent Gs activity was similar in follicle-enclosed and follicle-free oocytes. Thus, the maintenance of prophase arrest depends on the constitutive activity of GPR3 in the oocyte, and the follicle cell signal acts by a means other than increasing GPR3 activity.

Introduction

Meiosis begins early in oogenesis but does not proceed beyond prophase until luteinizing hormone from the pituitary acts on the somatic cells of the follicle surrounding the oocyte (Eppig et al., 2004). For the prolonged period of storage before oocyte growth and during the growth period, meiotic arrest is maintained by inherent factors in the oocyte (Eppig et al., 2004). However, once the oocyte has grown to its full size and an antral space has begun to form between the somatic cells of the follicle, a different mechanism for maintaining meiotic arrest comes into play. After this point, the oocyte will resume meiosis spontaneously if isolated from the follicle, leading to the conclusion that maintenance of meiotic arrest requires an inhibitory signal from the follicle cells (Pincus and Enzmann, 1935; Erickson and Sorensen, 1974).

The inhibitory signal has been proposed to be cAMP that enters the oocyte from the somatic cells (Anderson and Albertini, 1976; Piontkewitz and Dekel, 1993; Webb et al., 2002; Eppig et al., 2004). However, this long-standing hypothesis has recently been challenged by evidence that the regulation of meiotic arrest requires a G protein, a receptor, and adenylyl cyclase in the oocyte (Mehlmann et al., 2002, 2004; Horner et al., 2003; Kalinowski et al., 2004; Ledent et al., 2005). This has led to the concept that follicle cells control the oocyte cell cycle by regulating rates of cAMP production or degradation within the oocyte, but the communication pathway between follicle cells and the oocyte has not been determined.

The requirement for elevated cAMP in the oocyte to maintain prophase arrest was established by several findings (Conti et al., 2002; Eppig et al., 2004; Mehlmann, 2005). (1) After removal from the antral follicle, the oocyte’s cAMP content decreases in parallel with meiotic resumption (Tornell et al., 1990). (2) Isolated oocytes can be maintained in prophase arrest by elevating cAMP (for example, with membrane permeant cAMP analogues or cAMP phosphodiesterase [PDE] inhibitors; Cho et al., 1974; Schultz et al., 1983; Eppig et al., 1985). (3) Oocytes within complexes of follicular somatic cells, which maintain meiotic arrest in vitro, resume meiosis in response to a permeant cAMP antagonist (Eppig, 1991). Elevated cAMP maintains meiotic arrest by activating protein kinase A, which phosphorylates and, thus, inhibits the phos-
phatase CDC25B; this prevents CDC25B from dephosphorylating and activating CDK1 and, therefore, prevents the prophase-to-metaphase transition (Duckworth et al., 2002; Lincoln et al., 2002).

The cAMP that maintains meiotic arrest is produced in the oocyte by a pathway requiring the activity of the heterotrimeric G protein $G_s$ (Mehlmann et al., 2002; Kalinowski et al., 2004) and type 3 adenyl cyclase (Horner et al., 2003). Supporting these conclusions, the injection of follicle-enclosed oocytes with a $G_s$ inhibitory antibody or a dominant-negative form of $G_s$ causes meiosis to resume (Mehlmann et al., 2002; Kalinowski et al., 2004), as does the deletion of the gene for type 3 adenyl cyclase, which is expressed in the oocyte (Horner et al., 2003).

In addition to $G_s$, the maintenance of meiotic prophase arrest requires an orphan member of the G protein–coupled receptor family, GPR3 (Mehlmann et al., 2004; Ledent et al., 2005). GPR3 was identified in a mouse oocyte cDNA library by a search for seven-transmembrane proteins (Mehlmann et al., 2004) and was of particular interest because the overexpression of GPR3 in various cell lines had been shown to elevate cAMP (Eggerickx et al., 1995; Uhlenbrock et al., 2002; Bresnick et al., 2003). Gpr3 RNA is highly localized in the oocyte versus the surrounding somatic cells, and in Gpr3 knockout mice, the majority of oocytes in antral follicles resume meiosis spontaneously (Mehlmann et al., 2004; Ledent et al., 2005). Oocytes in Gpr3$^{-/-}$ preantral follicles remain arrested in prophase, but as antral spaces begin to form between the somatic cells (early antral stage), the oocytes resume meiosis independently of an increase in luteinizing hormone. As a consequence of this disruption of the normal coordination of meiotic progression and fertilization, Gpr3 knockout females are subfertile (Ledent et al., 2005). The spontaneous progression of meiosis that occurs in Gpr3 knockout oocytes can be reversed by an injection of Gpr3 RNA into preantral follicle-enclosed oocytes followed by culture to the early antral/antral stage (Mehlmann et al., 2004). It is thought that GPR3 in the oocyte maintains meiotic arrest by activating $G_s$; however, this has not been directly tested.

We investigated this hypothesis by taking advantage of two properties of $G_s$ that have been established in somatic cells: the activation of $G_s$ causes its $\alpha$ subunit to move from the plasma membrane to the cytoplasm (Rasenick et al., 1984; Ransnas et al., 1989; Levis and Bourne, 1992; Wedegaertner et al., 1996; Iiri et al., 1997; Thiyyagarajan et al., 2002; Yu and Ransnick, 2002; Hynes et al., 2004; Allen et al., 2005) and also to be degraded at an increased rate (see Discussion). We compared the localization of $G_s$, in follicle-enclosed oocytes from Gpr3$^{+/+}$ and Gpr3$^{-/-}$ mice by using immunofluorescence and Gao$\alpha$GFP and found that GPR3 causes the ratio of $G_s$ in the oocyte plasma membrane versus the cytoplasm to decrease. GPR3 also causes the total amount of $G_s$ protein in the oocyte to decrease. Both of these properties indicate that GPR3 activates $G_s$ in the oocyte. We then used the $G_s$ distribution to determine whether the presence of follicle cells is necessary to keep GPR3 active in the oocyte.

