Oocyte Maturation in Starfish Is Mediated by the βγ-subunit Complex of a G-protein

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Abstract. The stimulation of meiotic maturation of starfish oocytes by the hormone 1-methyladenine is mimicked by injection of βγ subunits of G-proteins from either retina or brain. Conversely, the hormone response is inhibited by injection of the GDP-bound forms of α_{i1} or α_t subunits, or by injection of phosducin; all of these proteins should bind free βγ. α-subunit forms with reduced affinity for βγ (α_{i1} or α_t bound to hydrolysis-resistant GTP analogs, or α_{i1}-GMPPCP treated with trypsin to remove the amino terminus of the protein) are less effective inhibitors of 1-methyladenine action. These results indicate that the βγ subunit of a G-protein mediates 1-methyladenine stimulation of oocyte maturation.

DURING oocyte maturation, a fully-grown oocyte resumes meiosis and also acquires the cytoplasmic and membrane properties necessary for successful fertilization (Masui and Clarke, 1979). Although maturation occurs in oocytes of all species, the stimuli for its occurrence vary. Hormones from the follicle cells surrounding the oocyte initiate maturation in starfish (1-methyladenine; Kanatani et al., 1969; Kanatani, 1985) and frogs (progesterone; Masui and Clarke, 1979; Smith, 1989). In mammals, however, the stimulus is thought to be the termination of an inhibitory message from the follicle cells (Schultz, 1991). In some other animals, such as clams, the sperm provides the signal for the oocyte to resume meiosis (Ruderman et al., 1991).

The action of 1-methyladenine on starfish oocytes has provided a particularly useful model for examining signaling pathways leading to oocyte maturation. The hormone acts on the external surface of the oocyte (Kanatani and Hiramoto, 1970; Yoshikuni et al., 1988) to cause a characteristic set of intracellular responses (Kanatani, 1985). The most prominent of these is the reinitiation of meiosis, involving breakdown of the nuclear envelope of the large germinal vesicle (GVBD).[1] This occurs at ~30 min after hormone addition at 20°C, and is followed by formation of two polar bodies and a female pronucleus as a consequence of the two meiotic divisions. To initiate these events 1-methyladenine must be present for ~5–20 min (Guerrier and Dorée, 1975; Chiba and Hoshi, 1989).

Accompanying nuclear maturation, the cytoplasm and plasma membrane of the oocyte also mature, in starfish (Kanatani, 1985) as well as other animals (Masui and Clarke, 1979). Before application of 1-methyladenine, sperm can fuse with the oocyte, but they cause very little release of calcium (Chiba et al., 1990) and little or no elevation of the fertilization envelope. Inseminated immature oocytes do not establish blocks to polyspermy, and do not cleave or develop. After hormone application, by the time of GVBD, the oocyte has acquired the ability to respond to sperm by releasing a much larger amount of calcium (Chiba et al., 1990) and elevating its fertilization envelope fully. Polyspermy does not occur, and the zygote cleaves and develops normally. Other maturation-induced changes include release of the follicle cells that surround the oocyte (Kishimoto et al., 1984), changes in cortical actin filaments and microtubules (Schroeder and Stricker, 1983; Otto and Schroeder, 1984; Schroeder and Otto, 1984), and decreases in potassium conductances in the oocyte plasma membrane (Miyazaki et al., 1975; Simoncini and Moody, 1990).

Like all stimuli that lead to cell division, 1-methyladenine causes the production in the starfish oocyte cytoplasm of maturation promoting factor (MPF). This in turn stimulates the reinitiation of meiosis, as well as at least some of the other events of oocyte maturation (Kishimoto and Kanatani, 1976; Kishimoto et al., 1984). A major component of MPF is the serine-threonine kinase composed of dephosphorylated p34cdc2 and a phosphorylated B-type cyclin; MPF may contain other components as well (Pondaven et al., 1990; Picard et al., 1991; Kobayashi et al., 1991). Additional kinases, a myelin basic protein kinase resembling MAP-2 kinase, and an S6 kinase, may function in a cascade leading

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1. *Abbreviations used in this paper:* GDP, guanosine-5'-diphosphate; GVBD, germinal vesicle breakdown; MPF, maturation promoting factor.

to GVBD (Pelech et al., 1988; Sanghara et al., 1991; Daya-Makin et al., 1991).

Although the receptor for 1-methyladenine has not been identified, the initial action of the hormone appears to be mediated by a heterotrimeric G-protein, because its action is inhibited by injection of pertussis toxin (Shilling et al., 1989; Chiba et al., 1992), and because high affinity binding of 1-methyladenine to starfish oocyte membranes is inhibited by GTP-γ-S (Tadenuma et al., 1992). The pertussis toxin inhibition can be bypassed by injection of MPF, in the form of cytoplasm from a maturing oocyte, indicating that the G-protein acts prior to the formation of MPF (Shilling et al., 1989). The pertussis substrate is a G_i-like protein, most similar in its α-subunit sequence to mammalian G_{i1} (Tadenuma et al., 1991; Chiba et al., 1992). Evidence that starfish G_i is activated by 1-methyladenine comes from the finding that 1-methyladenine treatment of oocyte membranes causes α_i to acquire the ability to be ADP-ribosylated by cholera toxin (Tadenuma et al., 1992). In the present paper, we describe the effects of injecting into starfish oocytes mammalian G-protein α and $\beta\gamma$ subunits and phosducin, a protein that binds G-protein $\beta\gamma$-subunits (Lee et al., 1987, 1992). Our results indicate that the $\beta\gamma$-subunit is the primary stimulus for starfish oocyte maturation.

