

Endocytosis in the mouse oocyte and its contribution to cAMP signaling during meiotic arrest

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Abstract

Mammalian oocytes are arrested at prophase I of meiosis until a preovulatory surge of LH stimulates them to resume meiosis. Prior to the LH surge, high levels of cAMP within the oocyte maintain meiotic arrest; this cAMP is generated in the oocyte through the activity of the constitutively active, G_s-coupled receptor, G-protein-coupled receptor 3 (GPR3) or GPR12. Activated GPRs are typically targeted for desensitization through receptor-mediated endocytosis, but a continuously high level of cAMP is needed for meiotic arrest. The aim of this study was to examine whether receptor-mediated endocytosis occurs in the mouse oocyte and whether this could affect the maintenance of meiotic arrest. We found that constitutive endocytosis occurs in the mouse oocyte. Inhibitors of receptor-mediated endocytosis, monodansylcadaverine and dynasore, inhibited the formation of early endosomes and completely inhibited spontaneous meiotic resumption. A red fluorescent protein-tagged GPR3 localized in the plasma membrane and within early endosomes in the oocyte, demonstrating that GPR3 is endocytosed. However, overexpression of G-protein receptor kinase 2 and β -arrestin-2 had only a modest effect on stimulating meiotic resumption, suggesting that these proteins do not play a major role in GPR3 endocytosis. Inhibition of endocytosis elevated cAMP levels within oocytes, suggesting that there is an accumulation of GPR3 at the plasma membrane. These results show that endocytosis occurs in the oocyte, leading to a decrease in cAMP production, and suggest that there is a balance between cAMP production and degradation in the arrested oocyte that maintains cAMP levels at an appropriate level during the maintenance of meiotic arrest.

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Introduction

Mammalian oocytes become arrested at prophase I of meiosis during embryonic development. Oocytes remain in prophase I until a preovulatory surge of LH from the pituitary signals to the oocyte, via the surrounding follicle cells, to resume meiosis and progress to metaphase II, where they undergo a second arrest. It is at the MII stage that fertilization occurs. Meiotic resumption is coordinated with the LH surge, such that by the time ovulation occurs the oocyte has undergone nuclear maturation as well as changes in intracellular organization (cytoplasmic maturation) that prepare the mature, MII-stage oocyte to be fertilized and initiate embryonic development.

It is well established that maintenance of meiotic arrest prior to the LH surge depends on high levels of cAMP in the oocyte (Conti *et al.* 2002, Mehlmann 2005a). In rodents, cAMP is produced in the oocyte by the activity of a constitutively active G-protein-coupled receptor 3 (GPR3; mouse) or GPR12 (rat; Mehlmann *et al.* 2004, Hinckley *et al.* 2005, Ledent *et al.* 2005, Vaccari *et al.* 2008), both of which stimulate a G_{αs}

G-protein to activate adenylate cyclase and thereby produce cAMP (Horner *et al.* 2003, Freudzon *et al.* 2005, Hinckley *et al.* 2005, Mehlmann 2005b). The removal or inactivation of any of the components of this signaling pathway causes the oocyte to undergo meiotic resumption in the absence of the LH surge (Mehlmann *et al.* 2002, 2004, 2005b, Horner *et al.* 2003, Ledent *et al.* 2005, DiLuigi *et al.* 2008, Vaccari *et al.* 2008).

Most GPRs are inactive until they are stimulated by a ligand. Ligand binding triggers a conformational change in the receptor and allows the phosphorylation by G-protein receptor kinases (GRKs) and subsequently the recruitment of β -arrestins, which cause receptor internalization through clathrin-coated pits. The receptor can then be degraded or recycled back to the membrane (Reiter & Lefkowitz 2006, Moore *et al.* 2007). GPR3 is unusual, as it is constitutively active and is likely to signal in the absence of a ligand (Freudzon *et al.* 2005, Yin *et al.* 2009). As a constitutively active receptor, it is unclear whether GPR3 is internalized in a similar fashion, and if so, the receptor continues to signal internally to provide a constant level of cAMP to the interior of the oocyte. Recently, the TSH and PTH

receptors, both of which act through the G_s signaling pathway, have been shown to continue to signal following endocytosis, within early endosomes (Calebiro *et al.* 2009, 2010, Ferrandon *et al.* 2009). In addition, GPR6, which is a constitutively active receptor closely related to GPR3 (Kostenis 2004), is localized within cells rather than on the cell surface, suggesting that it signals within endosomes (Padmanabhan *et al.* 2009). Signaling within endosomes has been associated with the ability of the cell to maintain a prolonged response to hormone following receptor internalization. Because prolonged cAMP signaling is crucial for the maintenance of meiotic arrest, it is possible that endosomal signaling is required for sustained cAMP production. Alternatively, it is possible that GPR3 must continuously remain in the oocyte plasma membrane in order to be active, so that it would either not recycle or be endocytosed and rapidly recycled back to the membrane. Depletion of GPR3 in the follicle-enclosed mouse oocyte by RNAi causes oocytes to undergo spontaneous maturation in the absence of LH (Mehlmann 2005b), indicating that GPR3 endocytosis and degradation occur in the oocyte, concomitant with synthesis of new GPR3 and insertion into the plasma membrane.

Receptor-mediated endocytosis is a critical process utilized by oocytes of non-mammalian species, such as insects and frogs, to import yolk protein precursors (Opresko & Wiley 1987). Import of vitellogenin occurs during oocyte growth through clathrin-mediated endocytosis following binding to a receptor. Although mammalian oocytes do not produce yolk, it is possible that endocytosis could be important during oocyte growth. In addition, endocytosis of cell surface receptors could be important for the regulation of meiosis. Indeed, receptor trafficking is critical for the regulation of meiotic maturation in oocytes of *Caenorhabditis elegans* (Cheng *et al.* 2008).

Previous studies have shown that vesicular trafficking at the plasma membrane has an important role in maintaining meiotic arrest in *Xenopus* oocytes (El-Jouni *et al.* 2007). Inhibiting exocytosis stimulated meiotic resumption in the absence of progesterone, whereas inhibiting endocytosis interfered with the ability of progesterone to stimulate meiotic resumption, indicating that some component that maintains meiotic arrest must be present in the plasma membrane. It was hypothesized that this component could be a constitutively active receptor, although the role of the receptor was not investigated. In this study, we examined whether active endocytosis occurs in the mouse oocyte and whether endocytosis impacts the maintenance of meiotic arrest. Our results demonstrate that trafficking at the membrane occurs and suggest that GPR3 signaling at the plasma membrane, rather than intracellularly, is necessary for the maintenance of meiotic arrest.