**Results**

The localization of $G_s$ in Gpr3 knockout and wild-type oocytes indicates that GPR3 activates $G_s$ in the oocyte

To investigate whether GPR3 activates $G_s$ in the mouse oocyte, we compared the plasma membrane-to-cyttoplasm distribution of $G_s$ in Gpr3$^{+/+}$ and Gpr3$^{-/-}$ oocytes. To avoid the problem that Gpr3$^{-/-}$ oocytes within early antral and antral follicles spontaneously resume meiosis, which would complicate a direct comparison with Gpr3$^{+/+}$ oocytes, we performed these experiments using preantral follicles (120–190-μm diam). These preantral follicles contained oocytes with ~70-μm diam that were not yet competent to resume meiosis when isolated (Erickson and Sorensen, 1974). At this stage, GPR3 is not required to maintain meiotic arrest (Mehlmann et al., 2004). However, we predicted that to ensure a continuously effective block to the progression of meiosis, the GPR3–$G_s$ regulatory system would need to be in place before the oocyte acquires the inherent factors that allow meiotic resumption, and results that are described below support this prediction.

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**Figure 1. Immunofluorescence localization of $G_s$ in a mouse ovary.** (A) Immunoblot demonstrating $G_s$ antibody specificity. 1 μg of ovari lystate [Gpr3$^{+/+}$] was loaded on the gel; both the 52- and 45KD splice variants of $G_s$ (Robishaw et al., 1986) were seen. (B) A section of a Gpr3$^{+/+}$ ovary labeled with an antibody against $G_s$. This ovary, which was obtained from a mouse that had not been injected with eCG to stimulate antral follicle formation, contained mostly preantral follicles and some early antral follicles. Follicles with 120–190-μm diam were classified as preantral, whereas those with ≥200-μm diam were classified as early antral (with multiple small antral spaces) or antral (with a single large antral space; Fig. 3 A). These categories were not completely distinct because some follicles with <200-μm diam showed a formation of antral spaces. (C) A control section labeled with nonimmune IgG. Confocal microscope settings and bars were the same for B and C.
By using an antibody against the COOH-terminal decapeptide of Goα (Simonds et al., 1989), which specifically recognized Goα, in mouse ovary (Fig. 1 A), we examined the distribution of Goα immunofluorescence in preantral follicle-enclosed oocytes in frozen sections of ovaries (Fig. 1, B and C) from Gpr3+/+ and Gpr3−/− mice. These sections showed that Goα was present at a high level in theca/interstitial cells that surround each follicle and in the oocyte. Goα was also present at lower levels in the follicular somatic cells.

In Gpr3+/+ oocytes, Goα fluorescence was present in both the plasma membrane and in the cytoplasm; within the cytoplasm, irregularly shaped bright spots up to several micrometers in diameter as well as diffuse fluorescence were visible in most sections (Fig. 2, A and C). In Gpr3−/− oocytes, Goα was more strongly localized in the plasma membrane (Fig. 2, B and C).

Intensity measurements (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1) showed that in Gpr3+/+ oocytes, the Goα fluorescence was higher in the plasma membrane and lower in the cytoplasm than in Gpr3−/− oocytes (Table I). For each oocyte, we determined the plasma membrane-to-cytoplasm fluorescence intensity ratio and found that the mean ratio was significantly smaller for Gpr3+/+ oocytes than for Gpr3−/− oocytes (Fig. 2 D). These results show that GPR3 causes Goα to move from the oocyte plasma membrane to the cytoplasm; the simplest interpretation of this observation is that GPR3 activates Goα.

The localization of Goα in oocytes from preantral and antral follicles indicates that GPR3 in the oocyte is already fully active at the preantral stage

To test whether the Goα distribution in oocytes in Gpr3+/+ preantral follicles was similar to that in oocytes in Gpr3+/+ early antral and antral follicles, we compared the oocyte plasma membrane-to-cytoplasm Goα fluorescence ratio for follicles in the 120–190-μm diam range (preantral) with that for follicles in the 200–475-μm diam range (early antral and antral; Fig. 3, A–D). The ratios were the same for oocytes within follicles of the two size ranges, indicating that the level of Goα activation in preantral follicle-enclosed oocytes is the same as in early antral and antral follicle-enclosed oocytes. These results indicated that the GPR3 in the oocyte is already fully active at the preantral stage. Consistent with this conclusion, oocytes from preantral versus early antral and antral follicles contained almost the same amounts of Gpr3 RNA, with slightly more Gpr3 RNA in the oocytes from preantral follicles (Fig. 3 E).

Additional evidence that GPR3 activates Goα in the oocyte

As another test of whether GPR3 activates Goα in the oocyte, we compared the amount of Goα protein in Gpr3+/+ with Gpr3−/− oocytes because the activation of Goα increases the rate of degradation of its α subunit (see Discussion). Oocytes were dissected from preantral follicles, and the amount of Goα protein in each sample was then analyzed by immunoblotting (Fig. 2 E). The amount of Goα was found to be 2.2–3.6 times greater in the...
Gpr3−/− oocytes versus the Gpr3+/+ oocytes (range for two independent experiments, each with two to three sets of 15 oocytes of each genotype).