Materials and Methods

Preparation of Gametes and Microinjection

Starfish (*Asterina miniata*) were obtained from Bodega Marine Laboratory (Bodega Bay, CA) and were maintained in recirculating natural sea water at 14°C. Ovaries were removed and cut apart in calcium free artificial sea water at 4°C. The oocytes that were released were washed several times in calcium free sea water until most of their follicle cells were removed; this process took about 20 min. The oocytes were then washed repeatedly in natural sea water at 20°C to remove all remaining follicle cells. Oocyte diameters were 180-190 μm.

For microinjection experiments, oocytes were held between two coverslips separated by two pieces of double stick tape (Kiehart, 1982; Terasaki and Jaffe, 1993). The oocytes were positioned within 200 μm of the coverslip edge, to allow rapid introduction of 1-methyladenine or sperm. All experiments were performed in natural sea water at 20°C. 1-methyladenine (Sigma Chemical Co., St. Louis, MO) was dissolved in natural sea water at a concentration of 1 μM. Sperm were obtained from a dissected testis; they were diluted approximately 1:1,000 in natural sea water before addition to the chamber containing oocytes.

Microinjections were made using a constriction pipet (Hiramoto, 1974; Kishimoto, 1986) filled with silicon oil (dimethylpolysiloxane, 100 centistokes, Sigma Chemical Co., St. Louis, MO). Injections were calibrated by measuring the diameter of an equivalent volume of oil, and ranged from 36 to 200 pl (1-5.6% of the 3,600 pl volume of an oocyte of 190-μm diam). Observations and photographs were made using 10X and 20X objectives with differential interference contrast optics (Carl Zeiss, Inc., Thornwood, NY), and Tech Pan film (Eastman Kodak Co., Rochester, NY).

Preparation of Proteins

Antibodies against G-protein subunit peptides were produced and affinity purified as previously described (Simonds et al., 1989; Murakami et al., 1992).

Transducin α and $\beta\gamma$ subunits were purified from bovine retina, and α_t-GppNHp was prepared as previously described (Ting et al., 1993). Purified proteins were stored in 50% glycerol at −20°C. Glycerol was removed by use of a Bio-Spin 6 column (BioRad, Richmond, CA) or a 30,000 MW Ultra-Spin filter (USA/Scientific Plastics, Ocala, FL); the buffer was exchanged to 100 mM K aspartate, 10 mM Hepes, pH 7.0. 1 mM MgCl₂ was included in the buffer for α-subunits. Results obtained with two different transducin $\beta\gamma$ preparations were the same.

Brain $\beta\gamma$ and $\beta_{36}\gamma_2$ were purified as previously described (Sternweis

and Robishaw, 1984; Muntz et al., 1992). "Brain $\beta\gamma$" was a preparation like that shown in Fig. 6 D of Sternweis and Robishaw (1984), and consisted of both 35 and 36 kD β's and multiple γ's. "Brain $\beta_{36}\gamma_2$" was derived from a heterotrimeric G-protein preparation like that shown in Fig. 6 B of Sternweis and Robishaw (1984). This preparation was enriched in the 36-kD β and one of multiple γ's (γ_2 refers to its position on the SDS gel in Fig. 6 B of Sternweis and Robishaw (1984), and does not denote the γ_2 DNA clone). Proteins were stored at −70°C in a buffer containing 0.8-1.0% cholate; injections were made in the cholate-containing buffer.

Recombinant nonmyristoylated α_{i1} was synthesized from a rat olfactory cDNA in which the amino-terminal glycine-encoding sequence had been changed to code for an alanine (Jones et al., 1990). Using a recombinant baculovirus, the protein was expressed in Sf9 cells and purified (Jones et al., 1993). The protein was treated with 1 mM GDP or GTP-γ-S and then extensively washed and concentrated; the procedure was the same as previously described except that no detergent was used (Yatani et al., 1987). Purified proteins were stored in 25% glycerol at −70°C; glycerol was removed and the buffer was exchanged as described for transducin subunits. Results obtained with three different α_{i1} preparations were the same.

The tryptic cleaved α_{i1} was prepared by resuspending the partially purified protein in a buffer containing 100 μM GMPPCP after the ammonium sulfate precipitation. Before chromatography with Blue Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), the sample was incubated at 4°C with 100 μM GMPPCP and TPCK trypsin in a ratio of 1:80 (trypsin: protein, by weight). After 10 min, soybean trypsin inhibitor was added at a ratio of 10:1 (inhibitor: trypsin, by weight) and the sample was immediately applied to the column. The purified protein was stored in 25% glycerol at −70°C; glycerol was removed and the buffer was exchanged as described for transducin subunits.

Phosducin was purified from bovine retina as described previously (Lee et al., 1987). Purified protein was stored in 40% glycerol at −20°C; glycerol was removed and the buffer was exchanged as described for transducin subunits. Results obtained with two different phosducin preparations were the same.

Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

Tryptic Cleavage of α-Subunits to Test Nucleotide Association

α_{i1}-GDP, α_{i1}-GTP-γ-S, α_t-GDP, and α_t-GppNHp were incubated at 4°C with trypsin (Boehringer Mannheim, Indianapolis, IN) in a ratio of 1:50 (trypsin: protein, by weight). After 60 min, SDS sample buffer was added, the samples were heated for 5 min at 95-100°C, and the cleavage products were separated by SDS gel electrophoresis.

