Regulation of *Xenopus* oocyte meiosis arrest by G protein βγ subunits

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Background: Progesterone induces the resumption of meiosis (maturation) in *Xenopus* oocytes through a nongenomic mechanism involving inhibition of an oocyte adenylyl cyclase and reduction of intracellular cAMP. However, progesterone action in *Xenopus* oocytes is not blocked by pertussis toxin, and this finding indicates that the inhibition of the oocyte adenylyl cyclase is not mediated by the α subunits of classical G_i-type G proteins.

Results: To investigate the possibility that G protein βγ subunits, rather than α subunits, play a key role in regulating oocyte maturation, we have employed two structurally distinct G protein βγ scavengers ($G_t\alpha$ and βARK-C_{CAAX}) to sequester free Gβγ dimers. We demonstrated that the injection of mRNA encoding either of these Gβγ scavengers induced oocyte maturation. The Gβγ scavengers bound an endogenous, membrane-associated Gβ subunit, indistinguishable from *Xenopus* Gβ1 derived from mRNA injection. The injection of *Xenopus* Gβ1 mRNA, together with bovine Gγ2 mRNA, elevated oocyte cAMP levels and inhibited progesterone-induced oocyte maturation.

Conclusion: An endogenous G protein βγ dimer, likely including *Xenopus* Gβ1, is responsible for maintaining oocyte meiosis arrest. Resumption of meiosis is induced by Gβγ scavengers in vitro or, naturally, by progesterone via a mechanism that suppresses the release of Gβγ.

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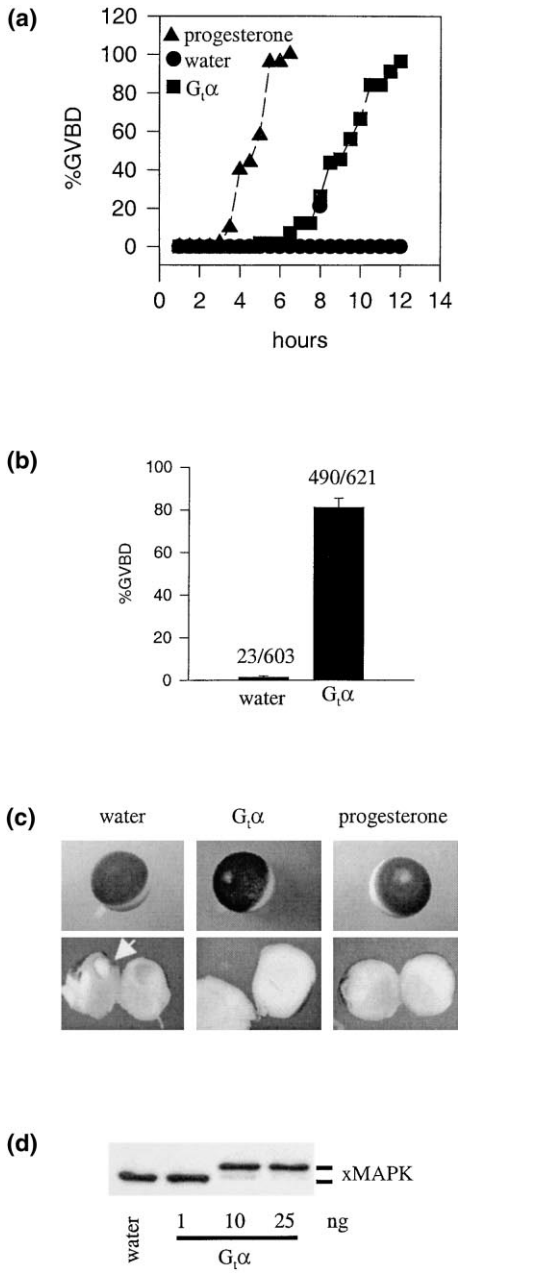
Introduction

Xenopus laevis oocytes naturally arrest at the G_2 phase of meiosis I. Progesterone triggers the resumption of meiosis or oocyte maturation (routinely defined by germinal-vesicle breakdown, or GVBD). Progesterone-induced oocyte GVBD has served as an experimental paradigm in many fundamental discoveries in cell cycle regulation and in signal transduction [1, 2]. In their seminal study that led to the discovery of maturation-promoting factor (MPF), Masui and Markert [3] demonstrated that progesterone could induce MPF activation in enucleated oocytes, and they thereby established the extranuclear nature of progesterone-signaling pathway in *Xenopus* oocytes.

It is likely that trimeric guanine nucleotide binding proteins (G proteins) play central roles in regulating oocyte maturation. Progesterone stimulation causes the inhibition of membrane bound oocyte adenylyl cyclase activity [4, 5] and a transient reduction of oocyte cAMP levels [6]. The importance of cAMP reduction in the resumption of meiosis is supported by studies demonstrating that the inhibition of cAMP-dependent protein kinase (PKA) causes hormone-independent GVBD and that the injection of active PKA inhibits progesterone-induced GVBD [7]. However, the inhibition of adenylyl cyclase or the induction of GVBD by progesterone is not blocked by Bordetella pertussis toxin (PTX) [8–10]. This finding suggests that the α subunits of classical G_i proteins are not involved in the inhibition of oocyte adenylyl cyclase. Indeed, the injection of activated α subunits of three mammalian G_i proteins (G_{i1}, G_{i2}, and G_{i3}) into *Xenopus* oocytes does not cause GVBD [11]. Although the injection of activated $G_o\alpha$ can cause GVBD, $G_o\alpha$-induced GVBD is thought to be mediated via its activation of oocyte protein kinase C but is not related to progesterone signaling [11].

These studies prompted us to explore the possibility that G protein βγ subunits (Gβγ) might play a role in oocyte maturation. It is well established that, upon G protein activation, not only do the α subunits bind GTP and signal downstream, but the released Gβγ complexes also often cooperate with the GTP bound α subunits or signal independently [12, 13]. In this study, we have employed two well-characterized Gβγ scavengers, the α subunit of bovine transducin ($G_t\alpha$) and the C-terminal Gβγ binding domain of bovine β adrenergic receptor kinase (βARK-C), to study the potential role of endogenous Gβγ in regulating oocyte maturation.

Figure 1



$G_t\alpha$ induces oocyte GVBD. **(a)** Groups of 50 or more oocytes were injected with water or mRNA encoding $G_t\alpha$. A third group of oocytes was incubated with progesterone (1 μM). At the indicated time following injection (or following the addition of progesterone), oocytes were scored for GVBD and expressed as a percentage of total treated oocytes. Shown is a representative of three independent experiments. **(b)** Oocytes were injected with water or $G_t\alpha$ mRNA. Injected oocytes were incubated for 15–24 hr before being scored for GVBD. Shown are the means (with errors) of 15 independent experiments with the actual numbers of GVBD-positive/total injected oocytes indicated on top. **(c)** Typical images of intact oocytes (top panels) and oocytes after they were fixed in 5% trichloroacetic acid and dissected (bottom panels). An arrow indicates a germinal vesicle in a GVBD-negative oocyte. **(d)** Groups of 20 or more oocytes were injected with water or the various amounts of $G_t\alpha$ mRNA (per oocyte). Injected oocytes were incubated for 15–24 hr before being lysed and subjected to immunoblotting with anti-xMAP kinase antiserum. Shown is a representative of three independent experiments.