Results

Components of endocytosis are expressed in mouse oocytes

Endocytosis of receptors in somatic cells occurs predominantly through the clustering and internalization of an activated receptor into clathrin-coated pits (Moore *et al.* 2007, Wolfe & Trejo 2007). However, little is known about the identity and function of recycling components in mammalian oocytes. Therefore, we first examined whether two of the most common proteins responsible for receptor endocytosis in somatic cells were expressed in mouse oocytes. Clathrin is a protein that plays an important role in the formation of coated pits in which receptors accumulate (Doherty & McMahon 2009). The heavy chain of clathrin is present in mouse oocytes, as determined by western blotting. Indeed, we were able to detect clathrin in as few as ten oocytes (Fig. 1A), indicating that it is abundant. Receptor endocytosis also requires the activity of dynamin, a GTPase that is necessary for excising clathrin-coated pits from the plasma membrane (Moore *et al.* 2007). We detected the expression of the ubiquitously expressed dynamin 2 in the oocyte. In contrast, neither dynamin 1, which is restricted to the nervous system, nor the more

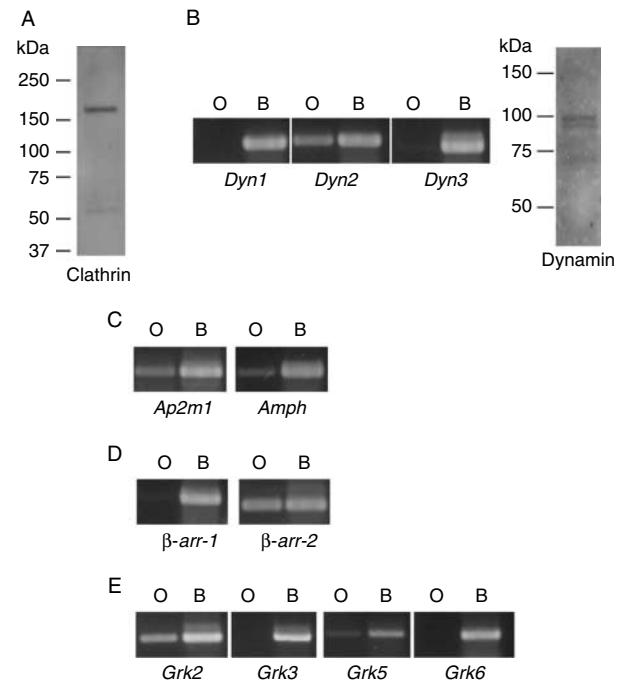


Figure 1 Components of receptor-mediated endocytosis are expressed in mouse oocytes. (A) Expression of clathrin protein, as assessed by western blot using lysate from ten oocytes. (B) Expression of dynamin in mouse oocytes, as assessed by RT-PCR. O, oocyte; B, brain, used as a positive control. (C) Western blot showing two bands at ~100 kDa in lysate from 300 oocytes, demonstrating that dynamin protein is present in mouse oocytes. (C, D and E) RT-PCRs showing expression of other components of endocytosis in mouse oocytes. *Ap2m1*, AP-2 μ 2 subunit; *Amph*, amphiphysin; β -*arr*, β -arrestin.

ubiquitously expressed dynamin 3, was detectable by RT-PCR using our cycling parameters (Fig. 1B). Western blot analysis of oocyte lysate detected two bands at the expected size of ~ 100 kDa, showing that the protein is expressed. The signal was not strong, even using lysate from 500+ oocytes, suggesting that the amount of dynamin in the oocyte is low. It is unclear why the protein was expressed as a doublet; it is possible that the two bands could represent different splice variants of dynamin 2 (Liu *et al.* 2008).

In addition to clathrin and dynamin, other major components of receptor recycling include the adaptor proteins AP-2 and amphiphysin, as well as the mediators of endocytosis, β -arrestins and GRKs. We examined the expression of mRNAs encoding these proteins using RT-PCR. mRNA for one of the major AP-2 subunits, $\mu 2$ (*Ap2m1*) subunit of AP-2, and amphiphysin were expressed (Fig. 1C). In addition, β -arrestin-2 and GRK2 and GRK5 were present (Fig. 1D and E), whereas β -arrestin-1 and *Grk3* and *Grk6* RNAs were not detectable in oocytes. The visual GRK1 and GRK7, and the testis-specific GRK4, were not examined.

Monodansylcadaverine and dynasore inhibit endocytosis in mouse oocytes

As a prelude to examining whether endocytosis could have a role in meiotic regulation, we first determined that inhibitors of receptor-mediated endocytosis could prevent endocytosis in mouse oocytes using the inhibitors monodansylcadaverine (MDC) and dynasore (Macia *et al.* 2006). We used pharmacological inhibitors because in initial experiments we found that we were unable to deplete the amount of clathrin protein using RNAi, nor were we able to significantly deplete the amount of dynamin 2 RNA within our culture period of 4 days using follicle-enclosed oocytes (not shown). We chose compounds that inhibit endocytosis through two different mechanisms: MDC inhibits the protein cross-linker, tissue transglutaminase (Davies *et al.* 1980), which is thought to inhibit the clustering and internalization of clathrin (Schlegel *et al.* 1982), whereas dynasore inhibits the GTPase activity of dynamin without affecting the activity of other small GTPases (Macia *et al.* 2006).

To confirm that MDC blocked endocytosis in oocytes, we treated oocytes with FM 1-43, a dye that becomes fluorescent when it incorporates into membranes and retains its fluorescence when membrane is endocytosed. Following a 3 h culture period in the presence of 100 μ M MDC and 10 μ M milrinone (to prevent spontaneous oocyte maturation), we incubated zona-free oocytes in FM 1-43 and examined them with a confocal microscope 1–2 h later. Zona-free oocytes were used because the FM 1-43 labeled transzonal processes in the zona pellucida, making plasma membrane fluorescence difficult to quantify. Untreated oocytes were fluorescent

in the plasma membrane and throughout the cytoplasm, often in punctate clusters (Fig. 2A). In contrast, oocytes preincubated in MDC were mainly fluorescent in the plasma membrane. The plasma membrane labeling was more intense than that in controls, suggesting that the treated oocytes had more membrane area due to the absence of endocytosis (Fig. 2A). The total plasma membrane to cytoplasmic fluorescence ratio was significantly higher in oocytes treated with MDC than in controls ($P < 0.0001$; Fig. 2B), demonstrating an inhibition of endocytosis. We also attempted to examine whether dynasore inhibits endocytosis in oocytes using FM 1-43. However, plasma membrane labeling in the presence of dynasore was very faint, indicating that dynasore might interfere with the incorporation of FM 1-43 into the membrane.