Consistent with the conclusion that GPR3 activates Gs in the oocyte, the injection of Gpr3 RNA increased the amount of cAMP in isolated Gpr3+/+ oocytes (≈1.7 times under the conditions of these measurements; supplemental Materials and methods, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1). However, the process of oocyte isolation, which is necessary for such assays, changes the amount of cAMP in the oocyte (Tornell et al., 1990); this limits the usefulness of biochemical measurements of cAMP to detect Gs activation by GPR3 in this system. Instead, we developed an optical method to monitor GPR3 activation in live oocytes.

Gaα,GFP as a monitor of GPR3 activation in live oocytes

To confirm our immunofluorescence results and to establish a method for investigating GPR3 activation in live oocytes, we examined the GPR3 dependence of Gaα distribution by using a GFP fusion protein. To validate the use of Gaα,GFP as a monitor of receptor activation, we showed that in oocytes expressing an exogenous β-adrenergic receptor and exposed to isoproterenol, Gaα,GFP moved from the plasma membrane to the cytoplasm, as previously described for somatic cells (supplemental Results and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1).

Preantral follicle-enclosed oocytes were injected with RNA encoding the α subunit of Gaα fused with GFP (Gaα,GFP; Hynes et al., 2004), were cultured overnight to allow protein expression, and were imaged with a confocal microscope while the oocytes were still within their follicles (Fig. 4 A). Under the conditions of these experiments, Gaα,GFP was expressed at a level only slightly greater than that of endogenous Gaα (Fig. 4 B). This level of Gaα,GFP expression did not disrupt the normal physiology of the oocyte, as indicated by the resumption of meiosis in Gaα,GFP-expressing oocytes that were isolated from antral follicles (supplemental Results).

In Gpr3+/+ oocytes, Gaα,GFP was present in both the plasma membrane and the cytoplasm; the cytoplasmic fluorescence included both diffuse and particulate components (Fig. 4 C). In contrast, in Gpr3−/− oocytes, Gaα,GFP was highly localized in the plasma membrane (Fig. 4 D). This difference in Gaα,GFP fluorescence distribution in the oocyte could not be attributed to an optical difference in the surrounding follicle cells because the follicles used were similar in size and optical density for the two genotypes. Quantitation of Gaα,GFP fluorescence (Fig. S1) showed that the plasma membrane-to-cytoplasm fluorescence ratio was smaller for Gpr3+/+ oocytes than for Gpr3−/− oocytes (Fig. 4 E), as was also observed with Gaα immunofluorescence (Fig. 2 D). Although the ratios that were obtained by the two methods were close, they were not identical; factors such as fixation or overexpression artifacts could possibly account for this lack of exact correspondence.

To determine whether GPR3 in the oocyte could account for the difference in Gaα,GFP localization in oocytes from Gpr3−/− and Gpr3+/+ mice, we injected Gpr3−/− oocytes within preantral follicles with a mixture of Gpr3 RNA and Gaα,GFP RNA, cultured the follicles overnight, and observed them with confocal microscopy. If GPR3 in the oocyte is responsible for causing the Gaα,GFP plasma membrane-to-cytoplasm ratio to decrease, we would expect that the injection of Gpr3−/− oocytes with Gpr3 RNA would decrease the relative amount of Gaα,GFP in the plasma membrane versus the cytoplasm.

In Gpr3−/− oocytes that had been injected with 7 pg of a control RNA (β-globin), Gaα,GFP was highly localized in the plasma membrane (Fig. 5, A and D), as in Gpr3−/− oocytes that were injected with Gaα,GFP RNA alone (Fig. 4, D and E). In contrast, in Gpr3−/− oocytes that had been injected with 7 pg of Gpr3 RNA, Gaα,GFP fluorescence was present in the cytoplasm.
and was not localized in the plasma membrane (Fig. 5 B). As the amount of Gpr3 RNA was reduced while keeping the amount of Gαs-GFP RNA constant, the fraction of Gαs-GFP in the plasma membrane increased. The injection of 7 fg Gpr3 RNA resulted in a plasma membrane-to-cytoplasm fluorescence ratio that was comparable with that in Gpr3+/+ oocytes (compare Fig. 5, C and D with Fig. 4, C and E). Similar results were obtained by using a GPR3-RFP construct, which allowed us to monitor the level of GPR3 expression as a function of the amount of RNA injected and to show that the amount of GPR3 protein correlates with the amount of Gαs-GFP internalization (supplemental Results and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1). Because an increase in the relative amount of Gαs in the cytoplasm versus the plasma membrane is an indicator of Gs activation, these findings confirm that within the oocyte, GPR3 activates Gs.

**GPR3 constitutively activates Gs in the oocyte independently of follicle cells**

One way in which the somatic cells of antral and early antral follicles could maintain prophase arrest in the oocyte is by producing a ligand that increases the activity of GPR3. Alternatively, the follicle cells could inhibit meiotic resumption by another pathway such as activating another Gs-linked receptor.
in the oocyte, inhibiting the breakdown of cAMP by a PDE in the oocyte, or supplying cAMP to the oocyte by way of gap junctions (see Discussion). To test the first of these possibilities, we examined whether the GPR3 stimulation of Go,GFP requires the presence of follicle cells.

