Roles of heterotrimeric and monomeric G proteins in sperm-induced activation of mouse eggs

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SUMMARY

Results of several lines of experimentation suggest that sperm-induced egg activation has several features in common with G protein-coupled receptor signal transduction mechanisms. We report that microinjection of GDPβS into metaphase II-arrested mouse eggs blocks sperm-induced egg activation. Since GDPβS inactivates both heterotrimeric and monomeric classes of G proteins, the involvement of members of each of these families in sperm-induced egg activation was evaluated. Neither pertussis toxin treatment of eggs nor microinjection of eggs with inhibitory antibodies toward Goα blocked sperm-induced egg activation. Nevertheless, microinjection of phosducin, a protein that binds tightly to free G protein βγ subunits, specifically inhibited second polar body emission, the fertilization evoked decrease of H1 kinase activity and pronucleus formation. Microinjection of phosducin, however, did not inhibit the fertilization-induced modifications of the zona pellucida and microinjection of βγ did not result in egg activation in the absence of sperm. Inactivation of the monomeric Rho family of G proteins with C3 transferase from Clostridium botulinum inhibited emission of the second polar body and cleavage to the 2-cell stage, but did not affect the modifications of the zona pellucida or pronucleus formation. Microinjection of Rasv¹², which is a constitutively active form of Ras, did not result in egg activation in the absence of sperm. Moreover, microinjection of either an anti-Ras neutralizing antibody (Y13-259) or a dominant negative form of Ras (Ras⁵) did not affect events of sperm-induced egg activation. In contrast, microinjection of Ras⁵ inhibited embryo cleavage to the 2-cell stage. These results suggest that both heterotrimeric and monomeric G proteins are involved in various aspects of sperm-induced egg activation.

Key words: mouse egg activation, fertilization, G proteins, heterotrimeric and monomeric

INTRODUCTION

Fertilization of the metaphase II-arrested eggs is accompanied by a rapid and transient increase in intracellular calcium (Miyazaki et al., 1993), which appears critical for a cascade of cellular events that lead to egg activation and subsequent embryonic development. One event of egg activation is cortical granule (CG) exocytosis, which results in modifications of ZP3 and ZP2, two glycoproteins that, in addition to ZP1, constitute the zona pellucida (ZP). These changes constitute the ZP block to polyspermy (Wassarman, 1990). Other events of egg activation include resumption of the cell cycle, emission of the second polar body, recruitment and translation of maternal mRNAs (Cascio and Wassarman, 1982), pronucleus (PN) formation and migration, and the initiation of DNA synthesis for the first time since entry of the oocyte into the first meiotic prophase (Howlett and Bolton, 1985).

The intracellular signaling mechanisms mediating sperm-induced egg activation are poorly understood. It has been postulated that this process is controlled by ligand-receptor-effector interactions that are similar to those occurring in many somatic cells. Little is known, however, about the identity of this putative egg-associated receptor for sperm and the mechanism by which this receptor initiates transmembrane signaling.

Guanine nucleotide-binding regulatory proteins (G proteins) play an essential role in somatic cell signaling by coupling cell-surface receptors to various intracellular effectors. A characteristic common to all G proteins is their ability to cycle between an inactive GDP-bound state and an active GTP-bound state. Two major classes of G proteins – heterotrimeric and monomeric – exist in most cell types. Heterotrimeric G proteins are composed of α (Mr=39,000-46,000), β (Mr=35,000-37,000) and γ (Mr=6,000-8,000) subunits. Receptor occupancy results in GDP-GTP exchange and subsequent dissociation of α from the βγ dimer. The dissociated subunits, either independently or simultaneously, can modulate the activity of different intracellular effectors, e.g., adenyl cyclase, phospholipase A₂, phospholipase C (PLC)-β, or ion channels (e.g., K⁺ channels, Ca²⁺ channels) (for review see Hepler and Gilman, 1992).