Gel Electrophoresis and Immunoblotting

Oocyte lysates were prepared by adding 1X SDS sample buffer to a pellet of defolliculated starfish oocytes. These lysates were then heated to 95-100°C for 5 min and subjected to 10% SDS-PAGE according to the method of Laemmli (1970). Molecular weight markers were from Bio-Rad Laboratories (Richmond, CA). After SDS-PAGE, the separated proteins in the gel were electrophoretically transferred to 0.2 μm nitrocellulose (Schleicher and Schuell, Keene, NH). The blot was briefly stained with a 0.2% Ponceau S solution (Harlow and Lane, 1988) to determine the position of the molecular weight markers and to cut it into individual sample strips. These strips were destained with water and then blocked by incubation for 1 h in TST-Blotto (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.075% Tween 20, 0.5% non-fat dried milk, and 0.05% sodium azide). The immunoblot strips were then matted dry and placed on a piece of parafilm. The primary antibody, diluted in TST-Blotto to 2.5-3.5 μg/ml, was slowly pipetted onto the strip, followed by an overlay of parafilm to form a sandwich. After an overnight incubation at room temperature, the immunoblots were washed four times with TST-Blotto and then incubated for 1 h with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Organon Teknika Corp., Durham, NC). The blots were washed four times with TST-Blotto, twice with TST (no milk), once with TSM (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, and 5 mM MgCl₂), and developed in TSM containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

The purified proteins used for microinjection and the G-protein α-subunits subjected to proteolysis were both analyzed by 10% SDS-PAGE using a Tris-tricine buffer system (Schagger and Von Jagow, 1987). This system enables one to examine both high and low molecular weight proteins on the

same gel. Upon completion of electrophoresis, the gels were stained with Coomassie blue and destained.
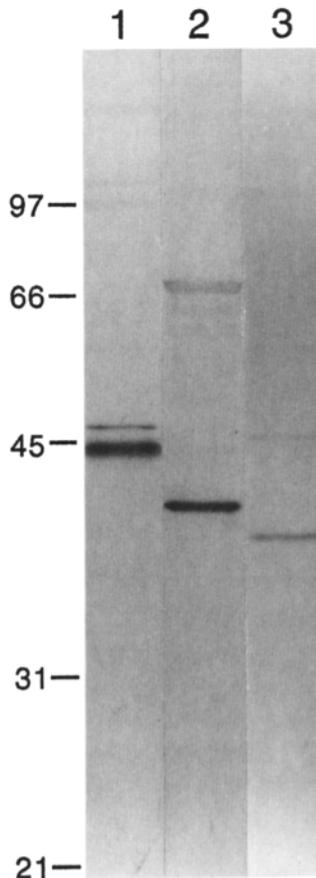
Results

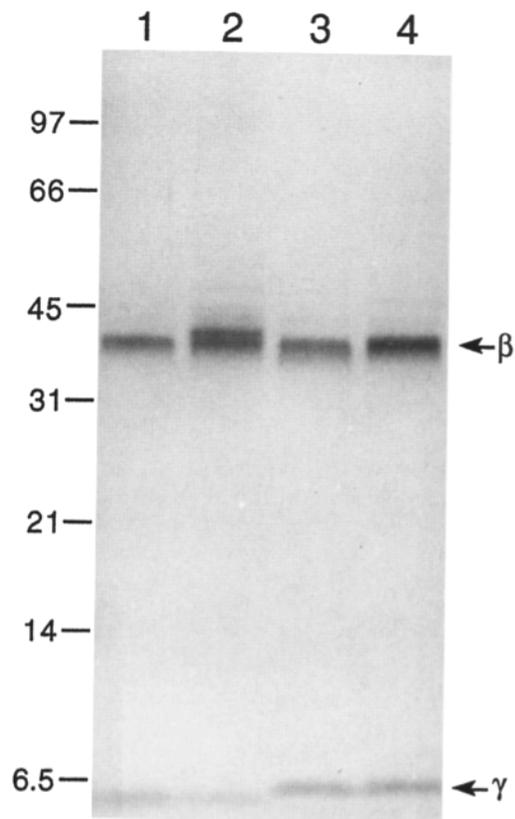### Identification of G-Protein Subunits in Starfish Oocytes

Immunoblots of lysates of *A. miniata* oocytes showed starfish proteins that cross-reacted with antibodies recognizing the mammalian G-protein subunits α_s, $\alpha_{i1,2}$, and β (RM, AS, and RA; Simonds et al., 1989; Murakami et al., 1992) (Fig. 1). The molecular weights of these proteins were 44, 39, and 37 kD, respectively, similar to the molecular weights of the corresponding mammalian proteins (Gilman, 1987), and identical to those of the previously described α_s, α_i, and β in *Asterina pectinifera* (Tadenuma et al., 1991, 1992). No cross-reactivity with α_{i3} or α_o antibodies (EC and GO; Simonds et al., 1989) was seen.

Transducin $\beta\gamma$-Subunits Stimulated Oocyte Maturation

$\beta\gamma$-subunits from bovine transducin (Fig. 2, lane *1*) were injected into starfish oocytes. A $\beta\gamma$ concentration in the cytoplasm of $\geqslant 1.8$ μM caused all of the oocytes to undergo GVBD (Figs. 3, 4). A $\beta\gamma$ concentration of $\leqslant 0.5$ μM did not cause GVBD. Oocytes that had been induced to undergo GVBD by injection of $\beta\gamma$-subunits were frequently observed to form polar bodies (Fig. 5 *A*) and a female pronucleus, al-


Figure 2. Tricine-SDS-PAGE of mammalian G-protein $\beta\gamma$-subunits used for injection. Samples were loaded onto a 10% polyacrylamide gel, subjected to Tricine-SDS-PAGE, and then Coomassie blue stained. (Lane *1*) bovine transducin $\beta\gamma$ (3 μg); (lane *2*) whole bovine transducin (6 μg); (lane *3*) bovine brain $\beta\gamma$ (3 μg); (lane *4*) bovine brain $\beta_{36}\gamma_2$ (3 μg).

Figure 1. Immunoblot of starfish oocyte lysate with affinity-purified antibodies raised against peptides of mammalian G-protein subunits. Lysate was loaded (70 μg/lane) onto a 10% polyacrylamide gel and subjected to SDS-PAGE and immunoblotted as described in Materials and Methods. (Lane *1*) RM antibody against mammalian α_s COOH-terminal decapeptide; (lane *2*) AS antibody against mammalian α_t COOH-terminal decapeptide, which shows immunoreactivity against α_{i1} and α_{i2}; (lane *3*) RA antibody against bovine transducin β-subunit amino acid residues 256–265.

though polar bodies were sometimes seen to form partially and then recede. Control injections of whole transducin (the $\alpha\beta\gamma$ complex, Fig. 2, lane 2), or of $\beta\gamma$ that had been heated at 90°C for 5–10 min, did not cause GVBD (Figs. 3 *B* and 4).