Because the immunofluorescence results described in Fig. 3 indicated that the GPR3–Gs complex is equally active in oocytes in preantral and in antral and early antral stage follicles, we performed these experiments using oocytes from preantral follicles, which have the important technical advantage of not resuming meiosis when isolated. This allowed us to measure the Gα,GFP distribution that was established after the injection of Gα,GFP RNA into isolated oocytes.

Oocytes were isolated from Gpr3+/+ and Gpr3−/− preantral follicles, injected with Gα,GFP RNA, cultured overnight, and imaged by confocal microscopy. The difference in Gα,GFP localization between Gpr3+/+ and Gpr3−/− oocytes was still evident, indicating that GPR3 activates Gs, even in the absence of follicle cells (Fig. 6).

A comparison of plasma membrane-to-cytoplasm fluorescence ratios from follicle-enclosed and isolated oocytes is complicated by optical factors (see next paragraph). However, by dividing the plasma membrane-to-cytoplasm fluorescence ratio for Gpr3−/− oocytes by the plasma membrane-to-cytoplasm fluorescence ratio for Gpr3+/+ oocytes, we obtained a measure of GPR3-dependent Gs activation that could be directly compared for follicle-enclosed and isolated oocytes (Table II, right column). This analysis indicated that the follicle cells do not increase the activation of Gs by GPR3.

The measurements in Table II also showed that the absolute values for plasma membrane-to-cytoplasm ratios of Gα,GFP fluorescence were greater in the isolated oocytes versus the follicle-enclosed oocytes regardless of the presence of GPR3. We suspected that this difference resulted, at least in part, because confocal imaging of an isolated oocyte involves light traveling through ~35 μm of optically clear medium to reach the plasma membrane versus ~35 μm of optically dense cytoplasm to reach the cytoplasmic imaging plane in the center of the oocyte. As a consequence, the plasma membrane of isolated oocytes appears artificially brighter (Fig. 7 A). A follicle-enclosed oocyte is free of this artifact because the paths to the cytoplasm and membrane both involve light travelling through cytoplasm (either follicle cell cytoplasm or oocyte cytoplasm; Fig. 7 A).

To determine the magnitude of this optical effect, we injected preantral follicle-enclosed oocytes with RNA-encoding YFP attached to the palmitoylation domain of neuromodulin (YFP-Mem) in order to introduce a fluorescent marker of the plasma membrane that should be independent of signals from the follicle cells. We also injected these oocytes with YFP protein, which stays in the cytoplasm, in an amount that resulted in a fluorescence ratio for the plasma membrane (YFP-Mem) to cytoplasm (YFP) that was similar to that of oocytes expressing Go,GFP. We then imaged their fluorescence before and after removal from the follicle (Fig. 7, B and C). These measurements indicated that to directly compare Go,GFP ratios in isolated oocytes with those in follicle-enclosed oocytes, we need to use a correction factor of 2.2 times (Fig. 7). This value fits well with another measurement of the absorbance of light by the mouse oocyte cytoplasm (Terasaki, 2005).

After correction for this optical factor, the plasma membrane-to-cytoplasm Go,GFP fluorescence ratios for follicle-enclosed and isolated oocytes were much closer (Table II, second and third columns). For the Gpr3−/− oocytes, a small but statistically significant difference remained, which could indicate some effect of the follicle cells on Gs activity. However, with regard to the Gpr3-dependent activation of Gs (Table II, third column), the presence of follicle cells had no detectable stimulatory effect. Therefore, although GPR3 and Gs are required

| Specimen                      | PM/CYTO (Gpr3+/+) | PM/CYTO (Gpr3−/+ | PM/CYTO (Gpr3−/−) |
|-------------------------------|------------------|------------------|------------------|
| Follicle-enclosed oocytes     | 3.1 ± 0.2        | 7.1 ± 0.4        | 2.3 ± 0.2        |
| Isolated oocytes             | 8.8 ± 0.8        | 21.8 ± 1.2       | 2.5 ± 0.3        |

Second and third columns indicate the ratios of plasma membrane Go,GFP fluorescence (PM) to cytoplasm Go,GFP fluorescence (CYTO) in the oocyte. Mean ± SEM (n = number of oocytes). Data for follicle-enclosed oocytes are from three mice of each genotype, and data for isolated oocytes are from two mice of each genotype. For each row, the last column indicates the value divided by the value listed in the second column. See the last section of Results and Fig. 7 for an explanation of the optical correction.

*These two values are not significantly different (t test, P = 0.08).
**These two values are significantly different (t test, P = 0.01).
†These two values are not significantly different (t test, P = 0.6).
‡Corresponds to Fig. 4 E.
§Corresponds to Fig. 6 C.
to maintain meiotic arrest in a follicle-enclosed oocyte (Mehlmann et al., 2002, 2004; Kalinowski et al., 2004; Ledent et al., 2005), the signal from follicle cells that maintains meiotic arrest acts by a mechanism other than providing a ligand that activates GPR3.