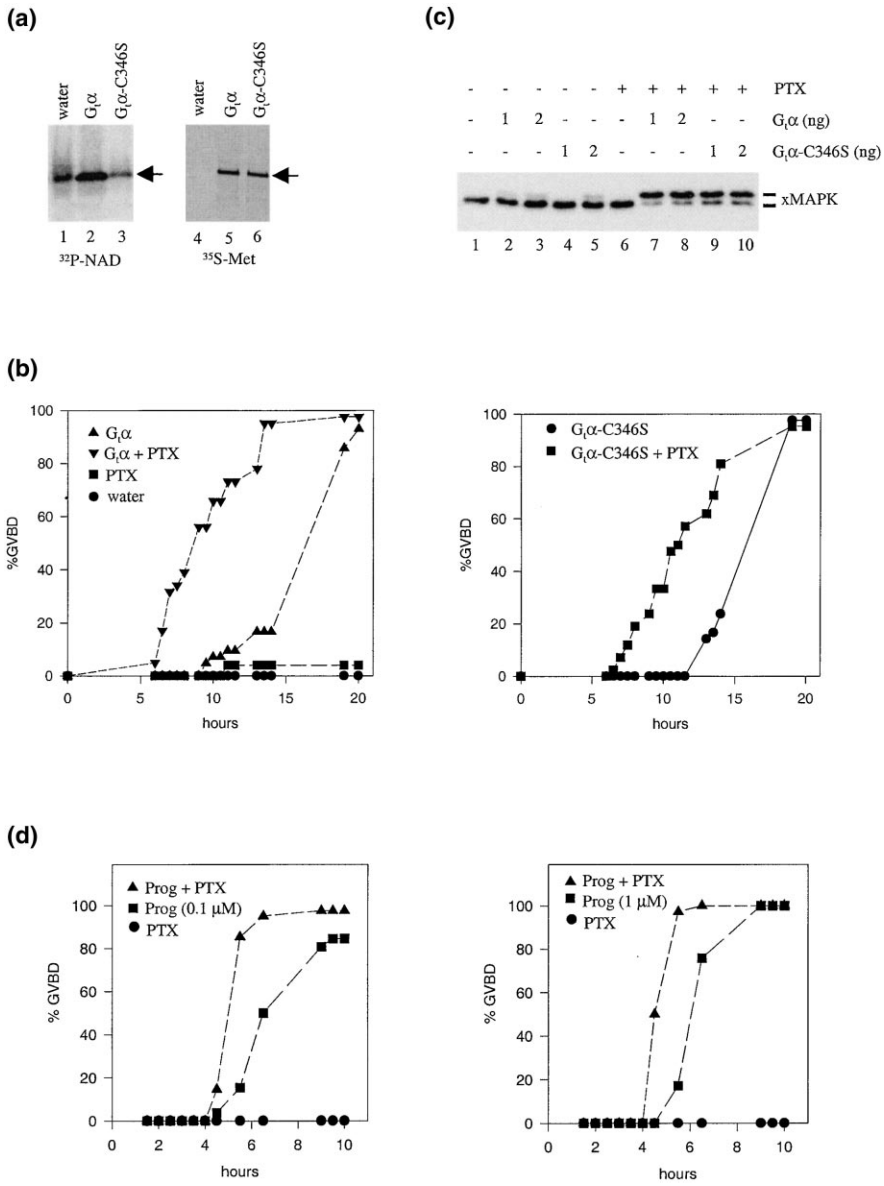
Results

G protein βγ scavengers induce GVBD

To examine the possible involvement of Gβγ in regulating oocyte maturation, we employed a well-characterized Gβγ scavenger, the α subunit of bovine transducin ($G_t\alpha$) [14, 15]. $G_t\alpha$ mRNA injection followed by incubation of oocytes in OR2 (oocyte incubation medium) caused a time-dependent induction of GVBD (Figure 1a). $G_t\alpha$-induced GVBD lagged behind GVBD induced by the natural hormone progesterone. This result presumably reflects the time required for translation and posttranslational modification of $G_t\alpha$. $G_t\alpha$ induction of GVBD was dose dependent; it required 10 ng mRNA per oocyte for efficient induction (Figures 1b,d). GVBD induced by $G_t\alpha$ mRNA injection was morphologically indistinguishable from that induced by progesterone (Figure 1c), and $G_t\alpha$-induced GVBD was accompanied by the characteristic *Xenopus* MAP kinase phosphorylation (Figure 1d) and activation (data not shown). As shown previously [16, 17], MAP kinase phosphorylation in *Xenopus* oocytes is an all-or-none event, and the ratio of phosphorylated (active) MAP kinase vs. unphosphorylated (inactive) MAP kinase therefore reflects the ratio of GVBD-positive versus GVBD-negative oocytes. Therefore, we used both GVBD and MAP kinase phosphorylation to measure oocyte maturation.

The overexpression of $G_t\alpha$, however, may result in a sufficient proportion exhibiting aberrant (receptor-independent) GTP binding to affect endogenous effector systems. To eliminate this possibility, we employed PTX in order to ADP-ribosylate $G_t\alpha$ and to inhibit its GTP binding [18]. As a control, we generated a point mutant ($G_t\alpha$-C346S) to eliminate the ADP-ribosylation site [18]. Like the wild-type $G_t\alpha$, $G_t\alpha$-C346S efficiently induced oocyte GVBD (see Figure 2b, right panel). Membrane extracts from oocytes injected with water or mRNA encoding $G_t\alpha$ or $G_t\alpha$-C346S were subjected to in vitro ADP-rybosylation assays. Figure 2a shows that PTX modified an endogenous protein, likely representing an α subunit(s) of the G_i class (lane 1), as reported previously [10]. Significantly higher levels of ADP ribosylation occurred in membrane extracts from oocytes injected with wild-type $G_t\alpha$ mRNA (lane 2) but not with $G_t\alpha$-C346S mRNA (lane 3). This finding indicates that the wild-type $G_t\alpha$, in addition to the endogenous Gα, was modified by PTX. Surprisingly, coinjection of PTX accelerated $G_t\alpha$-induced GVBD (Figure 2b, left panel). PTX similarly accelerated $G_t\alpha$-C346S–induced GVBD (Figure 2b, right panel). This synergism was also evident when suboptimal amounts of $G_t\alpha$ or $G_t\alpha$-