GVBD in response to $\beta\gamma$ injection occurred with a time course similar to the response to 1-methyladenine (measurements at 20°C). In oocytes from different animals, the time of GVBD in response to 1-methyladenine differs somewhat, so comparisons were made for oocytes from individual animals. For oocytes from an animal in which GVBD occurred at 38 $\pm$ 3 min (SD, $n = 19$ oocytes) after applying 1 μM 1-methyladenine, GVBD in response to injection of 1.5 μM $\beta\gamma$ occurred at 34 $\pm$ 3 min ($n = 11$). For oocytes from another animal, in which GVBD occurred at 21 $\pm$ 1 min ($n = 20$) after applying 1 μM 1-methyladenine, GVBD in response to injection of 1.5 μM $\beta\gamma$ occurred at 22 $\pm$ 3 min ($n = 9$). Similar results were obtained when the concentration of $\beta\gamma$ was increased twofold.
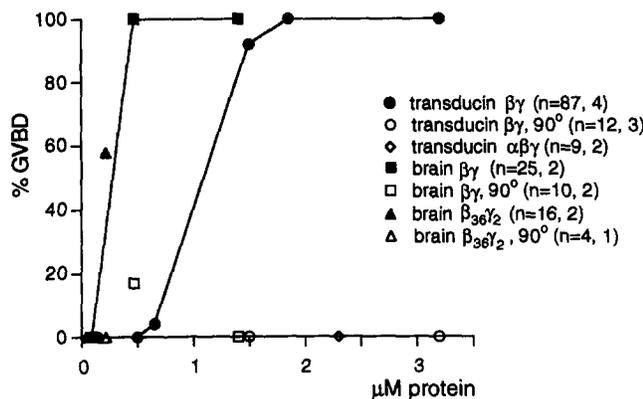
In addition to causing the reinitiation of meiosis, $\beta\gamma$ injection caused the oocytes to acquire the ability to respond to sperm by elevating a full fertilization envelope (Fig. 5 *A*). These fertilized eggs underwent cleavage (Fig. 5 *A*) and developed at least to blastulae. In contrast, control oocytes injected with heat-inactivated $\beta\gamma$ or whole transducin did not elevate fertilization envelopes or cleave in response to sperm (Fig. 5 *B*).
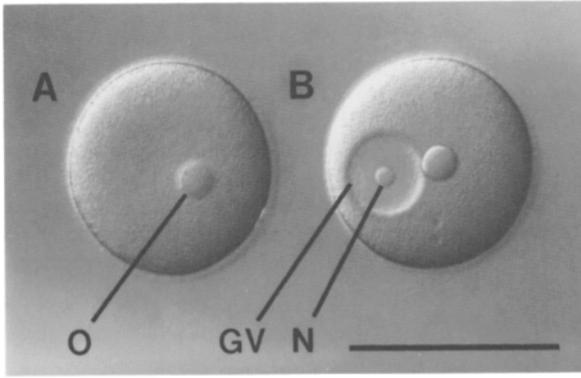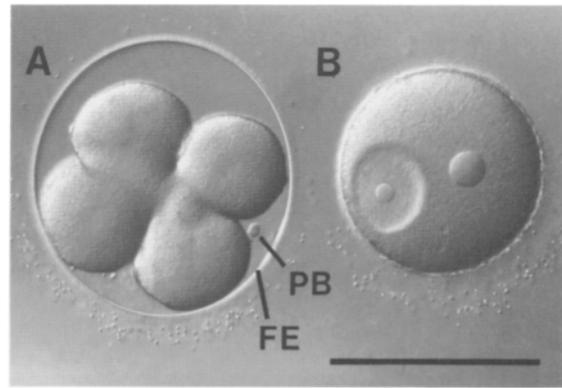
Figure 3. Stimulation of oocyte maturation by injection of transducin βγ subunits. (*A*) An oocyte 47 min after injection of 180 pl of 1.3 mg/ml transducin βγ, 1.5 μM final concentration in the oocyte. GVBD has occurred, as indicated by the absence of the germinal vesicle and nucleolus (compare to control in *B*). *O* indicates the oil drop left in the oocyte as a consequence of microinjection. (*B*) Control oocyte 95 min after injection of 180 pl of 3.8 mg/ml whole transducin (αβγ), 2.3 μM final concentration in the oocyte. The germinal vesicle (*GV*) and nucleolus (*N*) are intact. Bar, 200 μm.

Figure 5. Fertilization and cleavage of an oocyte inseminated after induction of maturation by injection of transducin βγ. (*A*) An embryo which developed from an oocyte injected with 1.5 μM transducin βγ and inseminated 1.7 h later. Photographed 3 h after insemination. The small dots beneath the embryo are sperm. The fertilization envelope (*FE*) has elevated, and the embryo has cleaved to form four cells. A polar body (*PB*) is also visible. (*B*) A control oocyte injected with 2.3 μM whole transducin and inseminated 2.5 h later. Photographed 3 h after insemination. The germinal vesicle is intact, the fertilization envelope has not elevated, and no cleavage has occurred. Bar, 200 μm.