**Discussion**

Optical methods have provided important insights about the function of G proteins and their receptors in intact cells; in this study, we applied such methods to study receptor–G protein interactions in a cell (the oocyte) within an intact tissue (the ovarian follicle). Our results showed that GPR3, which is a member of the G protein–coupled receptor family that is required to maintain meiotic arrest, constitutively activates Gs in the oocyte. The activation of Gs, causes its α subunit to dissociate from the plasma membrane, thus providing a way to examine the activation of Gi in single cells within tissues. Translocation of the activated α subunit of Gi into the cytoplasm has been demonstrated in somatic cells by fractionation of soluble and particulate components (Rasnias et al., 1984; Ransnas et al., 1989; Levis and Bourne, 1992; Wedegaertner et al., 1996; Jones et al., 1997; Huang et al., 1999; Thiyagarajan et al., 2002; Yu and Rasenick, 2002), by immunofluorescence microscopy (Wedegaertner et al., 1996; Iiri et al., 1997; Thiyagarajan et al., 2002), and, most recently, by GFP fusion protein microscopy (Yu and Rasenick, 2002; Hynes et al., 2004; Allen et al., 2005).

In particular, these microscopic methods have shown a consistent correlation between Gi activation and translocation of its α subunit from the plasma membrane into the cytoplasm whether Gs is activated by a receptor, by cholera toxin, or by point mutation. In addition, Goi, is not internalized in response to receptor stimulation if the α subunit is point mutated such that its activation is impaired (Iiri et al., 1997). We validated the use of Goi localization as an indicator of its activation in oocytes by showing that Goi,GFP moves from the oocyte plasma membrane to the cytoplasm when an exogenously expressed β-adrenergic receptor is stimulated with isoproterenol.

Incorporation of GFP into an internal sequence of Goi, does not prevent receptor-mediated activation of GTP-binding and adenylyl cyclase (Yu and Rasenick, 2002; Hynes et al., 2004). The Goi,GFP fusion protein that we used, which includes a linker sequence on either side of the inserted GFP, is almost identical to Goi, in terms of the receptor-mediated activation of adenylyl cyclase (Hynes et al., 2004). We used Goi,GFP as well as immunofluorescence to visualize the Goi, distribution within follicle-enclosed oocytes. Both localization methods showed that the ratio of Goi, in the plasma membrane versus cytoplasm is smaller in Gpr3+/+ oocytes than in Gpr3−/− oocytes, indicating that GPR3 activates Goi. The injection of Gpr3 RNA into Gpr3−/− oocytes restored the Goi,GFP distribution that is characteristic of Gpr3+/+ oocytes, showing that GPR3 in the oocyte is sufficient to maintain the activity of Gi in the oocyte.

The mechanism of Goi, internalization is incompletely understood (Hynes et al., 2004; Allen et al., 2005), but depalmitoylation of the activated α subunit is likely to be an important factor (Wedegaertner and Bourne, 1994; Wedegaertner et al., 1996; Thiyagarajan et al., 2002). Goi, is then repalmitoylated (Jones et al., 1997) and, as a consequence, is thought to associate with intracellular membranes for transport back to the plasma membrane (Wedegaertner et al., 1996; Hynes et al., 2004). Dissociation of α from βγ subunits could also contribute to Goi, internalization, but whether such dissociation occurs during Gi activation is unknown (Frank et al., 2005).

Bright spots of Goi, fluorescence within the cytoplasm of Gpr3+/+ oocytes are suggestive of an association of Goi, with clusters of intracellular membranes. Punctate localization of Goi, has been seen in somatic cells after receptor activation (Wedegaertner et al., 1996; Yu and Rasenick, 2002; Hynes et al., 2004; Allen et al., 2005), and, in these cells, colocalization of Goi, with the monomeric G protein RAB11 supports the idea that Goi, associates with vesicles for recycling to the plasma membrane (Hynes et al., 2004). Because the Goi, accumulations in oocytes...
are up to several micrometers in diameter and are irregular in shape, and because the fluorescence is present throughout the spots rather than at their peripheries, these spots are unlikely to be single vesicles; whether they function in the recycling of Goα to the oocyte plasma membrane remains to be determined.

As another test of whether GPR3 activates Go in the oocyte, we compared the amount of Goα protein in oocytes that had been isolated from Gpr3+/+ or Gpr3−/− follicles. The activation of Goα by cholera toxin, point mutation, or receptor activation increases its rate of degradation (Levis and Bourne, 1992; Moravcová et al., 2004). Thus, a decrease in Goα protein is an indicator of Gi activation. We found that the amount of Goα protein in Gpr3−/− oocytes was approximately three times that in Gpr3+/+ oocytes, indicating that the presence of GPR3 reduces the amount of Goα protein. This finding, as well as our finding that GPR3 overexpression elevates cAMP in the oocyte, are additional evidence that GPR3 activates Go in the mouse oocyte.

For somatic cells, it has been speculated that one function of the internalization and degradation of activated Go may be signal attenuation; internalization of Goα potentially separates it from the receptor that activates it and from adenyl cyclase, both of which are localized in the plasma membrane (Wedegaertner and Bourne, 1994). However, for the maintenance of meiotic arrest in oocytes, it is not clear why attenuation of the Gi signal would be useful. Another proposed function of internalization of Goα is to allow it to interact with molecules in the cytoplasm (Wedegaertner and Bourne, 1994). In the oocyte, much of the adenyl cyclase 3 protein is found in the cytoplasm (Horner et al., 2003), so the internalization of Goα could allow it to activate this pool of adenyl cyclase as well as regulate other cytoplasmic proteins such as tubulin (Roychowdhury et al., 1999).

These results tie together previous findings that both GPR3 (Mehlmann et al., 2004; Ledent et al., 2005) and G1 (Mehlmann et al., 2002; Kalinowski et al., 2004) are required to maintain meiotic arrest and support the conclusion that GPR3–Gi interaction is responsible for the activation of adenyl cyclase that maintains elevated cAMP in the oocyte, thus maintaining meiotic prophase arrest.