Endocytosed proteins enter early endosomes, after which they can be sorted into late endosomes and lysosomes, or can be recycled back to the membrane. We hypothesized that inhibiting endocytosis with MDC and dynasore would block the formation of early endosomes. For these experiments, we labeled early endosomes by immunofluorescence using a specific antibody against the early endosome marker, early endosome antigen 1 (EEA1; Fig. 2C). In control oocytes, early endosomes were abundantly present in both the cortex and throughout the cytoplasm, excluding the germinal vesicle (GV; Fig. 2C). The diameter of the fluorescent spots was generally much larger than the 50–400 nm diameter that characterizes endosomes in most somatic cells (Jovic *et al.* 2010), reaching sizes of up to ~ 5 μ m (Fig. 2C). MDC dose dependently decreased the number of endosomes present in the oocyte following a 3 h incubation, such that at a concentration of 100 μ M, early endosomes were completely absent (Fig. 2C). A similar reduction in early endosomes was seen using 80 μ M dynasore, a concentration commonly used to inhibit endocytosis in somatic cells (Macia *et al.* 2006, Newton *et al.* 2006, de Beco *et al.* 2009, Barrias *et al.* 2010; Fig. 2C). These results show that there is rapid endocytosis at the plasma membrane in mouse oocytes and that both MDC and dynasore effectively inhibit endocytosis.

Inhibiting endocytosis prevents spontaneous meiotic resumption in mouse oocytes

To examine the effect of inhibiting endocytosis on spontaneous meiotic resumption, oocytes were pre-treated with MDC or dynasore for 3 h in the presence of milrinone, then were washed out of milrinone while maintained in MDC or dynasore, and were periodically scored for the presence of a GV. MDC dose dependently inhibited spontaneous meiotic resumption for at least 7 h after oocytes were washed out of milrinone (Fig. 3A). Oocytes became unhealthy during overnight incubation in MDC, so later time points were not examined. Similar to MDC, 80 μ M dynasore dose

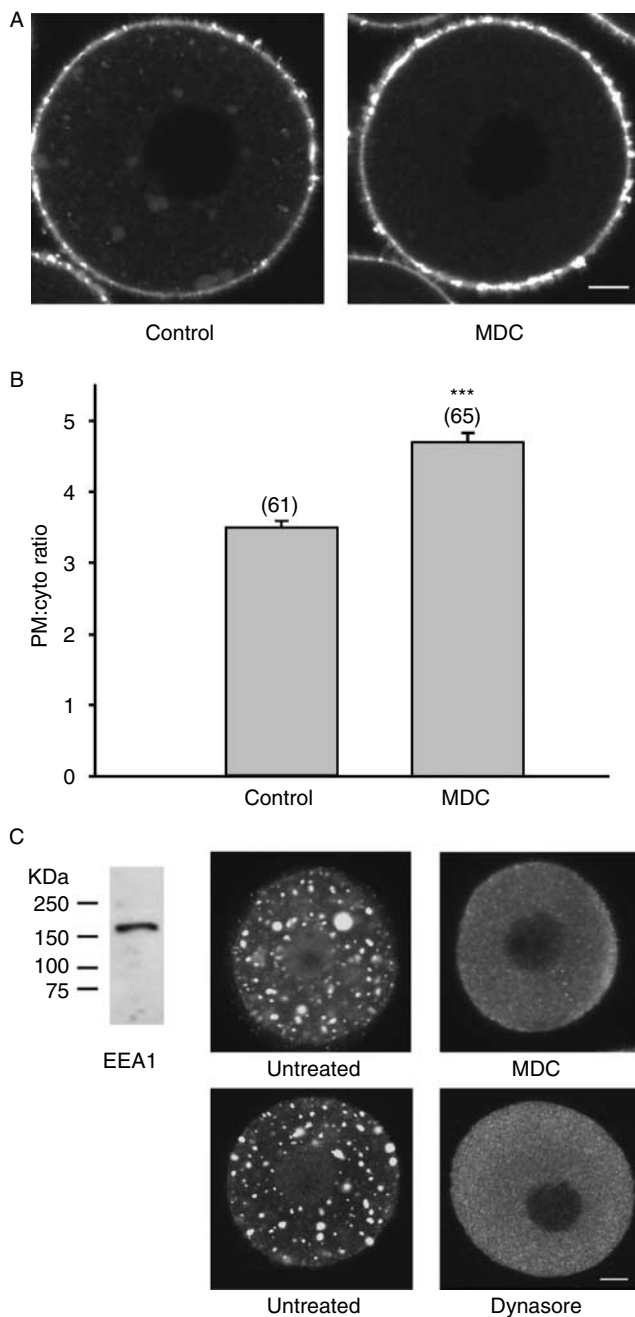


Figure 2 Inhibitors of clathrin-mediated endocytosis inhibit endocytosis in mouse oocytes. (A) MDC inhibits endocytosis. Isolated oocytes were treated with 100 μ M MDC, the zonae were removed, and oocytes were incubated in 2 μ M FM 1-43 and imaged 1–2 h later with a confocal microscope. (B) Quantification of membrane to cytoplasmic fluorescence in control and MDC-treated oocytes. The membrane to cytoplasm ratio was significantly higher in MDC-treated than in control oocytes ($***P < 0.0001$; error bars = mean \pm S.E.M.). The number of oocytes is indicated in parentheses. (C) MDC and dynasore block the formation of early endosomes. (Left) Antibody against EEA1 that was used for these experiments specifically recognizes a single band from mouse oocyte lysate. (Right) Early endosomes are present throughout the oocyte excluding the GV in control oocytes, whereas in oocytes treated with 100 μ M MDC or 80 μ M dynasore early endosomes were completely absent. Bars = 10 μ m.

independently inhibited spontaneous maturation in the absence of milrinone (Fig. 3B).

The effects of MDC and dynasore were reversible, as oocytes treated with MDC and dynasore for 3 h underwent spontaneous maturation following the removal of MDC, dynasore, and milrinone (Fig. 3C). There was a slight delay between the time the control oocytes underwent GVBD and those treated with MDC or dynasore. Although almost half of the control oocytes underwent GVBD within 1 h following milrinone removal, GVBD was only observed after 2 h in oocytes treated with MDC or dynasore. The overall rate of GVBD was slower in the group treated with dynasore than in the group treated with MDC. The reason for this delay is unclear. However, almost 100% of the oocytes in both treated groups underwent GVBD within 5 h after inhibitor washout, and 84% of the matured oocytes from the MDC group and 90% of matured oocytes from the dynasore group formed first polar bodies ($n = 25$ and 30 oocytes respectively).