Several lines of evidence suggest that heterotrimeric G
proteins play a role in fertilization. For example, microinjection of mammalian eggs with GTPγS, a hydrolysis-resistant G protein activator, results in the release of intracellular Ca²⁺ to variable extents and the likely exocytosis of CGs (Cran et al., 1984, 1988; Miyazaki, 1988; Swann et al., 1989; Swann, 1992). Conversely, microinjection of hamster eggs with the G protein antagonist, GDPβS, blocks the release of intracellular Ca²⁺ that occurs in response to the fertilizing sperm (Miyazaki, 1988).

Also consistent with a role for G proteins in mouse egg activation is the observation that acetylcholine treatment of mouse eggs expressing the mRNA encoding the human m1 muscarinic receptor, a member of the seven transmembrane family of G protein-coupled receptors, induces complete egg activation, including cleavage and activation of the embryonic genome (Williams et al., 1992; Moore et al., 1993). Similar results have been obtained using the same approach in echinoderm and amphibian eggs (Kline et al., 1988, 1990; Shilling et al., 1990). Moreover, fertilization of the sea urchin egg results in an increased turnover of phosphatidylinositol 1,4-bisphosphate (PIP₂) with the generation of inositol 1,4,5-trisphosphate (IP₃) and sn-1,2-diacylglycerol (DAG) (Turner et al., 1984; Ciapa and Whitaker, 1986; Ciapa et al., 1992), and fertilization of Xenopus oocytes produces an increase in the concentration of IP₃ (Stith et al., 1993). Receptor-mediated changes in phosphoinositide turnover can be mediated by a G protein-coupled pathway (Smrcka et al., 1991). PLC-catalyzed hydrolysis of PIP₂ may be involved in mouse egg activation since microinjection or treatment of mouse eggs with IP₃ or DAG, respectively, induces CG exocytosis and ZP modifications similar to those seen following fertilization (Endo et al., 1987a,b; Kurasawa et al., 1989; Ducibella et al., 1993). Moreover, microinjection of a monoclonal antibody to the IP₃ receptor (18A10), which does not interfere with IP₃ binding but blocks IP₃-induced Ca²⁺ release, inhibits the fertilization-associated increase in intracellular Ca²⁺ in hamster eggs (Miyazaki et al., 1992). In mouse eggs, microinjection of this antibody prior to insemination inhibits sperm-induced egg activation (Xu et al., 1994).

A second class of G proteins that couple cell surface receptors to intracellular effectors belongs to the monomeric Ras-related superfamily. This family consists of more than 50 members of low molecular weight (Mr=20,000-29,000) GTP-binding proteins that play critical roles in different intracellular processes that include regulation of the cell cycle and differentiation (Ras and Rap), intracellular vesicular trafficking (Rab and Arf), and control of cytoskeletal assembly (Rho and Rac) (for a review, see Bokoch and Der, 1993). Although several of these types of events are observed following fertilization, e.g., resumption of the cell cycle and changes in the cytoskeleton, the role of monomeric G proteins in egg activation is not known.

We report here that heterotrimeric and monomeric G proteins are likely to play a role in sperm-induced mouse egg activation. Microinjection of GDPβS, a hydrolysis-resistant analog of GDP, into metaphase II-arrested eggs blocks sperm-induced egg activation. Since GDPβS inactivates both heterotrimeric and monomeric G proteins, we evaluated the role of members of each family in sperm-induced egg activation. Inhibition of Gαᵣ family of heterotrimeric G proteins with pertussis toxin (PT) or blocking the activity of Gαᵣ with specific antibodies does not inhibit sperm-induced egg activation. Nevertheless, microinjection of phosducin, a protein that binds tightly free G protein βγ subunits, inhibits second polar body emission, the fertilization evoked decrease of H1 kinase activity and PN formation following insemination of these injected eggs. Phosducin, however, does not affect the ZP2 to ZP3 conversion. In contrast, microinjection of βγ does not induce egg activation in the absence of sperm. Inactivation of the Rho family of monomeric G proteins by microinjection of the C3 transferase from Clostridium botulinum inhibits the emission of the second polar body and cleavage to the 2-cell stage following insemination. Last, the Ras family of monomeric G proteins does not appear to be involved in sperm-induced egg activation events up to cleavage, as the constitutively active form of Ras, Ras¹va12, does not induce egg activation in the absence of sperm. Moreover, neither an anti-Ras neutralizing antibody (Y13-259) nor a dominant negative form of Ras (Ras¹) inhibits the events of sperm-induced egg activation. In contrast, however, microinjection of Ras¹ inhibits the cleavage to the 2-cell stage.