Figure 2

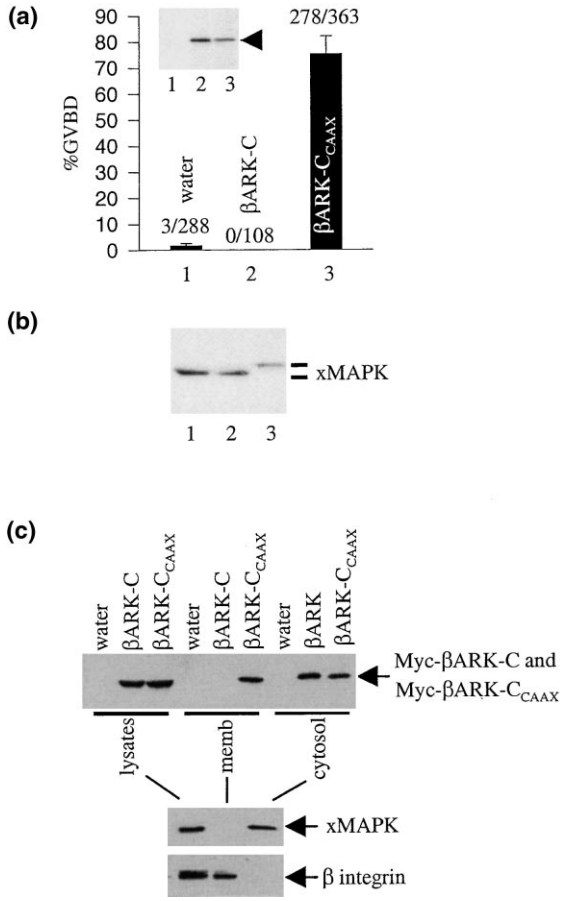


Synergism between $G_t\alpha$ and PTX. **(a)** Oocytes injected with water or with mRNA for $G_t\alpha$ or $G_t\alpha$-C346S were incubated overnight in OR2. Oocyte membranes were isolated and subjected to in vitro ADP-ribosylation assays. Samples were analyzed by SDS-PAGE followed by autoradiography (lanes 1–3). Shown is a representative of three independent experiments. As a control, 1 μg of each of the two types of mRNA were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Samples were analyzed by SDS-PAGE followed by autoradiography (lanes 4–6). **(b)** Oocytes (at least 50 per group) were injected with PTX (0.2 ng per oocyte), $G_t\alpha$ (or $G_t\alpha$-C346S) mRNA, or both, as indicated. At the indicated time following the injection, each group (plus a control group injected with water only) was monitored for GVBD. For clarity, the data are presented in two separate graphs. Shown is a representative of three independent experiments. **(c)** Groups of at least 50 oocytes were injected with PTX, $G_t\alpha$ (or $G_t\alpha$-C346S) mRNA (1 or 2 ng per oocyte as indicated), or both PTX and the mRNA. The injected oocytes were incubated in OR2 for 6–10 hr, by which time at least 50% of the oocytes coinjected with PTX and mRNA had undergone GVBD (data not shown). The oocytes were lysed for MAP kinase immunoblotting. Shown is a representative of three independent experiments. **(d)** Oocytes injected with water or PTX were incubated for 2 hr before being divided into groups of 30 oocytes each and incubated with the indicated concentrations of progesterone. At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of five independent experiments.

C346S mRNA were injected. In this case, coinjection of the mRNA and PTX, but not either alone, caused significant GVBD (data not shown) and MAP kinase activation (Figure 2c). The ability of PTX to accelerate $G_t\alpha$-C346S–induced GVBD suggests that ADP ribosylation of an endogenous G protein(s) (Figure 2a) is responsible. Interestingly, an earlier study reported the synergistic effect of PTX on progesterone-induced GVBD [19], although others have reported the opposite effect [10, 20]. We have consistently observed that PTX accelerates progesterone-induced GVBD (Figure 2d).

To further establish a role for Gβγ in regulating oocyte meiosis, we employed a structurally distinct Gβγ scavenger, the noncatalytic C-terminal region of β adrenergic receptor kinase (βARK-C) [21, 22]. The injection of βARK-C mRNA failed to induce oocyte GVBD (Figure 3a, lane 2). We reasoned that targeting βARK-C to the membrane might be necessary for its efficient binding to the membrane bound Gβγ. To create a membrane targeting βARK-C, we changed the C-terminal four amino acids of βARK-C to a geranylgeranylation site (CVLL). We termed this construct βARK-C_{CAAX} according to the original nomenclature for isoprenylation consensus [23]. An identical alteration of the full-length βARK results in its constitutive association with the membranes [24]. The injection of βARK-C_{CAAX} mRNA efficiently induced GVBD (Figure 3a, lane 3) and MAP kinase activation

Figure 3



βARK-C_{CAAX} induces oocyte maturation. **(a)** Oocytes were injected with water or mRNA for βARK-C or βARK-C_{CAAX}. Injected oocytes were incubated for 15–20 hr before GVBD scoring. Shown are the means (with errors) of 3–8 independent experiments. The actual numbers of GVBD-positive oocytes/total injected oocytes are indicated. Inset is an in vitro translation experiment in which 1 μg each of the two types of mRNA was used. **(b)** Following GVBD scoring (as in 3a), oocytes were lysed and subjected to xMAP kinase immunoblotting. Shown is a representative of three independent experiments. **(c)** Oocytes injected with water or Myc-βARK-C (or Myc-βARK-C_{CAAX}) mRNA were incubated overnight. Oocyte extracts and membrane and cytosol fractions (each lane contained the equivalent of 1/2 oocyte) were immunoblotted with anti-Myc antibodies (top panel). Extracts, membrane, and cytosol derived from water-injected oocytes were also immunoblotted with anti-xMAP kinase (middle panel) or anti-*Xenopus* β-integrin (bottom panel). Shown is a representative of three independent experiments.

(Figure 3b, lane 3). To facilitate subcellular localization and binding studies, we generated Myc-tagged versions of both βARK-C and βARK-C_{CAAX}. The presence of the Myc tag did not alter the ability of βARK-C_{CAAX} to induce GVBD (data not shown). Subcellular fractionation experiments confirmed that a significant proportion of Myc-βARK-C_{CAAX} had been targeted to the membrane, whereas Myc-βARK-C was exclusively found in the cytosol (Figure 3c).

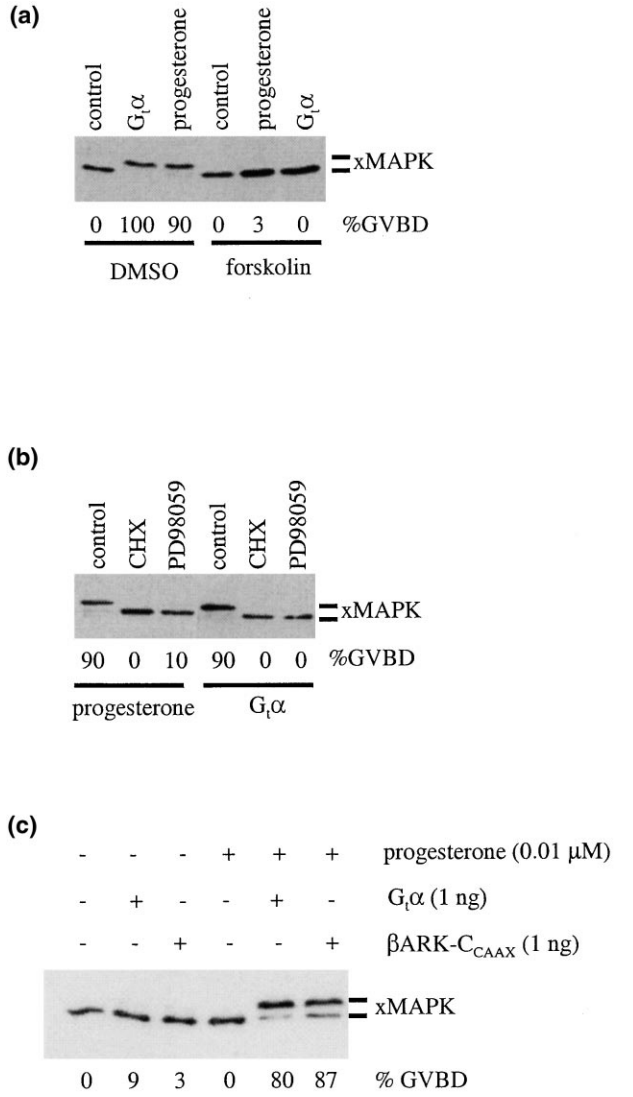
To determine whether $G_t\alpha$-induced GVBD shares the same characteristics as GVBD induced by the natural hormone progesterone, we tested three well-known GVBD inhibitors: the adenylyl cyclase activator forskolin [25], a protein synthesis inhibitor cycloheximide (CHX), and a MAP kinase kinase inhibitor, PD98059 [2]. All three blocked $G_t\alpha$-induced GVBD (Figures 4a,b). Expectedly, both $G_t\alpha$ and βARK-C_{CAAX} potentiated progesterone in the induction of GVBD and MAP kinase activation (Figure 4c).