Brain βγ Subunits also Stimulated Oocyte Maturation

Transducin βγ was used for the experiments described above, because no detergent is required to solubilize it. This property may be a consequence of the isoprenylation of transducin γ-subunits with a 15-carbon farnesyl group instead of a 20-carbon geranylgeranyl group which occurs on other γ-subunits (Fukada et al., 1990; Mumby et al., 1990; Spiegel et al., 1991). To test the generality of the stimulatory effect of βγ, we also used brain βγ, which was solubilized in a buffer containing 0.8–1.0% cholate (Fig. 2, lanes *3* and *4*). These cholate-containing solutions were used at volumes not exceeding 1–3% of the oocyte volume. Larger injections of cholate-containing control solutions sometimes caused a

gradual and partial shrinking of the germinal vesicle, but did not cause the breakdown of the nuclear envelope that characterizes the normal GVBD response.

Injection of 0.47 μM brain βγ caused GVBD in all oocytes; thus brain βγ was effective at a somewhat lower concentration than transducin βγ (Fig. 4). 0.09 μM brain βγ did not cause GVBD. Similar results were obtained with a preparation containing a mixture of the various forms of β and γ present in brain G-proteins, and with a preparation enriched in β_{36} and γ_2. Brain βγ that had been heated at 90°C for 10 min did not cause GVBD. Oocytes injected with brain βγ also formed polar bodies and acquired the ability to elevate a fertilization envelope in response to sperm.

Figure 4. Stimulation of GVBD by injection of βγ subunits. Percent of oocytes that underwent GVBD by 60 min after injection is plotted as a function of the final βγ-subunit concentration in the oocyte. 90°C indicates a sample that was heat-inactivated. Numbers in parentheses indicate the number of oocytes injected (total number for all concentrations), and the number of different animals from which the oocytes were obtained.

α_i-Subunits in the Guanosine-5'-diphosphate-Bound Form Inhibited Germinal Vesicle Breakdown in Response to 1-Methyladenine

Because βγ-subunits stimulated GVBD, we tested whether GVBD would be inhibited by injection of α-subunits. α-subunits can be bound to either GDP or GTP; the GDP-bound form, which is present in the absence of receptor stimulation, has high affinity for βγ. After receptor stimulation, GDP dissociates and GTP binds; the GTP-bound form has low affinity for βγ (Gilman, 1987). Because of these properties, we used α-subunits in the GDP-bound form, which would be expected to bind free βγ in the oocyte. We used the α-subunit of mammalian G_{i1}, because of its similarity to the G-protein that is apparently activated in starfish oocytes in response to 1-methyladenine (Tadenuma et al., 1991, 1992; Chiba et al., 1992). To allow free solubility of the α-subunit in the absence of detergent, we used a recombinant form of α_{i1} that had been modified to prevent myristoylation (Fig. 6, lane *1*) (Jones et al., 1990). This mutant α_{i1} can effectively combine with βγ-subunits, although its
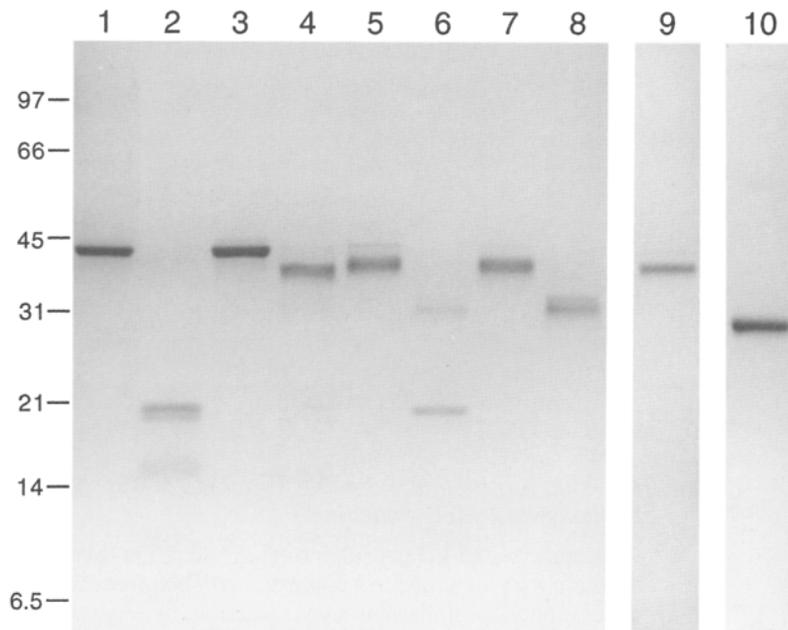
Figure 6. Tricine-SDS-PAGE of mammalian G-protein α-subunits and phosducin used for injection, and tests of nucleotide association by tryptic digestion. Samples were loaded (3 μg/lane) onto a 10% polyacrylamide gel, subjected to Tricine-SDS-PAGE, and then Coomassie blue stained. (Lane *1*) Recombinant nonmyristoylated α_{i1}-GDP; (lane 2) recombinant nonmyristoylated α_{i1}-GDP, treated with trypsin; (lane 3) recombinant nonmyristoylated α_{i1}-GTP-γ-S; (lane 4) recombinant nonmyristoylated α_{i1}-GTP-γ-S, treated with trypsin; (lane 5) α_t-GDP; (lane 6) α_t-GDP, treated with trypsin; (lane 7) α_t-GppNHp; (lane 8) α_t-GppNHp, treated with trypsin; (lane 9) recombinant nonmyristoylated α_{i1}-GMPPCP that was treated with trypsin and then isolated by column chromatography; (lane 10) phosducin.

affinity for βγ is lower than that of wild-type α_{i1} (Jones et al., 1990, 1993; see also Linder et al., 1991).

Injection of α_{i1}-GDP at a final concentration of ⩾2.2 μM inhibited GVBD in response to 1 μM 1-methyladenine (Figs. 7 *A* and 8). Injections were made 5–110 min before applying the hormone, and oocytes were observed at 60 min after hormone application to determine whether GVBD had occurred. With the light microscope, these oocytes showed no morphological changes and appeared normal. Injection of 0.4 μM α_{i1}-GDP did not inhibit GVBD in response to 1-methyladenine (Fig. 8).