To further characterize MDC and dynasore as reversible endocytosis inhibitors, we examined whether early endosomes reappear following MDC or dynasore washout. Oocytes were incubated in 100 μ M MDC or 80 μ M dynasore for 3 h in the presence of milrinone and then were washed into medium without MDC or dynasore. We kept milrinone in the culture medium for this experiment to rule out an effect of falling cAMP levels (caused by activation of phosphodiesterase (PDE; Norris *et al.* 2009)) on the localization of endosomes. Early endosomes, which were completely absent in oocytes treated with MDC or dynasore (Figs. 2D and 3D), began to reappear within 1 h following MDC or dynasore washout (Fig. 3D). Endosomes were generally smaller than those seen in controls and were often concentrated around the cortex, where early endosomes are formed. The formation of early endosomes preceded GVBD in oocytes treated with MDC or dynasore, indicating that cAMP levels are higher in the oocyte when endocytosis is blocked and then fall when endocytosis resumes.

To examine the possibility that falling cAMP levels due to removal of milrinone from medium that did not contain endocytosis inhibitors affects endosome localization, we collected oocytes in the presence of milrinone and labeled early endosomes 1 and 2 h following milrinone washout. Localization of endosomes was indistinguishable from those observed in the presence of milrinone (not shown), demonstrating that endosome formation is independent of cAMP levels in the oocyte.

GPR3 undergoes endocytosis in the oocyte

Meiotic arrest depends on high levels of cAMP in the oocyte (Mehlmann 2005a). Because GPR3 is responsible for maintaining meiotic arrest prior to the LH surge, we

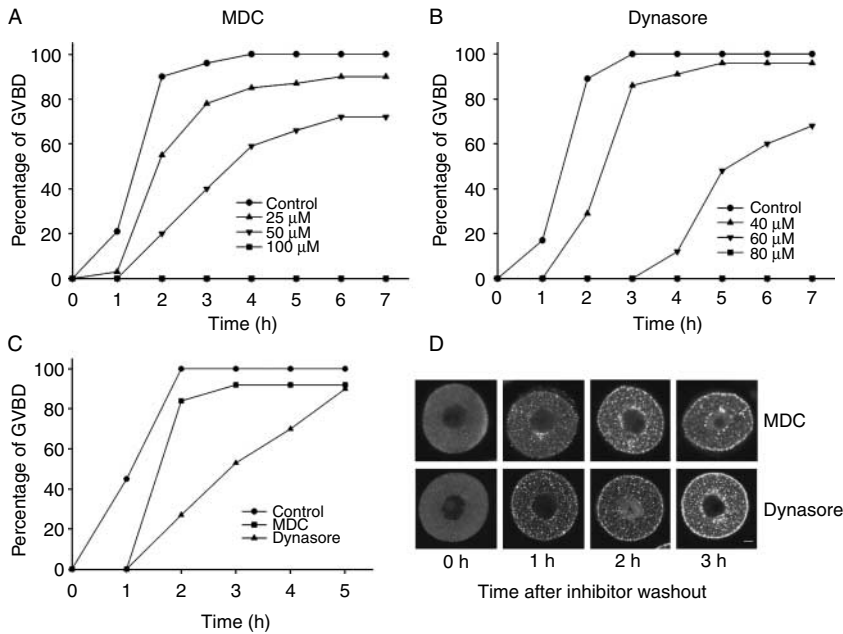


Figure 3 MDC and dynasore inhibit spontaneous meiotic resumption in oocytes. Isolated oocytes were treated with the indicated concentrations of MDC (A) or dynasore (B). After a 3 h incubation period, the oocytes were washed out of milrinone and periodically scored for the presence or absence of a GV. (C) The effects of MDC and dynasore on inhibiting GVBD are reversible. Oocytes were treated with MDC or dynasore for 3 h in the presence of milrinone and were washed out of all inhibitors. (D) Early endosomes, which were absent following a 3 h treatment with MDC or dynasore, re-form within 1 h after MDC or dynasore are removed from the culture medium. Bar=10 μm.

were interested in knowing whether its localization is important for the regulation of meiotic arrest. Most GPRs are internalized following agonist stimulation. However, as a constitutively active receptor, it is possible that GPR3 does not get internalized but remains at the plasma membrane and continuously signals. We examined GPR3 internalization in the oocyte by expressing GPR3 fused to red fluorescent protein (GPR3-RFP). Overexpressed GPR3-RFP localized both in the plasma membrane and in clusters throughout the cytoplasm of the oocyte (Fig. 4) when incubated in the presence of milrinone. Double labeling with EEA1 showed that GPR3-RFP co-localized within early endosomes. These results demonstrate that GPR3 is internalized in the mouse oocyte.

We attempted to examine the localization of GPR3-RFP in response to treatment of MDC and dynasore. However, there was no noticeable accumulation of GPR3-RFP in the plasma membrane in response to inhibitor treatment (not shown). This could be due to the large surface area of the oocyte and the high level of membrane labeling of overexpressed GPR3-RFP that would not permit the detection of small changes in plasma membrane fluorescence.

Overexpression of β -arrestin-2 and GRK2 into follicle-enclosed oocytes has a partial effect on stimulating GVBD

If GPR3 signaling at the plasma membrane is necessary to maintain meiotic arrest, then the removal of GPR3 from the membrane would be expected to cause GVBD. Receptor-mediated endocytosis generally occurs by phosphorylation of an activated receptor by GRKs, followed by binding to β -arrestins, which remove the

receptor from the membrane and sort it into clathrin-coated pits (Reiter & Lefkowitz 2006, Moore *et al.* 2007). We examined whether GRKs/ β -arrestins are involved in GPR3 endocytosis by determining the effect on GVBD of overexpressing β -arrestin-2 and GRK2 in follicle-enclosed oocytes. Overexpression of β -arrestins/GRKs hyperphosphorylates and enhances endocytosis of a constitutively active viral receptor, US28, in somatic cells and decreases its activity (Miller *et al.* 2003). We used follicle-enclosed oocytes for these experiments because unlike the above pharmacological inhibitors, which are cell permeable, the GRK2 and β -arrestin-2-GFP proteins were not permeable, thereby allowing us to discern an effect of the proteins on the oocyte as opposed to the follicle. In addition, use of follicle-enclosed oocytes simulates the native environment of the oocyte, rather than using oocytes incubated in inhibitors that artificially raise cAMP levels.