GPR3 activity in the oocyte is not increased by the presence of follicle cells

In somatic cells, GPR3 expression elevates cAMP apparently independently of an extracellular ligand (Eggerickx et al., 1995; Uhlenbrock et al., 2002; Bresnick et al., 2003). Likewise, our measurements of GoαGFP distribution indicate that even without the follicle cells, GPR3 activates Gi in the oocyte. Previous studies have also shown that Gi is active in follicle-free oocytes because the injection of a Goα inhibitory antibody or a dominant-negative form of Goα overcomes the inhibition of spontaneous meiotic resumption by a cAMP PDE inhibitor (Mehlmann et al., 2002; Kalinowski et al., 2004). However, this previous work did not quantitatively compare Gi activity in oocytes with and without the follicle cells. Based on the GoαGFP distribution, we now conclude that the follicle cells have no detectable effect on GPR3-dependent Gi activity in the oocyte. Instead, GPR3 appears to be either inherently active or activated by a molecule within the oocyte cytoplasm or plasma membrane.

How follicle cells signal to the oocyte to maintain meiotic arrest is unknown. Our results indicate that the target of the follicle cell signal is not GPR3 but do not exclude the possibility that the follicle cell signal activates a different, unidentified Gi-linked receptor that could be present in the oocyte membrane. The small but statistically significant difference in the plasma membrane-to-cytoplasm ratio of GoαGFP, comparing follicle-enclosed with isolated oocytes from Gpr3−/− mice, suggests a role for a second Gi-linked receptor. Another possible explanation of the meiosis-inhibiting effect of follicle cells is that additional cAMP enters the oocyte from follicle cells by way of gap junctions (Anderson and Albertini, 1976; Piontkewitz and Dekel, 1993; Webb et al., 2002; Eppig et al., 2004). Although this has been a long-standing hypothesis, it has not been definitively tested.

Alternatively, follicle cells could reduce cAMP PDE activity in the oocyte. Consistent with a role for a PDE inhibitor in maintaining meiotic arrest, oocytes from mice lacking the PDE3A isoform remain in prophase when isolated (Masciarelli et al., 2004). In addition, cilostamide-sensitive cAMP PDE activity in cumulus cell–oocyte complexes increases with time after isolation from the follicle; because cilostamide is an inhibitor of the oocyte-specific PDE3A isoform, this finding indicates that the presence of follicle cells may suppress oocyte PDE activity (Richard et al., 2001). Mouse follicular fluid contains the PDE inhibitor hypoxanthine, which is an effective inhibitor of meiotic resumption in isolated oocytes (Eppig et al., 1985). However, because the concentration of hypoxanthine in follicular fluid is not precisely known, its physiological significance is uncertain (Eppig et al., 1985; Eppig, 1991).

Although follicular fluid alone has some inhibitory effect on meiotic resumption in isolated oocytes, the inhibition is stronger when there is contact between the somatic cells and the oocyte (Tsafri and Channing, 1975; Racowsky and Baldwin, 1989). This suggests that somatic cell molecules that act on the oocyte are, at least in part, membrane associated rather than free in the follicular fluid. The somatic cells extend processes that contact the oocyte; these contacts may be the sites where the meiosis-inhibiting signal is transmitted to the oocyte (Anderson and Albertini, 1976).

Although our findings argue against stimulation of GPR3 activity in the oocyte by an agonist from the follicle cells, GPR3 activity could be inhibited by an inverse agonist when luteinizing hormone acts on the somatic cells to cause meiosis to resume in the oocyte (Eppig et al., 2004; Park et al., 2004; Mehlmann, 2005). GPR3 is related to the melanocortin receptors (Joost and Methner, 2002), and the melanocortin-4 receptor shows constitutive activity that is inhibited by an endogenous inverse agonist, agouti-related protein (Nijenhuis et al., 2001).

Two sequential but overlapping mechanisms for maintaining meiotic prophase arrest

The GPR3–Gi mechanism for maintaining meiotic prophase arrest becomes necessary only at the early antral stage; at the preantral stage, meiotic arrest does not require GPR3 (Mehlmann et al., 2004), or the presence of follicle cells (Erickson and Sorensen, 1974). Why fully grown oocytes within preantral folli-
cicles are incompetent to resume meiosis is unknown. Cell cycle regulatory protein levels have not been compared in oocytes from preantral with early antral follicles, but a comparison of ∼55-μm diam oocytes from 12-d-old mice with ∼80-μm diam oocytes from antral follicles of adult mice has shown that the small oocytes contain lower concentrations of cyclin B and CDK1 (Kanatsu-Shinohara et al., 2000). It is possible that the concentrations of these proteins, which are essential for meiotic resumption, stay low until the early antral stage.

Although the GPR3–Gs system is not required at the preantral stage, GPR3 is already present and activating Gs. Thus, as seems logical, the oocyte establishes the GPR3–Gs regulatory system before the oocyte becomes competent to resume meiosis, therefore ensuring that progression to metaphase does not precede the luteinizing hormone signal.

Materials and methods

Mice

Gpr3 knockout mice were obtained from Deltagen, Inc. and were genotyped as previously described (Mehlmann et al., 2004). Ovaries were collected from 20–25-d-old mice. For Fig. 3, one mouse was injected interperitoneally with 5 IU equine chorionic gonadotropin (eCG; provided by A.F. Parlow, National Hormone and Peptide Program, Torrance, CA). All animal studies were approved by the University of California Health Center Animal Care Committee.