Gβγ subunits inhibited Gβγ scavenger– or progesterone-induced GVDB

We tested two commercial rabbit polyclonal anti-β antibodies in an attempt to detect endogenous oocyte Gβ proteins. Sc378, which was raised against the C terminus of mouse Gβ1, detected a prominent protein of relative molecular mass of about 35 kDa in oocyte extracts (Figure 5a, lanes 1–3). This finding is consistent with the protein being an endogenous *Xenopus* Gβ (xGβ). The identity of a second protein, of slightly greater molecular mass, is unknown. Sc261, which was raised against the N terminus of bovine Gβ1, did not detect either of these proteins (not shown, but see Figure 6a). As expected, xGβ was associated with oocyte membrane (Figure 5b). To determine whether Myc-βARK-C_{CAAX} bound xGβ, we performed coimmunoprecipitation experiments. Anti-Myc antibodies pulled down similar amounts of Myc-βARK-C (Figure 5a, lower panel, lane 5) or Myc-βARK-C_{CAAX} (lane 6) from oocytes injected with the respective mRNA. However, xGβ was only pulled down with Myc-βARK-C_{CAAX} (Figure 5a, upper panel, lane 6) but not with Myc-βARK-C (lane 5). Anti-Myc also did not pull down xGβ in extracts made from water-injected oocytes (lane 4).

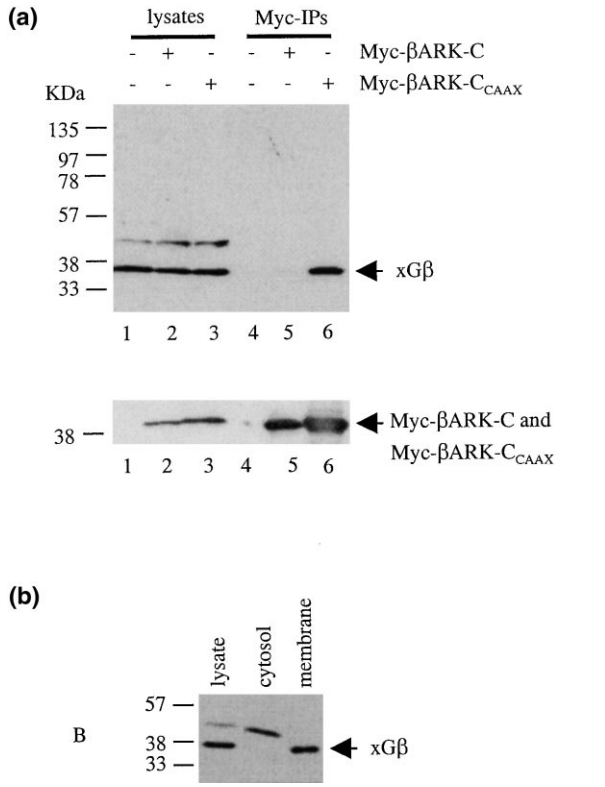
Our results suggest that the $G_t\alpha$ and βARK-C_{CAAX} induced GVBD by binding and sequestering an endogenous Gβγ complex. Although the identity of xGβ is not known, we believe that it is xGβ1, not only because it is recognized by anti-Gβ1 antibodies but also because xGβ1 is the only G protein β subunit identified in oocytes [26]. We amplified xGβ1 by PCR and injected the corresponding mRNA into oocytes, either alone or in combination with bovine Gγ2. Figure 6a shows that the overexpressed xGβ1 (lanes 5 and 6), like the endogenous xGβ (lane 4), was readily recognized by Sc378 anti-mouse Gβ1. The overexpressed xGβ1 was poorly, but reproducibly, recognized by Sc261 anti-bovine Gβ1 (Figure 6a, lanes 2 and 3). The weak cross-reactivity of Sc261 for xGβ1 may explain its inability to detect the much lower levels of endogenous protein. Coimmunoprecipitation experiments (Figure 6b) indicated that an increased level of Gβ became associated with Myc-βARK-C_{CAAX} in oocytes injected with xGβ1 mRNA (Figure 6b; compare lane 5 to 4). These results further support the notion that the endogenous xGβ is xGβ1.

Figure 4



Gβγ scavengers potentiate progesterone action in *Xenopus* oocytes. **(a)** Oocytes were incubated in OR2 (control) or OR2 containing 1 μM progesterone, or they were injected with $G_t\alpha$ mRNA. Either forskolin (50 μM) or an equivalent volume of dimethylsulfoxide (DMSO) was added to each group, as indicated. Oocytes were incubated for 15 hr before GVBD scoring followed by anti-xMAP kinase immunoblotting. Shown is a representative of three independent experiments. **(b)** Oocytes were treated with progesterone (1 μM) or injected with $G_t\alpha$ mRNA. Overnight incubation was carried out in OR2 (control) or OR2 containing 10 μg/mL CHX or 50 μM PD98059. Oocytes were scored for GVBD, then anti-xMAP kinase immunoblotting was performed. Shown is a representative of three independent experiments. **(c)** Oocytes were injected with water or mRNA (1 ng per oocyte) for $G_t\alpha$ or βARK-C_{CAAX}. Following an overnight incubation in OR2, the injected oocytes were further incubated for 10–15 hr in OR2 (lanes 1–3) or OR2 containing 10 nM progesterone (lanes 4–6) before GVBD scoring and anti-xMAP kinase immunoblotting were performed. Shown is a representative of three independent experiments.

Figure 5



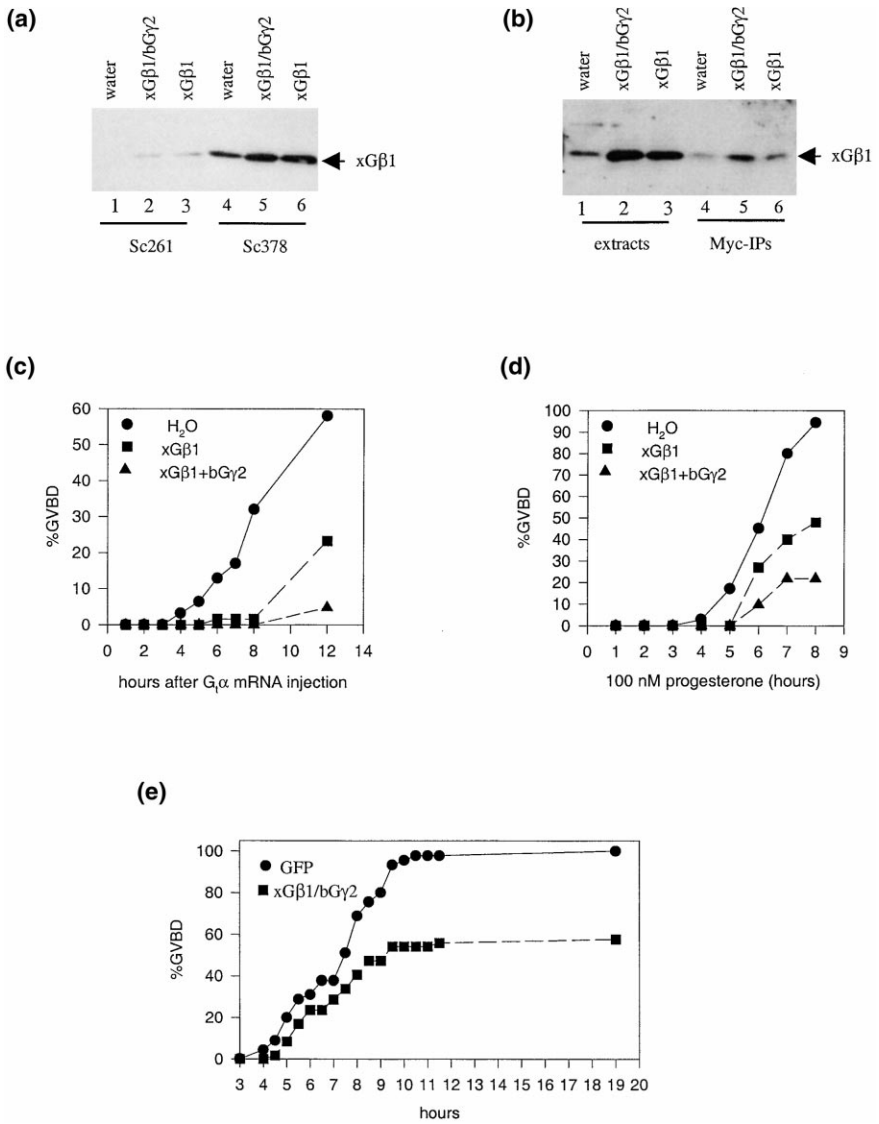
Association of Myc-βARK-C_{CAAX} with an endogenous Gβ. **(a)** Extracts (lanes 1–3, one oocyte equivalent) or anti-Myc immunoprecipitates (lanes 4–6, each derived from 20 oocytes) from oocytes injected with water (lanes 1 and 4) or mRNA encoding Myc-βARK-C (lanes 2 and 5) or Myc-βARK-C_{CAAX} (lanes 3 and 6) were immunoblotted with anti-Gβ1 (upper panel) or anti-Myc (lower panel). Shown is a representative of four independent experiments. **(b)** Extracts (one oocyte), supernatant (cytosol, two oocytes), or pellet (membrane, two oocytes), following centrifugation at 100,000 g, were immunoblotted with anti-Gβ1. Shown is a representative of three independent experiments.