α_i Subunit Forms with Lower Affinity for βγ Were Less Effective or Ineffective in Inhibiting Germinal Vesicle Breakdown in Response to 1-Methyladenine

As controls, we injected forms of α_{i1} that had reduced
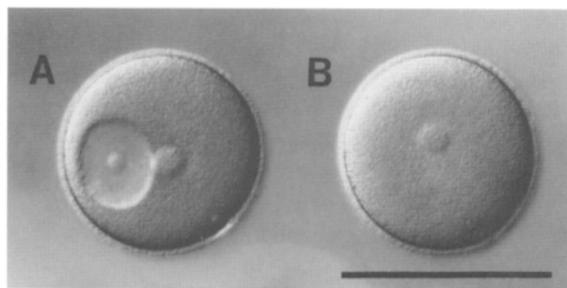
Figure 7. Inhibition of 1-methyladenine-induced oocyte maturation by injection of α_{i1}-GDP. (*A*) An oocyte injected with 200 pl of 1.6 mg/ml α_{i1}-GDP (final concentration in the cytoplasm 2.2 μM) and exposed to 1 μM 1-methyladenine starting at 35 min after injection. Photographed 60 min after application of 1-methyladenine. GVBD has not occurred. (*B*) A control oocyte injected with 200 pl of 1.6 mg/ml α_{i1}-GTP-γ-S (final concentration in the cytoplasm 2.2 μM) and exposed to 1 μM 1-methyladenine starting at 45 min after injection. Photographed 60 min after application of 1-methyladenine. GVBD has occurred. Bar, 200 μm.

affinity for βγ. α_i that has been activated by preincubation with a hydrolysis resistant GTP analog has reduced affinity for βγ (Kohnken and Hildebrant, 1989; Jones et al., 1990); correspondingly, α_{i1}-GTP-γ-S (Fig. 6, lane 3) was a less effective inhibitor of the 1-methyladenine response (Figs. 7 *B* and 8). α_{i1}-GTP-γ-S was, however, partially inhibitory at higher concentrations. This could be due to the dissociation of the GTP-γ-S from a fraction of the activated α-subunits, resulting in the introduction of some α_{i1}-GDP into the cell. To examine this possibility, we compared the tryptic cleavage patterns of the α_{i1}-GTP-γ-S and α_{i1}-GDP preparations (Fig. 6, lanes 2 and 4); if GTP-γ-S is bound, α_{i1} should be protected from multiple cleavages (Eide et al., 1987; Neer et al., 1988). The molecular weight of the major tryptic cleavage product of α_{i1}-GTP-γ-S was only slightly smaller than that of the original protein, indicating that the GTP-γ-S was bound to at least a significant fraction of the α_{i1} in the stock solution; however, some dissociation is likely to have occurred, because the apparent K_D of α_{i1}-GTP-γ-S has been measured to be ~30 nM (Carty et al., 1990). Based on this K_D and a concentration of 5 μM α_{i1}-GTP-γ-S after injection, we estimated that the amount of α_{i1}-GDP present would be ~0.4 μM, close to the concentration at which inhibitory effects of α_{i1}-GDP begin to appear. The value of 0.4 μM is only an estimate, since other factors such as cytoplasmic [Mg] might influence the K_D, and because some additional dissociation might have occurred during dialysis of the protein. Based on these considerations, the presence of α_{i1}-GDP seems to be a likely explanation of our results. These results were unrelated to the introduction of free GTP-γ-S into the cell, because injection of GTP-γ-S, at a concentration in the cytoplasm of 10 μM, neither stimulated nor inhibited GVBD.

α_i-subunits that have been incubated with a hydrolysis-resistant GTP analog and trypsin have reduced affinity for βγ, not only because they are activated, but also because they lack a 2-kD fragment from the amino terminus (Neer et al., 1988). We used such a form of α_{i1}, α_{i1}-GMPPCP-TR (Fig.
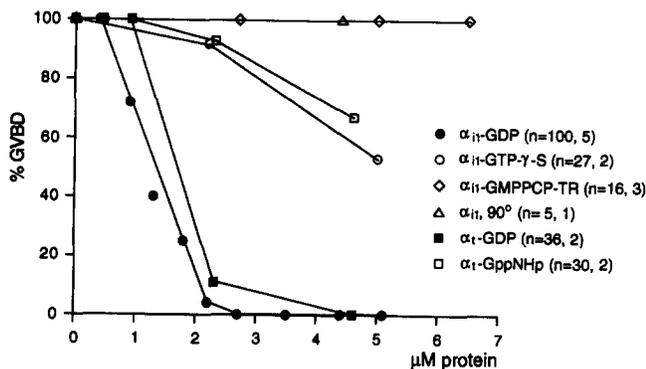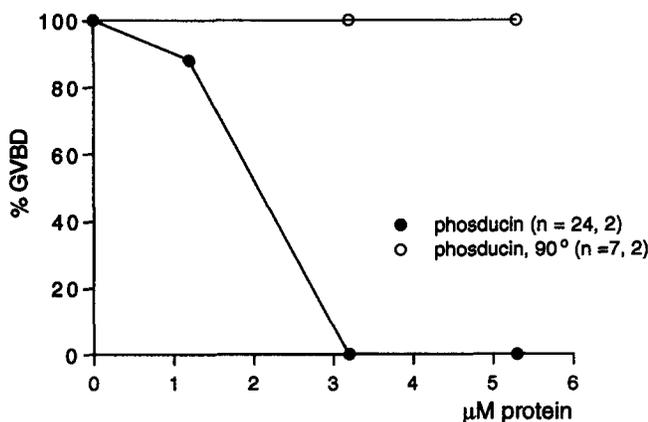
Figure 8. Inhibition of 1-methyladenine-induced GVBD in oocytes injected with α-subunits. Percent of oocytes that underwent GVBD by 60 min after application of 1-methyladenine is plotted as a function of the final α-subunit concentration in the oocyte. 90°C indicates a sample that was heat-inactivated. Numbers in parentheses indicate the number of oocytes injected (total number for all concentrations), and the number of different animals from which the oocytes were obtained.