MATERIALS AND METHODS

G protein reagents

GDPβS was purified prior to use by the method of Connolly et al. (1982). Ras antibody Y13-259 (in 50 mM sodium phosphate, pH 7.3) was purchased from Oncogene. Oncogenic Ras¹va12, Ras¹va12 and Ras¹ [in 25 mM Hapes, 1 mM EDTA, 1 mM DL-dithiothreitol (DTT), pH 7.4] were a gift from Dr Paul Sternweis (Univ. of Texas, Southwestern Medical Center, Dallas, Texas). These antibodies were purified on a protein G agarose column (Gibco). Phosducin (10 mM Hapes, 100 mM KCl, 0.1 mM DTT) was generously provided by Dr Rehwa Ho Lee (UCLA). Dr Yee-Kin Ho (Univ. of Illinois at Chicago, Health Science Center, Chicago, Illinois) and Dr Teresa L. Jones (NIH) provided βγ-subunit (10 mM MOPS, pH 7.5, 2 mM MgCl₂, 200 mM NaCl, 1 mM DTT).

Oocyte and egg collection and culture

Fully grown, germinal vesicle-intact oocytes from preovulatory antral follicles were obtained from gonadotropin-primed 6-week-old female CF-1 mice (Harlan) and the oocytes were freed of attached cumulus cells as previously described (Schultz et al., 1983). Cumulus cell-enclosed metaphase II-arrested eggs were obtained from superovulated mice and the cumulus cell masses were dispersed with hyaluronidase (0.05%) as previously described (Kurasawa et al., 1989). The collection medium was Waymouth medium (Gibco) supplemented with 5 mM NaHCO₃, 20 mM Hapes, pH 7.4, pyruvate (100 µg/ml), gentamicin (10 µg/ml), and fetal calf serum (FCS) (10%, Gibco) (Way/FCS). Oocytes were collected in Way/FCS containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) to prevent germinal vesicle breakdown (GVBD) (Schultz et al., 1983). Oocytes were matured in vitro in Way/FCS in the absence of IBMX and microinjected eggs or 1-cell embryos were cultured in CZB medium (Chatot et al., 1989) in a humidified atmosphere containing 5% CO₃/5% O₂/90% N₂ at 37°C.

Microinjection of eggs

Metaphase II-arrested eggs were microinjected with ~10 pl of the appropriate solution as previously described (Williams et al., 1992). Microinjected eggs were cultured in CZB medium or subjected to in vitro fertilization as described below.
In vitro fertilization
In vitro fertilization of ZP-intact-metaphase II-arrested eggs was performed as previously described (Moore et al., 1993). For in vitro fertilization of ZP-free eggs, cells were freed of the ZP by incubation for ~15 seconds in acidic Tyrodes solution (pH 2.5) and then washed once in phosphate-buffered saline containing 3 mg/ml polyvinylpyrrolidone (PBS/PVP), pH 7.4. Eggs were transferred to Whitten’s medium (Whitten, 1971) and incubated for 1 hour at 37°C in an atmosphere of 5% CO2/5%O2/90% N2. Ten eggs were transferred into 10 µl drops of Whitten’s medium containing capacitated sperm (2.5×10^9/ml). After 1 hour, the embryos were washed in CZB medium and incubated as previously described (Moore et al., 1993). Second polar body emission, the extent of ZP2 to ZP2f conversion and PN formation were evaluated during the following 18 hours.