We microinjected RNA encoding a fluorescently tagged β -arrestin-2 (β -arrestin-2-GFP) and GRK2 into oocytes from follicles ≥ 320 μm in diameter; such follicles contain oocytes that are competent to resume meiosis. After a 17–24 h incubation to allow time for the proteins to be expressed, oocytes were removed from their follicles and the meiotic status evaluated. By the end of the culture period, 13% of the injected oocytes had undergone GV breakdown (GVBD) compared with 0% of control oocytes ($n=24$ and $n=23$ respectively; Fig. 5A), despite the proteins being robustly expressed as determined by western blot for GRK2 (Fig. 5B) and fluorescence microscopy for β -arrestin-2-GFP (not shown).

To confirm that our β -arrestin-2 and/or GRK2 proteins were active, we examined the ability of β -arrestin-2-GFP to translocate from the cytoplasm to the plasma membrane following stimulation of an exogenously

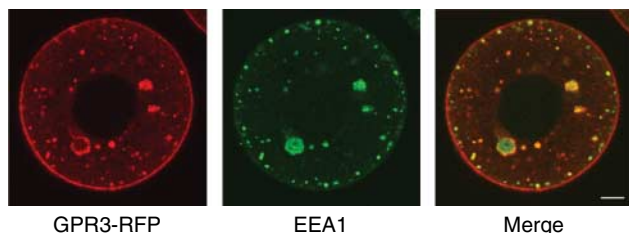


Figure 4 GPR3-RFP localizes in the plasma membrane in oocytes and co-localizes with early endosomes. Isolated oocytes were injected with RNA encoding GPR3-RFP and cultured overnight prior to fixation. Early endosomes were labeled using immunofluorescence. GPR3-RFP (left). Early endosomes (middle). Merge (right). Bar = 10 μ m.

expressed G_s -coupled receptor. To do this, we co-injected isolated oocytes with RNA encoding the β_2 adrenergic receptor (β_2 AR), GRK2, and β -arrestin-2-GFP. Following an overnight culture to allow time for the oocytes to synthesize the proteins, we examined the oocytes before and after stimulating the receptor with 10 μ M isoproterenol. Before adding the isoproterenol, oocytes were fluorescent throughout the cytoplasm, excluding the GV (Fig. 5B). Within 2 min of adding isoproterenol, the β -arrestin-2-GFP translocated to the plasma membrane (Fig. 5C). The localization to the membrane was transient, and fluorescence began to be lost from the membrane and become punctate within 10 min after adding agonist (Fig. 5C), indicating that the receptor was rapidly removed from the membrane. These results show that mouse oocytes are capable of undergoing GRK/ β -arrestin-dependent receptor recycling but suggest that if GRKs/ β -arrestins have a role in clathrin-mediated receptor endocytosis of GPR3, it is not likely to be the primary mechanism by which this occurs in the oocyte.

Inhibiting endocytosis raises cAMP levels in the oocyte

If GPR3 signaling at the cell membrane is necessary for maintaining meiotic arrest, we would expect to see an increase in cAMP concentration in oocytes in which endocytosis is inhibited. To examine the possibility that GPR3 activity at the plasma membrane increases cAMP levels, we measured cAMP levels in oocytes treated with dynasore. We used dynasore for this experiment because we found in preliminary experiments that MDC interfered with the cAMP sensor, whereas dynasore did not (not shown). To measure cAMP levels, we used a fluorescence resonance energy transfer (FRET)-based assay that has previously been described (Nikolaev *et al.* 2004, Norris *et al.* 2009). This assay uses an indicator consisting of yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) fused to the cAMP-binding domain of EPAC2 (Epac2-camps300). cAMP levels are inversely proportional to FRET when CFP is excited with a laser (see Materials and Methods).

We microinjected the cAMP indicator protein, Epac2-camps300, into oocytes and measured the YFP/CFP ratio following excitation of CFP, after treatment with dynasore. We found that oocytes held in the presence of milrinone throughout the course of the experiment maintained a constant fluorescence ratio (Fig. 6). When oocytes were washed out of milrinone, the YFP/CFP ratio increased by $\sim 20\%$, demonstrating that cAMP levels decreased. The baseline ratios that we observed, and the increase in FRET seen following removal of milrinone, are very similar to those reported previously in follicle-enclosed oocytes, in which cAMP levels corresponded to ~ 700 nM prior to adding LH and dropped to ~ 140 nM following LH treatment (Norris *et al.* 2009). Oocytes treated with dynasore displayed a gradual decrease in FRET that was statistically significant after 2 h of treatment (Fig. 6). The YFP/CFP ratio continued to decrease even after oocytes were washed out of milrinone but maintained in dynasore. These results show that inhibiting endocytosis increases cAMP levels and suggest that signaling at the plasma membrane is needed to maintain meiotic arrest prior to the LH surge.

Discussion

Endocytosis is a ubiquitous process in somatic cells and is critically important for yolk production in the oocytes of non-mammalian species. However, endocytosis in

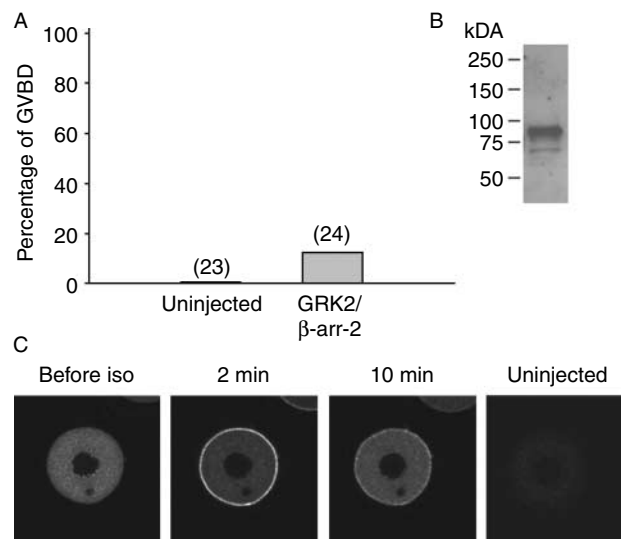


Figure 5 Effect of GRK2 and β -arrestin 2 on meiotic resumption. (A) Follicle-enclosed oocytes were injected with *Grk2*/ β -arrestin-2-GFP mRNA. Follicles were cultured for 17–24 h, and the oocytes were isolated to examine meiotic status. The number above each bar is the total number of follicles per group. The experiment was repeated three times. (B) Immunoblot showing expression of GRK2 in follicle-enclosed oocytes. (C) GRK2 and β -arrestin-2-GFP are active in mouse oocytes. Isolated oocytes were co-injected with GRK2, β -arrestin-2-GFP, and the β_2 -adrenergic receptor. After an overnight culture, oocytes were imaged with a confocal microscope before and after adding 10 μ M isoproterenol (iso). Bar = 10 μ m.