Injection of xGβ1 mRNA significantly reduced the ability of $G_t\alpha$ (Figure 6c) or βARK-C_{CAAX} (data not shown) to induce GVBD. Coinjection of bovine Gγ2 mRNA further enhanced the inhibitory effect of xGβ1. Importantly, the injection of xGβ1 mRNA, alone or in combination with bovine Gγ2 mRNA, also reduced the ability of progesterone to induce GVBD (Figure 6d). The possibility of non-specific effects on oocyte protein synthesis due to mRNA injection was eliminated since injection of an equal amount of a control mRNA, green fluorescence protein (GFP) [27], did not affect progesterone-induced GVBD (Figure 6e).

Overexpression and activation of human β_2AR inhibits progesterone- and Gβγ scavenger–induced GVBD

Our data implicate an endogenous G protein βγ complex as a key inhibitor of oocyte meiosis. How is this Gβγ

Figure 6



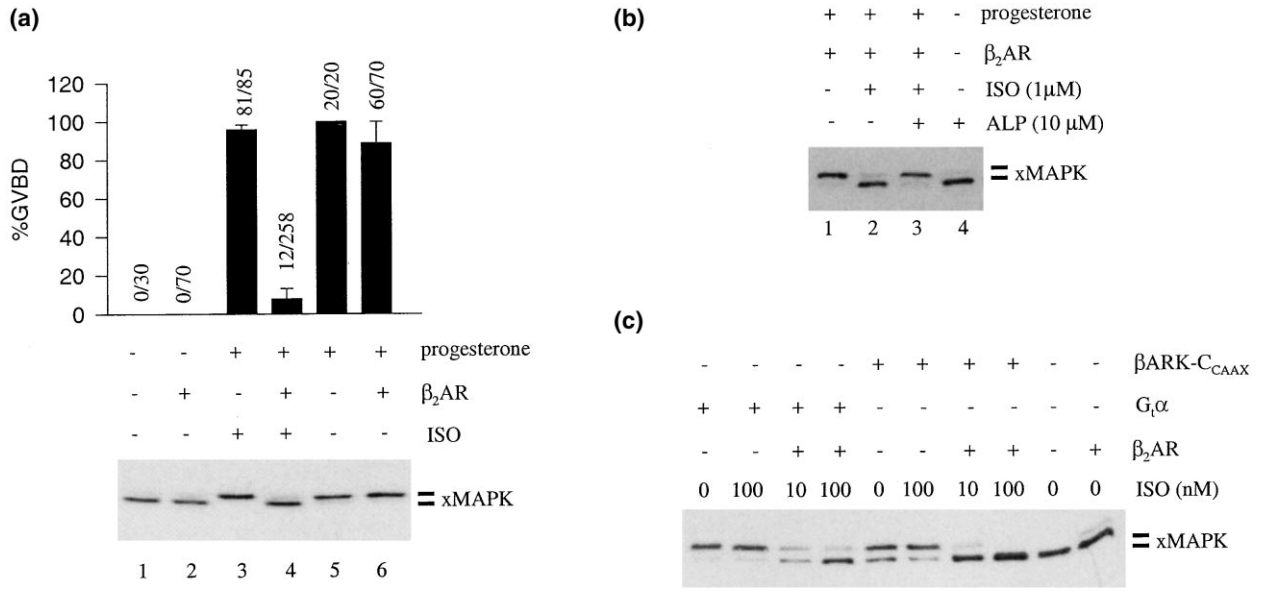
Overexpression of Gβγ blocks GVBD. **(a)** Oocytes were injected with water (lanes 1 and 4), xGβ1 mRNA (lanes 3 and 6), or a combination of xGβ1 mRNA and bovine Gγ2 mRNA (lanes 2 and 5). Following an overnight incubation in OR2, oocyte extracts were prepared and immunoblotted with Sc261 (lanes 1–3) or Sc378 (lanes 4–6) anti-Gβ1 antibodies. Shown is a representative of three independent experiments. **(b)** Oocytes were injected with Myc-βARK-C_{CAAX} mRNA together with water (lanes 1 and 4), xGβ1 mRNA (lanes 3 and 6), or a combination of xGβ1 mRNA and bovine Gγ2 mRNA (lanes 2 and 5). Following an overnight incubation in OR2, oocyte extracts were prepared and immunoprecipitated with anti-Myc. The extracts (lanes 1–3) and immunoprecipitates (lanes 4–6) were blotted with anti-Gβ1 antibodies. Shown is a representative of two independent experiments. **(c)** Oocytes injected with water or the indicated mRNA (xGβ1, bGγ2, or both) were incubated overnight in OR2 before a second injection with $G_t\alpha$ mRNA. At the indicated time following $G_t\alpha$ mRNA injection, oocytes were scored for GVBD. Each group contained at least 30 oocytes. Shown is a representative of three independent experiments. **(d)** Oocytes injected with water or the indicated mRNA (xGβ1, bGγ2, or both) were incubated overnight in OR2 before the addition of progesterone (100 nM). At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of three independent experiments. **(e)** Oocytes injected with a control mRNA (GFP, 10 ng per oocyte) or a mixture of xGβ1 and bGγ2 (5 ng each per oocyte) were incubated overnight in OR2 before the addition of progesterone (1 μM). At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of two independent experiments.

generated in G_2-arrested oocytes? We reasoned that there might be a constitutively active G protein–coupled receptor (GpCR) in G_2 oocytes that is responsible for activating a trimeric G protein and releasing the inhibitory Gβγ. The inhibition of this putative GpCR might therefore inhibit the release of the Gβγ and hence cause GVBD. A survey of well-characterized GpCR antagonists (those specific for β adrenergic receptors, acetylcholine receptors, dopamine receptors, or serotonin receptors) found that only very high and nonphysiological concentrations (500 μM to 1 mM) of propranolol or alprenolon, both antagonists for β adrenergic receptors, were able to induce GVBD (data not shown). Interestingly, an earlier study [28] has reported similar findings, although it has concluded that the ability of these antagonists to induce GVBD is the result of their nonspecific interaction with other membrane components [28]. Indeed, whereas the agonist for β adrenergic receptor, isoproterenol (ISO), had no effect on progesterone-induced GVBD or MAP kinase activation (Figure 7a, lane 3), ISO blocked both in oocytes that had been injected with human β_2 adrenergic receptor (β_2AR) [13] mRNA (lane 4). The inhibitory effect of β_2AR/ISO was completely reversed by 10 μM alprenolon (ALP, Figure 7b, lane 3), which otherwise had no effect on oocyte maturation (Figure 7b, lane 4). Similarly, the β_2AR/ISO combination also effectively blocked Gβγ scavenger–induced MAP kinase activation (Figure 7c) and GVBD (data not shown). These results indicate that oocytes do not have a functional β adrenergic–receptor system.