6, lane 9) as an additional control, and found that it did not inhibit the 1-methyladenine response (Fig. 8). Also, 4.4 μM α_{i1} that had been heated at 90°C for 5 min did not inhibit GVBD in response to 1-methyladenine (Fig. 8).

Neither of the activated α_i subunits, α_{i1}-GTP-γ-S or α_{i1}-GMPPCP-TR, at 5.0 or 6.5 μM respectively, stimulated GVBD.

Transducin α Subunits in the Guanosine-5'-diphosphate-bound Form also Inhibited Germinal Vesicle Breakdown in Response to 1-Methyladenine

Because α-subunits can in some cases bind $\beta\gamma$ without a high degree of specificity (Hekman et al., 1987; Iniguez-Lluhi et

Figure 9. Inhibition of 1-methyladenine-induced GVBD in oocytes injected with phosducin. Percent of oocytes that underwent GVBD by 60 min after application of 1-methyladenine is plotted as a function of the final phosducin concentration in the oocyte. 90°C indicates a sample that was heat-inactivated. Numbers in parentheses indicate the number of oocytes injected, and the number of different animals from which the oocytes were obtained.

al., 1992), we tested whether transducin α (Fig. 6, lane 5) would inhibit GVBD in response to 1-methyladenine. Injection of α_t-GDP at a final concentration of $\geqslant 2.3$ μM inhibited the response to 1-methyladenine; 0.9 μM α_t-GDP was not inhibitory (Fig. 8). Thus, similar concentrations of α_t-GDP and α_{i1}-GDP inhibited the response to the hormone. Injection of α_t that had been activated with the hydrolysis-resistant GTP analog GppNHp (Fig. 6, lane 7), and thus having reduced affinity for $\beta\gamma$ (Navon and Fung, 1987; Phillips and Cerione, 1991), was less effective in inhibiting GVBD (Fig. 8). We confirmed that GppNHp was bound to α_t by tryptic digestion (Fung and Nash, 1983) (Fig. 6, lanes 6, 8).

We also observed that α_t-GppNHp, at 4.6 μM, did not stimulate GVBD.

Phosducin Inhibited Germinal Vesicle Breakdown in Response to 1-Methyladenine

Phosducin is a 33-kD phosphoprotein from retina that binds transducin $\beta\gamma$ subunits; in a reconstituted phototransduction system, phosducin disrupts signal coupling by sequestering transducin $\beta\gamma$ subunits (Lee et al., 1987, 1992). Because of its ability to bind $\beta\gamma$, we tested whether GVBD in starfish oocytes would be inhibited by injection of phosducin.

Injection of phosducin (Fig. 6, lane 10) at a final concentration of $\geqslant 3.2$ μM inhibited GVBD in response to 1-methyladenine (Fig. 9). Injections were made 15–60 min before applying the hormone, and oocytes were observed to determine whether GVBD had occurred at 60 min after hormone application. With the light microscope, these oocytes showed no morphological changes and appeared normal. Injection of 1.2 μM phosducin did not inhibit GVBD in response to 1-methyladenine (Fig. 9). As a control, we injected 5.3 μM phosducin that had been heated at 90°C for 10 min; this heat-treated phosducin did not inhibit GVBD in response to 1-methyladenine (Fig. 9).

Discussion

G-Protein Actions Mediated by βγ-Subunits

Although most actions of G-proteins have been attributed to the interaction of the α-subunit with an effector molecule, recent evidence indicates that the $\beta\gamma$-subunit complex can also activate effector molecules. These actions include stimulation of type II adenylylcyclase, potentiation of α_s stimulation of types II and IV adenylylcyclases, and inhibition of α_s or calmodulin stimulation of type I adenylylcyclase (Katada et al., 1987; Tang et al., 1991; Tang and Gilman, 1991; Gao and Gilman, 1991; Federman et al., 1992; Iniguez-Lluhi et al., 1992; Mangels et al., 1992; Taussig et al., 1993), activation of phospholipase A₂ (Jelsema and Axelrod, 1987; Kim et al., 1989), opening of muscarinic K⁺ channels (Logothetis et al., 1987; Ito et al., 1992), activation of phospholipase C (Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992a,b; Katz et al., 1992; Carozzi et al., 1993), promoting the membrane association of β-adrenergic receptor kinase and thus, indirectly, increasing the kinase activity (Pitcher et al., 1992), and stimulating muscarinic acetylcholine receptor kinase (Haga and Haga, 1992). $\beta\gamma$ also binds phosducin (Lee et al., 1987) and calmodulin (Katada et al., 1987; Mangels et al., 1992), and mediates responses

to mating pheromones in yeast (Blumer and Thorner, 1991; Leberer et al., 1992a,b). Injection of βγ subunits into frog oocytes has been reported to either enhance (Dascal et al., 1986) or inhibit (Moriarty et al., 1988) agonist-induced opening of calcium-dependent Cl⁻ channels.

A Role for βγ in Stimulation of Meiotic Maturation

The stimulation of meiotic maturation of starfish oocytes by the hormone 1-methyladenine is mimicked by injection of βγ subunits of G-proteins from either retina or brain. Conversely, the hormone response is inhibited by injection of the GDP-bound forms of α_{i1} or α_t subunits, or by injection of phosducin; all of these proteins should bind free βγ. α-subunit forms with reduced affinity for βγ (α_{i1} or α_t bound to hydrolysis-resistant GTP analogs, or α_{i1}-GMPPCP treated with trypsin to remove the amino terminus of the protein) are less effective inhibitors of 1-methyladenine action. These results indicate that the βγ subunit of a G-protein mediates 1-methyladenine stimulation of oocyte maturation.