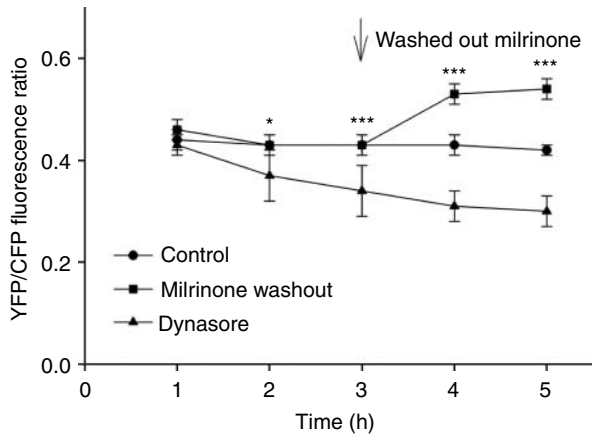


Figure 6 Inhibiting endocytosis with dynasore increases cAMP levels in oocytes. Measurement of cAMP levels in oocytes using the FRET-based cAMP sensor, Epac2-camps300. Isolated oocytes were injected with 5 μ M Epac2-camps300 protein, cells were visualized with a confocal microscope at hourly intervals, and the YFP/CFP ratio was measured. The 1 h time point corresponds to a 1 h treatment with dynasore. Control oocytes were maintained in milrinone throughout the course of the recordings. The arrow shows the point at which milrinone was washed out of the culture medium in the milrinone washout and dynasore-treated groups. Each point represents mean \pm s.d. Statistical significance relative to controls is indicated by asterisks (* P <0.05; *** P <0.0001).

mammalian germ cells and its possible contribution to their unique physiology is poorly understood. The aim of this study was to examine whether endocytosis occurs in the mouse oocyte and whether this affects the signaling properties of GPR3. We found that active, robust endocytosis occurs in the isolated mouse oocyte. Inhibiting endocytosis had an effect on the oocyte's meiotic status, inhibiting spontaneous meiotic resumption in the absence of PDE inhibitors. We also found that GPR3 localizes in the oocyte plasma membrane as well as in early endosomes, indicating that it is actively endocytosed in the oocyte. Inhibiting endocytosis stimulated an increase in oocyte cAMP, suggesting that signaling at the plasma membrane, possibly through an accumulation of GPR3, is responsible for maintaining meiotic arrest.

Mouse oocytes express several of the major proteins involved in receptor endocytosis, including clathrin, dynamin 2, AP-2, amphiphysin, β -arrestin-2, and GRK2 and GRK5. The well-characterized inhibitors of receptor-mediated endocytosis, MDC and dynasore, effectively prevented endocytosis in oocytes. These inhibitors blocked the formation of both endocytic vesicles and early endosomes, as well as spontaneous meiotic resumption. The effect of MDC is likely to be related to inhibition of the protein cross-linker, tissue transglutaminase (Davies *et al.* 1980), which is thought to inhibit the clustering and internalization of clathrin (Schlegel *et al.* 1982). Indeed, MDC does not inhibit endocytosis in cells lacking transglutaminase (Davies *et al.* 1984).

A previous study in mouse oocytes showed the presence of active tissue transglutaminase that was inhibited by MDC (Kim *et al.* 2001), and in that study, MDC inhibited GVBD in a dose-dependent manner. However, endocytosis itself was not examined. As a second method of blocking endocytosis in oocytes, we used dynasore. In contrast to MDC, dynasore specifically inhibits dynamin by inhibiting its GTPase activity without affecting the activity of other small GTPases (Macia *et al.* 2006). Dynasore may therefore be the more specific inhibitor of endocytosis. The results showing that dynasore inhibits endocytosis in oocytes support the data using MDC, confirming that endocytosis was prevented using both inhibitors.

Dynasore caused an increase in cytoplasmic cAMP levels in oocytes, suggesting that GPR3 signaling at the plasma membrane could be responsible for this increase. Recently, it has been shown that some GPRs are able to signal within endosomes (Calebiro *et al.* 2009, 2010, Ferrandon *et al.* 2009, Mullershausen *et al.* 2009) and that this signaling is necessary for prolonged cAMP signaling initiated by various hormones. Therefore, it is possible that GPR3 likewise signals within endosomes, perhaps to provide cAMP to the interior of the oocyte, which has a relatively large volume. Although our results do not exclude the possibility that endosomal signaling occurs within arrested oocytes, they support the hypothesis that signaling at the plasma membrane and not within endosomes is sufficient and necessary to maintain meiotic arrest.

If signaling at the plasma membrane alone is needed to maintain meiotic arrest, how is cAMP able to transmit its signal to the interior of the oocyte, which has a large volume? Cyclic AMP is a freely diffusible molecule (Bacskai *et al.* 1993, Chen *et al.* 1999), but its diffusion is restricted in many cases by the localized pools of PDEs and protein kinase A (PKA) tethered to the plasma membrane and various intracellular membranes by the A kinase anchoring proteins (AKAPs; Dodge *et al.* 2001). It is thought that PDE activity stimulated by PKA is necessary for establishing cAMP microdomains (Fischmeister *et al.* 2006, Houslay *et al.* 2007). For example, stimulation of the β -AR in neonatal cardiac myocytes generates microdomains of high concentrations of cAMP that act within a range as small as $\sim 1 \mu$ m, while cAMP diffuses freely in cells that have been treated with PDE inhibitors (Zaccolo & Pozzan 2002). On the other hand, β_1 -ARs generate cAMP signals capable of diffusing over 10 μ m throughout large adult ventricular cardiomyocytes even in the absence of PDE inhibitors (Nikolaev *et al.* 2006). AKAPs have been identified in mouse oocytes (Brown *et al.* 2002, 2003, Kovo *et al.* 2006, Newhall *et al.* 2006) and the RI- and RII-type PKAs are tethered in the plasma membrane and the cytoplasm respectively (Brown *et al.* 2002, Newhall *et al.* 2006, Webb *et al.* 2008). However, PDE activity is kept low in oocytes by cGMP, which is produced in the

somatic cells and diffuses into the oocyte through gap junctions (Norris *et al.* 2009). It is not until the surge of LH that cGMP levels in the oocyte fall, allowing the activation of PDE3A (Norris *et al.* 2009, Vaccari *et al.* 2009). Therefore, it is possible that cAMP is able to readily diffuse throughout the oocyte cytoplasm prior to LH stimulation to activate intracellular PKAs. In support of this, the catalytic subunit of PKA is present both in the plasma membrane and throughout the cytoplasm (Brown *et al.* 2002, Webb *et al.* 2008).