Regulation of oocyte cAMP by $G_t\alpha$ and Gβγ

We wished to determine whether Gβγ scavengers, like progesterone, reduced oocyte cAMP levels [1, 2]. It is

Figure 7



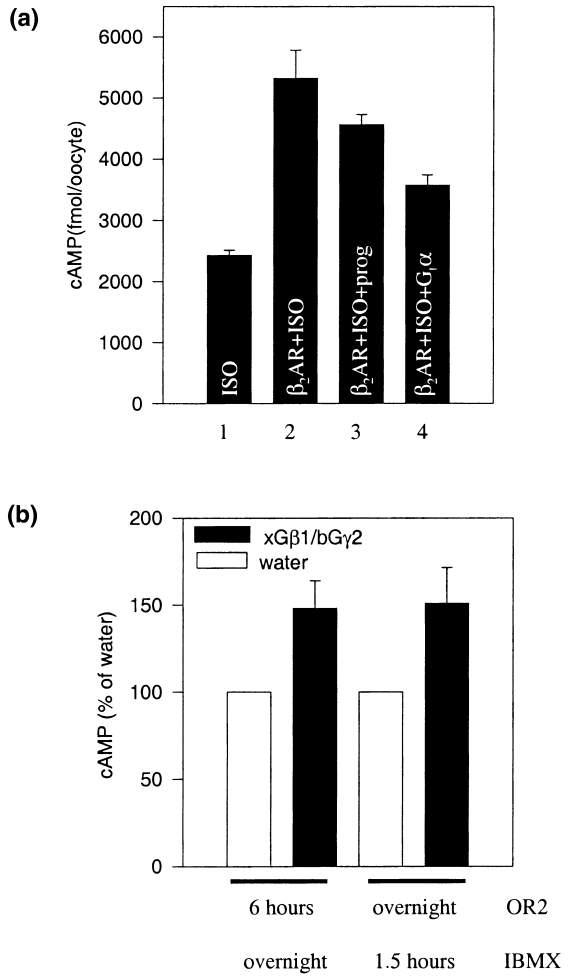
Human β_2AR overexpression blocks GVBD. **(a)** Oocytes were injected with water (lanes 1, 3, and 5) or human β_2AR mRNA (lanes 2, 4, and 6) and then incubated overnight in OR2. ISO (final 1 μM) was added to lanes 3 and 4. One hour following the addition of ISO, progesterone was added to the indicated groups. All groups were further incubated for at least 15 hr before GVBD scoring. Shown are means of three to seven independent experiments; the actual numbers of GVBD-positive oocytes/total treated oocytes are indicated. A typical xMAP kinase immunoblot is shown below. **(b)** Groups of at least 20 oocytes were injected with human β_2AR mRNA and then incubated overnight in OR2. Where indicated, ISO (1 μM), ALP (10 μM), or both were added. Oocytes were further incubated for at least 15 hr before being scored for GVBD (data not shown) and being lysed for anti-xMAP kinase immunoblotting. **(c)** Oocytes were injected with water or β_2AR mRNA followed by incubation in OR2 for 6 hr. Oocytes were injected again with water, $G_t\alpha$ mRNA, or βARK-C_{CAAX} mRNA. Following the second injection, oocytes were immediately transferred to OR2 containing the indicated concentrations of ISO, and incubation continued overnight. Oocytes were scored for GVBD (data not shown) and lysed for anti-xMAP kinase immunoblotting. Shown is a representative of three independent experiments. Each group contained at least 20 oocytes.

generally agreed that progesterone stimulation causes a transient and moderate (about 20%) reduction of oocyte cAMP levels. However, the direct demonstration of such a reduction is often difficult [6], presumably due to the significant variations of cAMP levels among individual oocytes [29]. In contrast, if oocytes are first treated with forskolin, which significantly raises cAMP levels, progesterone reproducibly blunts the forskolin-dependent cAMP elevations [25]. Taking a similar approach, we used overexpression and activation of human β_2AR [13] to elevate oocyte cAMP (Figure 8a, lane 2). Under these conditions, $G_t\alpha$ (lane 4) or progesterone (lane 3) clearly reduced oocyte cAMP levels. In contrast, the injection of xGβ1/bGγ2 significantly increased oocyte cAMP levels in the presence of cAMP phosphodiesterase inhibitor 3-isobutyp-1-methylxanthine (IBMX) (Figure 8). IBMX alone did not change oocyte cAMP levels (data not shown). These results suggest that *Xenopus* oocytes contain a Gβγ-responsive adenylyl cyclase.

Discussion

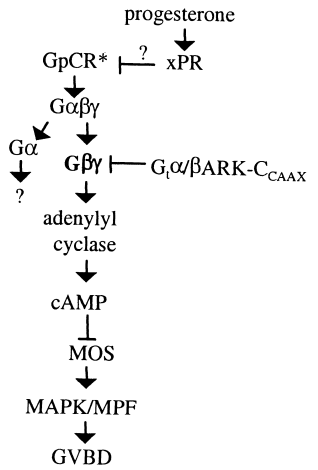
$G_t\alpha$ and βARK-C (βγ scavengers) are thought to specifically inhibit G protein function that involves Gβγ heterodimers as regulators of cellular effector systems. The specificity of Gβγ scavengers in blocking Gβγ function is supported by many studies in which they do not interfere with the signaling of many classical G proteins (G_s [21, 30], G_q, or G_o [31, 32]) that are dependent on GTP binding to the α subunits. In this study, we demonstrated that both $G_t\alpha$ and a membrane-targeting version of βARK-C, βARK-C_{CAAX}, were able to induce hormone-independent oocyte GVBD. The results of this study therefore implicate an endogenous Gβγ heterodimer in maintaining meiosis arrest. In a recent study [33], Lutz et al. demonstrated that the injection of mRNA encoding $G_t\alpha$ or the GRK minigene (identical to βARK-C) accelerates progesterone-induced GVBD but does not induce hormone-independent GVBD. Whereas the inability of the GRK minigene to induce GVBD [33] can be explained by its requirement of membrane association (Figure 3), the reasons for the discrepancy concerning $G_t\alpha$ are not known. It is possible that Lutz et al. did not obtain the relatively high concentrations of $G_t\alpha$ required to induce GVBD (see Figure 1d). It is also possible that oocytes that were isolated via collagenase treatment, as was the case in the study by Lutz et al. [33], were less responsive in GVBD induction than were manually isolated oocytes (in this study). Treating oocytes with commercial collagenase

Figure 8



Regulation of oocyte cAMP by Gβγ. **(a)** Oocytes were injected with water (lane 1) or β_2AR mRNA (lanes 2–4). Six hours later, one group (lane 4) was further injected with $G_t\alpha$ mRNA. Two hours following the $G_t\alpha$ injection, all groups were treated with ISO (1 μM) except for group 3, which was treated with a combination of ISO and 1 μM progesterone. One hour later, oocytes were lysed and subjected to cAMP assays. Shown are means (with standard errors) of two independent experiments done in duplicate determinations. **(b)** Oocytes were injected with water or a mixture of xGβ1 and bGγ2 mRNA (5 ng each mRNA per oocyte). Following incubation in OR2 for the indicated period of time, IBMX (0.5 mM) was added, and the incubation continued for the indicated length of time. Oocytes were than lysed and subjected to cAMP assays. The levels of cAMP are expressed as a percentage of levels in water-injected oocytes. Shown are means (with standard errors) of three to four independent experiments of duplicate determinations.