Isolation and culture of follicles and oocytes

Preantral follicles (120–190-μm diam) and early antral/antral follicles (250–430-μm diam) were dissected from ovaries by using Dumont mini forceps (11200-14; Fine Science Tools). For some experiments, oocytes were isolated from the dissected follicles by using 30-gauge needles; residual follicle cells were removed by mouthpipetting through a micropipette that had been cut with a diamond knife (10100-00; Fine Science Tools) such that the inner diameter at the end was ∼60 μm. All manipulations were performed in MEMα medium (12000-022; Invitrogen) supplemented with 25 mM NaHCO3, 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, and 5% FBS (16000-044; Invitrogen).

For overnight culture of preantral follicles, the medium also contained 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (all from Sigma-Aldrich), and 10 ng/ml ovine follicle-stimulating hormone (National Hormone and Peptide Program). Preantral follicles were placed on Millicell culture plate inserts (PicolMRG005; Millipore) inside coverslip-bottomed dishes (P35G-0-20-C-IV, MatTek Corp.) containing 1.6 ml of medium. The dishes were thoroughly rinsed with MEMα before use. The cultures were maintained at 37°C with 5% CO2 in humidified air for 20–34 h before imaging. Isolated oocytes were cultured in 200 μM MEMα drops containing 5% FBS under light mineral oil (Fisher Scientific) for 21–28 h before imaging.

Immunolabelling

An ovary was homogenized in 100 μl PBS with protease inhibitors by using a 0.1 ml Dounce homogenizer. Oocytes were isolated from preantral follicles as described above. An affinity-purified antibody against the COOH-terminal decapetide of Gαs (RM) was provided by A. Spiegel (National Institutes of Health, Bethesda, MD) and used at 1.7 μg/ml; blots were developed by using ECL Plus reagents (GE Healthcare).

Immunofluorescence microscopy

Ovaries were fixed in 4% PFA (Electron Microscopy Sciences) in PBS for 3 h at 4°C, rinsed in PBS, incubated in 30% sucrose for 1–2 h, and transferred to freezing medium (HTFM; Triangle Biomedical Sciences, Inc.) in 6-mm–diam gelatin capsules (5214; Ernst F. Fullam, Inc.). The samples were frozen in isopentane that was cooled with dry ice, and 8–10-μm sections were cut using a cryostat. The sections were blocked with 1% BSA and 5% normal goat serum in PBS, stained using 3 μg/ml Gαs antibody or 3.6 μg/ml nonimmune rabbit IgG (MP Biomedicals) and 20 μg/ml of a fluorescein-labeled goat anti-rabbit secondary antibody (MP Biomedicals), and mounted with Vectashield mounting medium (Vector Laboratories). The sections were imaged by using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.) with the pinhole set for an optical section thickness of 3 μm. Fluorescence was excited with the 488-nm line of an argon laser and was detected by using a 500–550-nm emission filter. Tile scans as shown in Fig. 1 (B and C) were collected by using a 20× NA 0.75 Fluor objective (Carl Zeiss MicroImaging, Inc.) and used to identify follicles for imaging with a 40× NA 1.2 water immersion objective (C-Apochromat; Carl Zeiss MicroImaging, Inc.). For these and all other confocal images, the photomultiplier amplifier offset was adjusted so that the baseline was above zero, and the gain was adjusted so that peak intensities were not saturated.

Plasma membrane and cytoplasmic fluorescence intensities were measured by using MetaMorph software (Molecular Devices) as described in Fig. S1 and in supplemental Materials and methods. For the analysis of Gαs immunofluorescence images, we subtracted a background value that was determined by measuring the fluorescence intensity of oocytes in sections that were processed in parallel but incubated with nonimmune IgG instead of the anti-Gαs primary antibody. These background intensity values, which, for different sets of sections, ranged from three to seven on a scale of 0–255, have been subtracted from the Gαs fluorescence intensity values shown in Table I, but the figures are shown without background subtraction.

Microinjection

Preantral follicle-enclosed oocytes and isolated oocytes were injected while held between coverslips that were separated by a piece of double stick tape that formed a 100-μm spacer (Vidéo 1, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1; Jaffe and Terasaki, 2004; Mehlmann et al., 2004). The preantral follicles (120–190-μm diam) were partially pulled into the coverslip space by applying suction with a mouth-controlled pipette and were pushed in further using the microinjection pipette. This flattened the follicles such that the oocytes could be seen clearly for injection. After injection, the follicles were swept out of the coverslip space by using the microinjection pipette such that they fell into the reservoir of medium that was formed by the injection slide. All injection volumes were 15 pl and were calibrated as previously described (Jaffe and Terasaki, 2004). For experiments in which follicle-enclosed oocytes were injected with RNA-encoding YFP-Mem, a second injection of YFP protein was performed after overnight culture; this was accomplished by putting the follicle-enclosed oocyte back into the coverslip space on an injection slide.