If signaling at the plasma membrane is needed for maintenance of meiotic arrest, it is not clear why GPR3 might be targeted for endocytosis, or the mechanism(s) by which endocytosis occurs. Many GPRs undergo a conformational change upon stimulation that allows the phosphorylation by GRKs and subsequent binding and internalization by β -arrestins. In this study, we found that overexpression of β -arrestin-2 with GRK2 caused GVBD in a small percentage of oocytes within our culture period, suggesting that GRKs and β -arrestins could interact with GPR3. However, because the percentage of oocytes that underwent GVBD was not significantly different from controls, it is not likely to be the primary mechanism by which endocytosis occurs. Other GPRs (e.g. the PAR1 and M₂ muscarinic receptors (Pals-Rylandsdam *et al.* 1997, Paing *et al.* 2002) have been shown to be endocytosed through β -arrestin/GRK-independent mechanisms; it is possible that GPR3 endocytosis occurs through similar mechanisms.

Another constitutively active receptor, the herpes virus chemokine receptor US28, signals in the absence of an agonist (Casarosa *et al.* 2001, Fraile-Ramos *et al.* 2001) and could perhaps serve as a model for the regulation of the signaling of constitutively active receptors, including GPR3. US28 is constitutively phosphorylated when overexpressed in cultured cells (Mokros *et al.* 2002), and this phosphorylation leads to an increase in GRK/ β -arrestin-induced receptor internalization (Miller *et al.* 2003). In addition, overexpressing GRKs and/or β -arrestins causes a decrease in US28 activity (Miller *et al.* 2003). Furthermore, mutation of serines at the C-terminus impairs phosphorylation and receptor internalization, leading to an increase in surface expression and consequently to an increase in activity (Mokros *et al.* 2002, Miller *et al.* 2003). Taken together, these results show that surface expression of a constitutively active GPR is necessary for intracellular signaling. Interestingly, the internalization of US28 is enhanced by, but not dependent on, β -arrestins, although it is dependent on a clathrin-mediated pathway (Fraile-Ramos *et al.* 2003, Droese *et al.* 2004). It will be interesting to determine whether GPR3 activity is regulated by similar mechanisms.

In summary, our results show that vesicular trafficking actively occurs at the mouse oocyte plasma membrane and that trafficking is a regulator of meiotic arrest. Although we were unable to observe an accumulation of

overexpressed GPR3 at the plasma membrane following inhibition of endocytosis, our results showing an increase in cAMP concentration following inhibition of endocytosis are consistent with this possibility. In addition, our results suggest that even if GPR3 normally signals within endosomes, that signaling at the plasma membrane is sufficient for the maintenance of meiotic arrest prior to the LH surge, as cAMP levels increased following endocytosis inhibition, when endosomes were absent. It should be noted that due to the nature of the chemical inhibitors we used, which were membrane permeable, we were limited to examining isolated, rather than follicle-enclosed, oocytes. It would be interesting to determine whether inhibiting endocytosis affects LH-induced meiotic resumption in follicle-enclosed oocytes. Future experiments will be needed to examine the mechanisms by which endocytosis occurs in oocytes and how GPR3 might be regulated in order to maintain the proper concentration of cAMP that is necessary to maintain meiotic arrest in the oocyte.

Materials and Methods

Media and reagents

Except where noted, all chemicals were obtained from Sigma Chemical Co. The medium used to collect isolated oocytes was MEM α (Invitrogen), supplemented with 20 mM HEPES, 75 μ g/ml penicillin G, 50 μ g/ml streptomycin, 0.1% polyvinyl alcohol, and 10 μ M milrinone to inhibit spontaneous meiotic resumption. For extended culture, oocytes were placed in bicarbonate-buffered MEM α as above, in which the HEPES was replaced with 25 mM sodium bicarbonate. The medium used to collect and culture follicle-enclosed oocytes was MEM α supplemented with 75 μ g/ml penicillin G, 50 μ g/ml streptomycin, and 5% fetal bovine serum (#12000-022, Invitrogen).

Mouse GPR3-RFP was provided by Y Saeki (Ohio State University) in pHGCY and was linearized with PacI prior to *in vitro* transcription using the Superscript III kit from Invitrogen. GRK2 was provided by R Lefkowitz (Duke University Medical Center) in pRK5 and was subcloned into the EcoRI and BamHI sites of pBSSK⁺ vector. RNA was linearized with BamHI prior to *in vitro* transcription. β -Arrestin-2-GFP was obtained from M Caron (Duke University Medical Center) in pS65T and was subcloned into pSP64.5 and was linearized with Sall prior to *in vitro* transcription. Rat β_2 AR was obtained from N Ancellin (University of CT Health Center) in pBSSK⁺ and was linearized with NotI prior to *in vitro* transcription. MDC was prepared as a 150 mM stock in DMSO. Dynasore (Tocris Bioscience, Ellisville, MO, USA) was prepared as a 5 mM stock in ethanol. FM 1-43 (Invitrogen) was prepared as a 2 mM stock in water.

Mouse oocyte and follicle isolation and culture

All experiments were done with prior approval of the Animal Care and Use Committee at the University of Connecticut Health Center.

Fully grown, GV-stage mouse oocytes were obtained from the ovaries of 6- to 12-week old CF-1 mice (Harlan Sprague-Dawley, Indianapolis, IN, USA) that had been primed with 10 IU eCG (Sigma or Calbiochem) 40–46 h prior to collection. Cumulus cells were removed by repeated pipetting through a small-bore pipette. Oocytes were cultured in 200 μ l drops of medium under light mineral oil on a warming tray set to 37 °C (when in HEPES-buffered medium) or in a humidified atmosphere at 37 °C with 5% CO₂ and 95% air (when in bicarbonate-buffered medium). In some cases, the zonae pellucidae were removed using 10 μ g/ml α -chymotrypsin (type II).