Direct and indirect immunofluorescence
For actin or DNA staining, eggs or embryos were fixed in 3.4% paraformaldehyde (in PBS pH 7.4) for 1 hour at 4°C and then permeabilized in PBS/PVP containing 0.1% Triton-X 100 for 30 minutes. Cells were washed in PBS/PVP and then incubated for 45 minutes in fluorescein-conjugated phalloidin (0.1 mM, Sigma) to localize actin filaments, or in 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml, Sigma) to stain DNA. Phalloidin and DAPI staining were detected using a Zeiss Universal fluorescence microscope equipped with fluorescein and DAPI filter cassettes, respectively. Cells were photographed with Kodak T-MAX film (32,000 ASA). For detection of endogenous Ras or microinjected Ras<sup>val12</sup>, the eggs were fixed in 100% methanol at ~20°C for 20 minutes, incubated at room temperature with anti-Ras antibody Y13-259 (10 µg/ml) for 1 hour, washed in PBS/PVP and then incubated with an affinity-purified fluorescein-conjugated mouse anti-rat IgG (1:500 dilution, Sigma). Indirect immunofluorescence detection of Ras was performed using a Bio-Rad MRC500 laser-scanning confocal microscope.

Microinjection of C3 transferase from Clostridium butulinum (C3-CB) and quantification of ADP-ribosylation
In order to evaluate the ability of C3-CB to catalyze the in situ ADP-ribosylation of the Rho family of monomeric G proteins in mouse eggs, 50 eggs were microinjected with C3-CB (final concentration 100 ng/ml) and another 50 uninjected eggs were used as the control. Eggs in each group were cultured for ~4 hours, washed 3 times in PBS/PVP and transferred to microcentrifuge tubes containing 10 µl of PBS, pH 7.2, 1% Lubrol PX, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 20 µM phenylmethylsulfonyl fluoride and 2 µM p-aminobenzamidine. The effectiveness of the in situ C3-CB catalyzed ADP-ribosylation was then monitored by measuring the extent of the in vitro C3-CB-catalyzed [<sup>32</sup>P]ADP-ribosylation of Lubrol PX egg extracts (Ridley and Hall, 1992). Briefly, in vitro [<sup>32</sup>P]ADP-ribosylation was initiated by the addition of 40 µl of 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 0.3 mM GTP with 0.5 mCi [<sup>32</sup>P]NAD (400 Ci/mmol) and 50 ng C3-CB to the egg extracts. The samples were then incubated for 60 minutes at 37°C and the reaction terminated by the addition of 2x sample buffer (Laemmli, 1970). The samples were subjected to SDS-PAGE (12.5% gel) and autoradiography was performed at ~80°C using Kodak X AR5 X-ray film. The autoradiogram was used to locate the [<sup>32</sup>P]ADP-ribosylated proteins in the gel; these bands were then cut out and the radioactivity was quantified by liquid scintillation counting.

Zona pellucida isolation, biotinylation and electrophoretic analysis
Isolation of the ZP and biotinylation were performed as previously described (Kurasawa et al., 1989; Moos et al., 1994). Briefly, ZP were isolated and transferred to microcentrifuge tubes containing 20 mM Hepes, pH 7.4, 150 mM NaCl and 3 mg/ml PVP. Biotin (Immuno-ure NHS-LC Biotin, Pierce in 100 mM NaHCO<sub>3</sub>, pH 8.3) was added to a final concentration of 417 µg/ml. The ZP were incubated at room temperature for 60 minutes and then washed in bicarbonate-free minimal essential medium (Earle’s salts) supplemented with PVP. Individual ZP were transferred into microcentrifuge tubes containing 2X concentrated sample buffer (Laemmli, 1970) and subjected to SDS-PAGE (9% gel) under reducing conditions. Proteins were transferred to Immobilon (Millipore), blocked in 6% low fat milk for 60 minutes and incubated with a Vectastain ABC kit following the manufacturer’s recommendations. Blots were developed using ECL detection reagents (Amersham) and exposed to Kodak X AR5 X-ray film.