The α-subunit and phosducin inhibition results could also be explained by the alternative hypothesis that the activation of an effector in response to 1-methyladenine is mediated by an α-subunit. Injected α-subunits or phosducin could sequester βγ freed by an initial cycle of G-protein activation, thus depleting the βγ available to an endogenous α-subunit, and preventing the reactivation of the endogenous α-subunit by the receptor. This mechanism could only be significant if individual G-proteins underwent multiple cycles of activation in response to 1-methyladenine. Also, if α was the primary subunit of the G-protein that mediates 1-methyladenine action, our βγ results could only be explained as an unnatural consequence of the high concentrations of βγ employed. This is a possible interpretation, although seemingly less likely.

For transducin βγ, stimulation of oocyte maturation requires ∼2 µM, and for brain βγ, ∼0.5 µM is required. By comparison, maximal βγ effects on adenylylcyclase occur at ∼20 nM-0.5 µM (Katada et al., 1987; Tang et al., 1991; Tang and Gilman, 1991; Gao and Gilman, 1991; Iniguez-Lluhi et al., 1992; Mangels et al., 1992), and maximal βγ effects on K⁺ channels occur at ∼30 nM (Ito et al., 1992). Maximal βγ effects on muscarinic acetylcholine and β-adrenergic receptor kinases are seen at concentrations of 30 nM-6 µM (Haga and Haga, 1992; Pitcher et al., 1992). Maximal stimulation of phospholipase C requires about 0.5-4 µM βγ (Blank et al., 1992; Camps et al., 1992a,b; Carozzi et al., 1993). These comparisons indicate that the stimulation of starfish oocyte maturation by βγ-subunits occurs in a concentration range similar to or somewhat higher than that seen for βγ stimulation of various responses in mammalian systems.

Although it seems unlikely to us that βγ plays no natural role in mediating the action of 1-methyladenine, it is very possible that the α-subunit of G_i also contributes to the action of the hormone. This question should be addressed by injection of activated α_i from starfish oocytes. Our results show that activated mammalian α_{i1} does not stimulate GVBD; although rat α_{i1} and starfish α_i are 89% identical (Chiba et al., 1992), the differences could be significant. The protein used in our experiments was nonmyristoylated; however, α_{i1} expressed in *Escherichia coli* is nonmyristoylated (Duronio et al., 1990) and is still capable of at least some

effector interactions (Yatani et al., 1988; Linder et al., 1990).

Possible Targets of βγ Action

The target of βγ in the oocyte is unknown. βγ could act either by binding to a G-protein α-subunit, or by binding to an effector enzyme. If in addition to G_i, there was in the oocyte another G-protein that had an action opposing maturation, the βγ released by 1-methyladenine could counteract the action of this other G-protein by binding to its α-subunit. This mechanism could only be significant if the βγ bound to the α-subunit in its GDP-bound form and interrupted its GDP/GTP cycle.

One possibility for the action of βγ pertains to the hypothesis that oocyte maturation is mediated by a fall in cAMP (see Meijer and Arion, 1991). The evidence for a role of a decrease in cAMP in mediating 1-methyladenine action is that cAMP in the oocyte has been measured to decrease by 10-30% in response to 1-methyladenine (Meijer and Zarutskie, 1987), that injection of the catalytic subunit of cAMP-dependent protein kinase increases the amount of 1-methyladenine required to cause maturation (Dorée et al., 1981), and that application of forskolin or injection of cAMP-S delays GVBD in response to the hormone (Meijer and Zarutskie, 1987; Meijer et al., 1989). However, none of these agents inhibit the 1-methyladenine response completely; in particular, oocytes treated with forskolin and 1-methyladenine have a measured cAMP content about 10 times the level in untreated oocytes, but GVBD is delayed only by ∼10 min (Meijer and Zarutskie, 1987). Thus, a fall in cAMP is probably one, but not the only, factor in mediating 1-methyladenine action. βγ-subunits have been demonstrated to inhibit type I adenylylcyclase, when the adenylylcyclase has been activated by α_s or calmodulin (Tang et al., 1991). Such an inhibitory action of βγ, either by way of interaction with α_s or by way of interaction with activated adenylylcyclase, could be occurring when starfish oocytes are stimulated by 1-methyladenine.

Other possible targets for βγ are kinases and phosphatases. Cyclin phosphorylation and tyrosine phosphorylation of a 155-kD cortical protein begin to increase within 2 min after applying 1-methyladenine (Pondaven et al., 1990; Peaucellier et al., 1990). By 4 min, tyrosine dephosphorylation of the p34cdc2 protein kinase is occurring (Pondaven et al., 1990). In other cells, the activity of at least two other kinases (muscarinic acetylcholine receptor kinase and β-adrenergic receptor kinase) is stimulated by way of βγ-subunits (Haga and Haga, 1992; Pitcher et al., 1992) and recent evidence suggests that βγ may activate a protein kinase in yeast (Leberer et al., 1992b). βγ is unlikely to be acting by way of stimulating phospholipase C, because the response to 1-methyladenine is not mediated by an increase in intracellular calcium (Witchel and Steinhardt, 1990; Kikuyama and Hiramoto, 1991). Another possibility is that βγ could stimulate a protease, because application of protease inhibitors inhibits production of MPF in 1-methyladenine treated starfish oocytes, and the activity of a 650-kD proteasome increases within 5 min after hormone addition (Kishimoto et al., 1982; Takagi Sawada et al., 1992). As the targets of βγ-subunits become better understood, a link should be found between the release of βγ-subunits in response to 1-methyladenine and the activation of the various

enzymes associated with the reinitiation of meiosis. The role of G-proteins as regulators of oocyte maturation in other species remains to be examined, although there are indications that both heterotrimeric and small molecular weight GTP-binding proteins may be important in oocyte maturation in frogs (Sadler and Maller, 1985; Smith, 1989).

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