Antral follicles ($\geq 320 \mu$ m in diameter) were dissected from the ovaries of 23- to 25-day old B6SJL/F1 mice (Jackson Laboratories) as described previously (Jaffe *et al.* 2009). Following isolation, follicles were plated on Millicell culture plate inserts (PICMORG50, Millipore, Billerica, MA, USA) and cultured in a humidified atmosphere at 37 °C with 5% CO₂ and 95% air. After a 3 h culture period, follicle-enclosed oocytes were examined under an upright microscope for the presence of a GV. Only follicles containing oocytes with readily visible GVs were selected for use in these experiments.

Microinjection

Microinjection of isolated and follicle-enclosed oocytes was carried out as described previously (Jaffe *et al.* 2009, Kline 2009). For isolated oocytes, oocytes were placed in HEPES-buffered MEM α for microinjection. For overnight culture, oocytes were incubated in bicarbonate-buffered MEM α containing milrinone. Follicles were loaded into an injection chamber between two coverslips spaced $\sim 200 \mu$ m apart. Following microinjection, follicles were plated on Millicell membranes and incubated for 17–24 h, the oocytes were removed, and examined for their meiotic status. Quantitative microinjection was carried out using pipettes backfilled with mercury and concentrations of injected substances were calculated based on an oocyte volume of 200 pl.

Immunoblotting and immunofluorescence

For western blots, oocyte samples were made by washing oocytes in PBS containing 0.1% polyvinyl alcohol (PVA) to remove serum. Oocytes were transferred to microcentrifuge tubes, gently pelleted, and excess culture medium was removed. Oocytes were frozen in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$ until use. Western blotting was performed as described previously (Mehmann *et al.* 1998). Except for anti-EEA1, which was obtained from Cell Signaling Technology (Danvers, MA, USA), primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blots were developed using ECL Plus reagents (Amersham).

For immunofluorescence, oocytes were fixed for 1 h in 2% formaldehyde in 100 mM HEPES, 50 mM EGTA, 10 mM MgSO₄, and 0.2% Triton X-100 at 37 °C. After fixation, oocytes were incubated in blocking buffer (PBS containing 0.01% Triton X-100, 0.1% PVA, and 3% BSA), then in primary antibody overnight, diluted 1:100 in blocking buffer, at room temperature. Oocytes were then washed in blocking buffer and were incubated in secondary antibody and finally in PBS

containing 0.1% PVA. The secondary antibody was Alexa Fluor 488-conjugated anti-rabbit (Invitrogen). Oocytes were observed with a 40 \times , 1.2 NA lens (C-Apochromat; Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) on either a Zeiss 510 or a Zeiss Pascal confocal microscope.

RT-PCR

RNA was extracted from oocytes or mouse brain using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. cDNA was produced using the Superscript III Reverse Transcriptase kit (Invitrogen) using oligo-dT as the primer. A total of seven oocyte equivalents or 125 ng brain cDNA was used for each PCR reaction. The cycling parameters were an initial denaturation of 2 min, 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. PCR products were electrophoresed on 1.5% agarose gels, visualized by staining the gel with SYBRGold (Invitrogen), and photographed with a digital camera (Canon Power Shot A650). The primer sets used for each experiment are shown in Table 1.

FM 1-43 membrane labeling

To examine endocytosis, zona-free oocytes were incubated in 2 μ M FM 1-43 (Invitrogen). FM 1-43 was diluted in Ca²⁺/Mg²⁺-free Hank's buffered salt solution (HBSS; Gibco) containing 0.1% PVA and oocytes were examined with a Zeiss LSM 510 confocal microscope after ~ 1 –2 h. Fluorescence was excited at 488 nm and was detected at 560 nm. Images were collected using a 40 \times NA 1.2 water immersion objective. The method for quantifying plasma membrane and membrane to cytoplasmic ratios has previously been described in detail (supplemental material in Freudzon *et al.* (2005), using MetaMorph Software (Molecular Devices Corp., Downingtown, PA, USA) and Microsoft Excel.

Table 1 Primer sets used for RT-PCR.

Dynamin 1	5'-ATCTGAAGCTGCGTGATGTG-3' 5'-GCCGATTCTTCCATCAGTGT-3'
Dynamin 2	5'-GACCTCCCAGGCATCACTAA-3' 5'-ACTCTCGGATGTGGTTGGTC-3'
Dynamin 3	5'-GCTTGTGTGCTGTGCTTTGT-3' 5'-ATCGCTGATGGATCACCTTC-3'
AP-2	5'-CTCAAGCCCTCACTTCTGG-3' 5'-TACTTGGACCTGTGGGAAGG-3'
Amphiphysin	5'-GGATTCTCAGGCACTCTCG-3' 5'-TGCTCTTCCCTCTTCGTA-3'
β -arrestin-1	5'-CCCATGTGTGAAGGGCTAGT-3' 5'-AATGACTTCACGGGACAAGG-3'
β -arrestin-2	5'-CAACGTCCATGTCACCAACAA-3' 5'-ACCACCAGCTTCACCTTGA-3'
GRK2	5'-ACCAGGGAAGTGTACCGCAAC-3' 5'-TGCGTTCCTTGATCTGTGTC-3'
GRK3	5'-AAGTTCGACAACCAAGAGGA-3' 5'-GTGTGGAAGGCGTAGGTCAT-3'
GRK5	5'-GGTGTGGAGACTGAGGAAG-3' 5'-CCGTAGGTCCGAACACATT-3'
GRK6	5'-ACTCTTCAGTCGCCAAAGGA-3' 5'-GCCCTAGAAGCCTGGAGTTT-3'

cAMP measurements

cAMP measurements were made using a cAMP sensor (Epac2-camps300) that has previously been described (Nikolaev *et al.* 2004, Norris *et al.* 2009). The sensor is FRET based and consists of YFP and CFP linked by the cAMP-binding domain of EPAC2. Epac2-camps300 protein (5 μ M final concentration in the oocyte) was injected into isolated oocytes cultured in the presence of milrinone. Fluorescence was excited at 435 nm and was detected at 535/50 nm (YFP) and at 480/40 nm (CFP). Images were collected using a 40 \times NA 1.2 water immersion objective on a Zeiss Pascal confocal microscope. YFP and CFP intensities were quantified within a circular region of interest that was slightly smaller than the oocyte diameter using the Zeiss Pascal program. Data are reported as the ratio of YFP to CFP fluorescence after subtracting the background value from an uninjected oocyte and correcting for spectral bleed-through of CFP into the YFP channel (Norris *et al.* 2009).

Statistical analysis

Student's *t*-tests or one-way ANOVA followed by Tukey's multiple comparison post-test were performed to determine statistical significance; $P < 0.05$ was considered to be significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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