Histone H1 kinase activity
Histone H1 kinase activity in single eggs was assessed by the method of Moos et al. (unpublished data). Briefly, eggs or embryos were collected, washed in PBS/PVP and individual eggs or embryos then transferred into microcentrifuge tubes containing 25 mM Hepes, pH 7.4, 80 mM β-glycerophosphate, 100 µM NaVO<sub>4</sub>, 15 mM p-nitrophenylphosphate, 10 mM EGTA, 10 mM MgCl<sub>2</sub>, 10 µg/ml leupeptin, 10 µg/ml aprotenin, 10 µg/ml PMSF, 100 mM ATP, 2.2 µM protein kinase A inhibitor peptide (Sigma) and 250 µg/ml histone (type III-S from calf thymus, Sigma), and then stored at ~80°C. When the assay was performed the extracts were thawed and [<sup>32</sup>P]ATP (3,000 Ci/mm) was added to a final concentration of 100 µC/mm in a final volume of 10 µl. The reaction was allowed to proceed for 30 minutes at 30°C and then stopped by adding 2x sample buffer (Laemmli, 1970). The extracts were subjected to SDS-PAGE (12% gel). Gels were stained with Cosmamise blue (0.1%), dried and then subjected to autoradiography at ~80°C using Kodak X AR5 X-ray film. Films were analyzed using scanning densitometer and Image I software (Interactive Video System, Inc., Concord, MA.).

RESULTS
Effect of GDPβS on the ZP2 to ZP2f conversion and pronucleus formation following in vitro fertilization
In the cell, G proteins exist in a dynamic equilibrium between the inactive GDP- and active GTP-bound forms. GDPβS, a hydrolysis resistant analog of GDP, competes with GTP for the nucleotide-binding site of G proteins and thereby prevents their activation. In order to determine whether G proteins play a role in sperm-induced egg activation, metaphase II-arrested eggs were microinjected with varying concentrations of GDPβS, inseminated 2 hours later and then examined for end points of egg activation.

Microinjection of GDPβS inhibited the extent of the ZP2 to ZP2f conversion and PN formation in a concentration-dependent manner (Fig. 1). Consistent with this inhibition, which should result in a loss of the ZP polyspermy block, was the observation that these microinjected eggs had a higher number of sperm present in the perivitelline space (Fig. 2B), which should result in a loss of the ZP polyspermy block, was the observation that these microinjected eggs had a higher number of sperm present in the perivitelline space (Fig. 2B), as well as in the cytoplasm (data not shown) when compared to buffer-injected eggs (Fig. 2A).

GDPβS has been found to have nonspecific effects on egg activation in other species that could potentially complicate the interpretation of the inhibitory effect of this nucleotide on sperm-induced mouse egg activation (Crossley et al., 1991). It is unlikely, however, that the inhibitory effect of GDPβS on sperm-induced activation of mouse eggs was nonspecific. Tetradecanoylphorbol myristate 13-acetate (TPA) induces CG exocytosis and the ZP2 to ZP2f conversion (Endo et al., 1987a; Ducibella et al., 1993) and appears to act distally to G protein...
activation (Williams et al., 1992). TPA treatment of these GDPβS-injected eggs resulted in an extent of ZP2 to ZP2f conversion similar to that produced by TPA alone (data not shown); the eggs were treated for 1 hour with 20 ng/ml of TPA 2-3 hours postmicroinjection of GDPβS, whose final concentration was 3 mM. In addition, although we observed that GDPβS inhibited protein synthesis by ~30% (Moore et al., unpublished observations), this inhibition could not account for the inhibition of PN formation (Fig. 1), since complete inhibition of protein synthesis by cycloheximide did not block PN formation (Moore et al., 1993). Last, following a 20-24 hour culture of these GDPβS-microinjected eggs, the eggs displayed a normal morphology (data not shown).