RNAAs and protein used for injection

RNA-encoding Gαs/GFP was transcribed from a cdNA that was identical to α1C,F, as described previously by Hynes et al. (2004), except that ECFP was replaced by ECFP. This construct, which was in the vector pCDNA3-Amp (Invitrogen), was linearized with XbaI and RNAs were transcribed using 17 polymerase (mMessage mMachine; Ambion). The cdNA for mouse Gpr3 (D21062) in pHGCX was as described previously by Saeki et al. (1993) and Mehlmann et al. (2004). The cdNA for mouse Gpr3-RFP consisted of the monoclonal form of RFP (mRFP1, A506027.1; Campbell et al., 2002) fused to the COOH-terminus of mouse Gpr3 and inserted into the vector pHGCX. The cdNA was prepared by N. Ancellin (GlaxoSmithKline, Les Ulis, France) and linearized with NotI; RNA was transcribed using 17 polymerase. pS64X-b, which is a plasmid containing Xenopus laevis β-globin cdNA, was obtained from D.A. Melton (Harvard University, Cambridge, MA) and linearized with PstI; RNA was transcribed using SP6 polymerase. The cdNA for the rat β2 adrenergic receptor (Adrb2b in pBluescript SK−) was obtained from N. Ancellin (GlaxoSmithKline, Les Ulis, France) and linearized with NotI; RNA was transcribed using 17 polymerase. YFP membrane marker pEYFP-Mem (BD Biosciences) was subcloned into pBluescript II SK− and linearized with SphiI; RNA was transcribed using 17 polymerase.

Except where indicated, an ∼2000-bp poly(A) tail was added to RNAs by incubation with Escherichia coli poly(A) polymerase I (Ambion; Aida et al., 2001) followed by LiCl precipitation and resuspension in nuclease-free water. RNA concentrations were estimated by comparison with RNA standards (Ambion) that were run on an agarose gel. Except where indicated, the Gαs/GFP RNA concentration was adjusted to ∼1 μg/μl such that a 15-pl injection introduced 1.5 pg/oocyte. YFP-Mem RNA was used at ∼1 pg/oocyte. Amounts of other RNAs are indicated in the text. YFP protein was made in bacteria by using EFPY (BD Biosciences) subcloned into pSETA (Invitrogen), and the protein was purified on a Ni-nitrilotriacetic acid column (Qiagen) and a Hitrap Q HP column (GE Healthcare), concentrated using a 10,000 MWCO centrifugal filter device (Amicon Ultra-4; Millipore), and used at a concentration of 4 μg in the oocyte.

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Imaging of live follicles and oocytes

Follicles were imaged while held on a Millicell membrane over a coverslip-bottomed dish (see Isolation and culture...oocytes). Isolated oocytes were imaged while held in 5 μl droplets under light mineral oil on a coverslip-bottomed dish. The dishes were placed within a covered microscope stage (Carl Zeiss Microlmaging, Inc.) maintained at 37°C; water-saturated 5% CO2 in air was passed through the chamber. The follicles and oocytes were imaged by using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.), with the pinhole set for an optical section thickness of 5 μm, and a 40× NA 0.8 water immersion objective (Achroplan; Carl Zeiss Microlmaging, Inc.). GFP fluorescence was excited by the 488-nm line of an argon laser and was detected by using a 500–550-nm emission filter. YFP fluorescence was excited with the 514-nm line of an argon laser and was detected by using a longpass 530-nm emission filter. RFP fluorescence was excited with the 543-nm line of a HeNe laser and was detected by using a longpass 560-nm emission filter.

Plasma membrane and cytoplasm intensities were measured by using MetaMorph software (Fig. S1 and supplemental Materials and methods). For both GFP and YFP images, controls using uninjected oocytes within preantral follicles showed no detectable difference in intensity when comparing the oocyte and surrounding follicle cells. Therefore, intensity measurements in follicle-enclosed oocytes expressing GFP or YFP were referenced with respect to the baseline fluorescence intensity in the surrounding follicle cells. Controls using uninjected oocytes that had been isolated from their follicles sometimes showed slightly higher autofluorescence than the surrounding medium (depending on the detector gain, a mean of zero to two units on a scale of 0–255). For those cases in which the oocyte autofluorescence was greater than one unit, it was subtracted from the fluorescence intensity values before calculating the plasma membrane-to-cytoplasm fluorescence ratio; figures are shown without background subtraction.

Statistics

Tests of statistical significance were performed by using Instat (GraphPad Software). For Table II, the SEM resulting from the division of one mean ± SEM divided by another mean ± SEM was calculated by using an equation for error propagation (http://www.njit.edu/~uphysics/uncertainties/Uncertaintiespa2.html).

Online supplemental material

Video 1 shows the microinjection of a preantral follicle-enclosed mouse oocyte. The supplemental Materials and methods section describes the use of MetaMorph software to measure plasma membrane and cytoplasm intensities (Fig. S1) as well as methods for RT-PCR and cAMP measurements. The supplemental Results section shows that GFP, expression does not prevent the spontaneous resumption of meiosis in oocytes that were isolated from antral follicles, that Gaαi GTP moves from the oocyte plasma membrane to the cytoplasm when an exogenously expressed β-adrenergic receptor is stimulated with isoproterenol (Fig. S2), and that the amount of GPR3 protein expression correlates with the amount of Gaαi internalization (Fig. S3). Fig. S1 shows the quantitation of plasma membrane/cytoplasm fluorescence ratios. Fig. S2 shows the distribution of Gaαi in an oocyte coexpressing the βαi-adrenergic receptor before and 30 min after exposure to 10 μM isoproterenol. Fig. S3 shows images of preantral follicle-enclosed Gpr3-/- oocytes coexpressing GPR3-RFP and Gaαi-GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1.

Submitted: 30 June 2005
Accepted: 20 September 2005

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