Figure 9



A working model. Details are explained in the text.

preparations is known to significantly reduce the oocytes' metabolic capacities [34].

We have further identified an endogenous xGβ that bound Myc-βARK-C_{CAAX} but not Myc-βARK-C. The lack of coimmunoprecipitation between the endogenous, membrane bound xGβ and the cytoplasmic Myc-βARK-C argues strongly that the coimmunoprecipitation of the xGβ and Myc-βARK-C_{CAAX} was the result of the two forming a complex at the membrane in intact oocytes. Although our data are consistent with assigning the endogenous xGβ as xGβ1, we cannot rule out the possible involvement of other Gβ isoforms since the anti-Gβ1 antibodies (both sc378 and sc261) are known to cross-react with other mammalian Gβ proteins.

Consistent with the notion that endogenous free Gβγ dimer functions to inhibit oocyte maturation, we have shown that the overexpression of xGβ alone or in combination with bovine Gγ2 significantly reduced the ability of Gβγ scavengers or progesterone to induce MAP kinase activation or GVBD. Similarly, Lutz et al. [33] have demonstrated that the injection of mRNA encoding bovine Gβ1 and Gγ2 partially inhibits progesterone-induced GVBD. Although Gβγ dimers function as single entities in all systems that have been studied, others have shown that overexpression of Gβ subunits alone have similar, albeit less potent, effects as overexpression of both Gβ and Gγ subunits [35, 36]. The overexpressed Gβ subunits may mimic native Gβγ dimers in interacting with cellular effectors [37], or they may increase the level of Gβγ dimers by promoting dimer formation, increasing Gγ protein synthesis, and/or increasing Gγ protein stability [38].

Our data implicate endogenous G protein βγ subunits as the natural inhibitors of oocyte meiosis in *Xenopus laevis* (Figure 9). Interestingly, earlier studies by others [39, 40] have suggested that an endogenous G protein βγ dimer functions to promote oocyte maturation in starfish. Whereas progesterone is the natural inducer of *Xenopus* oocyte maturation, starfish oocyte maturation is induced by the ovarian hormone 1-methyladenine [41]. Receptors

for 1-methyladenine, although not yet identified, are thought to signal through a PTX-sensitive starfish G_i protein, in particular its Gβγ [39, 40, 42]. Furthermore, the main effector for the starfish Gβγ appears to be phosphatidylinositol 3′-kinase [43,44], which is not required for progesterone-induced GVBD [45, 46].

How might a role for Gβγ be consistent with the nongenomic action of progesterone in inducing *Xenopus* oocyte GVBD? We suggest that an endogenous G protein–coupled receptor (GpCR) regulates the release of Gβγ (Figure 9). The released Gβγ activates adenylyl cyclase, which in turn maintains elevated cAMP levels and G_2 arrest. In this scenario, the GpCR is constitutively active (GpCR*) in G_2 oocytes (e.g., by an autocrine loop in which its ligand is secreted by the oocytes or via a ligand-independent mechanism). We have recently cloned the first amphibian progesterone receptor (xPR). xPR is clearly the homolog of mammalian and avian (nuclear) progesterone receptor. However, in *Xenopus* oocytes, xPR resides extranuclearly and appears to be the long-sought-after receptor responsible for progesterone-induced oocyte maturation [47]. We speculate that xPR negatively regulates this oocyte GpCR signaling function and therefore results in adenylyl cyclase inhibition and the release of G_2 arrest or GVBD. The synergistic effect of PTX may be attributed to its ability to convert endogenous $G_i\alpha$ or $G_o\alpha$ into Gβγ inhibitor since ADP ribosylation of the α subunit prevents its dissociation from Gβγ [48]. Alternatively, PTX may target the α subunit of the G protein depicted in Figure 9 and therefore inhibit the release of the inhibitory Gβγ.

Although the identity, and indeed the validity, of this GpCR is currently unknown, we suggest that it exerts its physiological effect through the release of Gβγ dimers since the injection of Gβγ scavengers alone induced GVBD. In contrast, Gallo et al. [49] demonstrated that the injection of antibodies against mammalian $G_s\alpha$ induced oocyte GVBD, and this finding suggests that the endogenous *Xenopus* $G_s\alpha$ may function in G_2 arrest. It is possible that the sequestration of oocyte Gβγ may interfere with endogenous $G_s\alpha$ signaling by preventing the formation of trimeric G_s complexes. The ability of a GTP binding–deficient mutant of $G_{\alpha i2}$ to inhibit G_q signaling in COS cells is interpreted to mean that the mutant $G_{\alpha i2}$ sequesters Gβγ that is required to form functional trimeric G_q complexes [50]. Alternatively, we postulate that a combination of $G_s\alpha$ and Gβγ is required to maintain high levels of cAMP and G_2 arrest. Interestingly, a very similar regulatory system (so-called conditional stimulation of adenylyl cyclase by Gβγ) involving adenylyl cyclase type II (ACII) has been described for mammalian systems [13]. In the presence of activated $G_s\alpha$, but not in its absence, certain Gβγ complexes (those released from activated G_i proteins) profoundly activate ACII activity [30, 51]. So far, the conditional stimulation of ACII by Gβγ has only been demonstrated in vitro with purified components [51] or in cells overexpressing ACII and other heterologous genes [30]. Our demonstration that the overexpression of xGβ1/bGγ2 elevated oocyte cAMP levels in the presence of cAMP phosphodiesterase inhibitor (IBMX) would suggest that the Gβγ dimer increased cAMP by activating an oocyte adenylyl cyclase rather than by affecting cAMP hydrolysis. This would imply that oocytes contain a Gβγ-responsive adenylyl cyclase or ACII.

Conclusion

We conclude that an endogenous Gβγ dimer, likely including xGβ1, is responsible for maintaining *Xenopus* oocyte G_2 arrest. The binding and sequestering of this Gβγ dimer by $G_t\alpha$ or βARK-C_{CAAX} releases G_2 arrest and results in oocyte maturation. The nongenomic action of progesterone, mediated via the extranuclear xPR, is likely to interfere with the activation of the trimeric G protein and hence the release of Gβγ dimer. This scheme, though not yet proven, would reconcile two well-known observations: that the nongenomic action of progesterone in *Xenopus* oocytes involves inhibition of adenylyl cyclase and that PTX does not block progesterone-induced adenylyl cyclase inhibition or oocyte maturation.

Materials and methods

Reagents

Pertussis toxin A protomer (PTX) was purchased from Calbiochem, dissolved in water to 100 μg/mL, and stored as single-use aliquots at −70°C. Rabbit anti-Gβ1 antibodies (sc378 and sc261) were purchased from Santa Cruz Biotechnology and were used at 1 μg/mL for immunoblotting. Unless otherwise indicated, sc378 was used for the anti-Gβ antibodies. Rabbit anti-xMAP serum was produced in house with a coupled peptide [52] kindly provided by J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, Washington). Anti-*Xenopus* β-integrin monoclonal antibodies (8C8) were purchased from Developmental Studies Hybridoma Bank at the University of Iowa. Other chemicals were purchased from Sigma.

Construction of various plasmids for in vitro mRNA synthesis

The bovine $G_t\alpha$ subunit in pML 52 vector [14] was a gift from Dr. Heidi Hamm. We linearized the $G_t\alpha$ construct with PstI and treated it with T4 polymerase to generate a blunt end. The linearized plasmid was then digested with NcoI. The coding sequence of $G_t\alpha$ was then inserted into pSP64TM [17] that had been previously treated with BglII/Klenow and NcoI. The resultant plasmid encoded wild-type untagged $G_t\alpha$. A PTX-insensitive mutant of $G_t\alpha$ was generated by PCR amplification from the pSP64TM-$G_t\alpha$ construct with SP6 primer (5′) and a 3′ primer that contained the C346S mutation (5′-TAT CCA TGG/TCA GAA GAG CCC GGA GTC TTT GAG G-3′) [18]. The PCR product was digested with NcoI and then inserted into pSP64TM that had been previously digested with NcoI.

A minigene encoding the bovine β adrenergic receptor kinase (βARK) C terminus (amino acids 495–689) was a gift from Dr. Robert J. Lefkowitz [21]. The βARK-C minigene was excised from the pRK5 vector with EcoRI and XbaI, treated with Klenow, and then ligated into pSP64TM [17] that had been previously treated with EcoRV. To generate a Myc-tagged version, we used NcoI for the partial digestion of pSP64TM-βARK-C, and we inserted the 800 bp NcoI fragment into pCS2+MT [53] that had been previously digested with NcoI. The resultant plasmid encoded five copies of the Myc tag followed by the same sequence in

pSP64TM-βARK-C. To change the C-terminal ANGL of βARK into a geranylgeranylation site (CVLL) [24], we PCR-amplified the βARK sequence (minus ANGL) from pSP64TM-βARK-C by using an SP6 primer (5′) and a 3′ primer that included CVLL instead of ANGL (5′-TAT CCA TGG TCA AAG CAG CAC GCA ACT GCC GCG CTG GAT-3′). The PCR product was digested with NcoI and inserted into the NcoI site of pSP64TM or pCS2+MT. This generated untagged or Myc-tagged versions of βARK-C_{CAAX}, respectively.

The coding sequence of human β_2 adrenergic receptor (β_2AR) full-length cDNA was excised from a mammalian pRK5 expression vector [54] (a gift from Dr. Susanna Cotecchia) by EcoRI and SalI. Klenow treatment subsequently generated blunt ends. The cDNA was then inserted into pCS2+ [53] that had been previously digested with StuI. The resulting plasmid encoded wild-type β_2AR without any sequence tag. xGβ1 sequence was PCR-amplified from an oocyte cDNA library [55] with the following primers: forward primer, 5′-TAT CTC GAG AAA ATG AGT GAA CTA GAT CAG C-3′; and reversed primer, 5′-TAT CTC GAG TTA GTT CCA GAT CTT GAG GAA G-3′. The amplified fragment was digested with XhoI and inserted into pCS2+ that had been previously digested with XhoI. The resulting plasmid encoded full-length xGβ1 [26]. Bovine Gγ2 coding sequence, a gift from Melvin I. Simon [56], was excised by EcoRI and inserted into pCS2+ that had been previously digested with EcoRI. The resulting plasmid encoded full-length Gγ2.

Oocyte isolation and mRNA injection

Sexually mature *Xenopus laevis* females were purchased from NASCO and maintained at 18°C. 3–7 days following gonadotropin priming (pregnant mare serum gonadotropin, 50 IU per frog), ovarian fragments were retrieved under hypothermia. Stage VI oocytes were manually removed from the follicles and stored, injected, and incubated in OR2 (83 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES [pH 7.8]).

In vitro transcription was performed with Ambion's MessageMachine kit with SP6 polymerase. mRNAs were dissolved in water to 1 mg/mL. Ten nanograms mRNA (in 10 nL) were injected per oocyte unless otherwise indicated. The injected oocytes were incubated at 18°C overnight before the addition of progesterone or the injection of a second mRNA.

Preparation of total oocyte extracts, membrane, and cytosol fractions

We lysed oocytes by forcing them through pipette tips in PBS lysis buffer (10 mM sodium phosphate buffer [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 μg/mL leupeptin, 100 μM phenylmethylsulfonyl fluoride; 10 μl per oocyte). Following centrifugation (15,000 g for 15 min), the clarified extracts were removed and mixed with SDS-sample buffer for electrophoresis. To prepare total membrane and cytosol fractions, we lysed oocytes in homogenization solution (10 mM Hepes [pH 7.5], 83 mM NaCl, 1 μg/mL leupeptin, 100 μM phenylmethylsulfonyl fluoride; 10 μl per oocyte). Following two rounds of low-speed centrifugation (900 g for 5 min), the clarified supernatants were subjected to high-speed centrifugation (100,000 g for 60 min). The pellets were designated as membrane and the supernatants as cytosol. All centrifugations were performed at 4°C. Samples were directly dissolved in SDS sample buffer for immunoblots except for conditions in which antibodies against *Xenopus* β-integrin were used, in which case samples were dissolved in SDS sample buffer without β-mercaptoethanol since the antibodies (8C8) do not recognize reduced integrin proteins [57].

Coimmunoprecipitation experiments

Ascites fluids from mice injected with 9E10 hybridoma (anti-Myc) were fractionated by 25% saturated ammonium sulfate followed by 50% saturated ammonium sulfate. The precipitates from 50% saturated ammonium sulphates were dissolved in PBS (10 mM sodium phosphate buffer [pH 7.5], 150 mM NaCl). A total of 20 mg of Myc-specific IgG (at least 80% pure judged by SDS-PAGE and Coomassie Blue staining) were coupled to 2 ml of CNBr-activated sepharose beads (Pharmacia) according to the manufacturer's instructions. Extracts (prepared in PBS lysis buffer as described above) from 20–30 control oocytes or oocytes injected with Myc-βARK-C or Myc-βARK-C_{CAAX} mRNA were incubated with 10 μl of the anti-Myc beads for 90 min at 4°C. The beads were washed three times with PBS lysis buffer. We eluted bound proteins by boiling them in SDS sample buffer and analyzed them by immunoblotting with polyclonal anti-Gβ.

In vitro ADP-ribosylation assay

The procedure was modified from Kopf and Woolkalis [58]. We incubated oocytes injected with water (control) or with $G_t\alpha$ or $G_t\alpha$-C346S mRNA overnight to allow protein synthesis. Total membranes (100,000 g pellets, as described above) from 20 oocytes were resuspended in 48 μl of assay buffer (0.1 M Tris-HCl [pH 7.6] and 20 mM dithiothreitol). To initiate the ADP-ribosylation reaction, we added 1 μl [^{32}P]NAD (1000 Ci/mmol, 10 mCi/mL) and 1 μl (100 ng) of PTX to the membrane extracts. The reaction was carried out at room temperature for 30 min and stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-PAGE and autoradiography.

cAMP assays

We lysed 20 oocytes, by forcing them through pipette tips, in 500 μl of 95% ethanol. Extracts were centrifuged at 15,000 g for 15 min. The ethanol extracts were transferred to new tubes and dried under vacuum. cAMP was resuspended and subjected to acetylation, followed by radioimmunoassays with a kit from Calbiochem (rabbit anti-cAMP) according to their provided protocol.

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