**Effect of pertussis toxin catalyzed ADP-riboseylation on sperm-induced egg activation**

The inhibitory effects of GDPβS suggested that sperm-induced egg activation is mediated by G proteins. Since activation of the heterotrimeric Gs family of G proteins following cholera-toxin catalyzed ADP-riboseylation does not activate mouse eggs (Williams et al., 1992), we determined whether inactivation of the heterotrimeric Gi family of G proteins with PT affects sperm-induced egg activation.

GV-intact oocytes were collected in medium containing 0.2 mM IBMX and the cells were matured in vitro in the presence or absence of PT (1 μg/ml) as previously described (Moore et al., 1993); we have previously shown that, under these conditions, PT catalyzes the in situ ADP-riboseylation and functional inactivation of >95% of the endogenous Gαi substrate (Moore et al., 1993). Metaphase II-arrested eggs were subjected to in vitro fertilization and the extent of the ZP2 to ZP2f conversion and PN formation were evaluated ~18 hours later. In vitro fertilization of either PT-treated or untreated eggs resulted in a similar extent of ZP2 to ZP2f conversion (>67%) or PN formation (>75%). These data suggested that sperm-induced egg activation did not appear to be mediated through the family of PT-sensitive G proteins.

**Effect of microinjection of antibodies directed against the Gαq subunit on sperm-induced egg activation**

Gq represents a member of the family of PT-insensitive heterotrimeric G proteins that is present in mouse eggs (Williams et al., 1992). Since Gq can stimulate PLC-β1 in somatic cells (Smrcka et al., 1991; Kozasa et al., 1993) and this enzyme catalyzes the hydrolysis of PIP2 to produce DAG and IP3, two second messengers that induce early events of egg activation (Endo et al., 1987a,b; Ducibella et al., 1993), experiments were designed to test whether Gq was involved in the sperm-induced egg activation.

Two antibodies directed to Gαq/Gα11 were microinjected into mouse eggs and their effects on sperm-induced egg acti-
subunits generated in response to sperm-formation. Eggs were microinjected with buffer (open bars), 5 µM phosducin (stippled bars), or 5 µM phosducin inactivated at 90°C for 10 minutes (solid bars) and then inseminated as described under Material and Methods. Polar body emission and PN formation were evaluated ~18 hours after insemination and individual ZP were isolated and analyzed. The data are expressed as the mean ± SEM. The difference in polar body emission and PN formation between buffer- and phosducin-microinjected samples of the combined experiments is significant (unpaired t test, *P<0.01; **P<0.05).

![Graph Fig. 3. Effect of microinjected phosducin on the extent of the ZP2 to ZP2f conversion, second polar body emission and pronucleus formation. Eggs were microinjected with buffer (open bars), 5 µM phosducin (stippled bars), or 5 µM phosducin inactivated at 90°C for 10 minutes (solid bars) and then inseminated as described under Material and Methods. Polar body emission and PN formation were evaluated ~18 hours after insemination and individual ZP were isolated and analyzed. The data are expressed as the mean ± SEM. The difference in polar body emission and PN formation between buffer- and phosducin-microinjected samples of the combined experiments is significant (unpaired t test, *P<0.01; **P<0.05).

![Graph Fig. 4. Relationship between the final concentration of microinjected phosducin and the effects on ZP2 to ZP2f conversion (●), second polar body emission (Δ) and pronucleus formation (○) following insemination. Eggs were microinjected and inseminated as described under Materials and Methods. Polar body emission and PN formation were evaluated ~18 hours after insemination and individual ZP were isolated and analyzed. A minimum of 24 eggs were analyzed at each concentration point. The experiment was performed two times and since similar results were obtained, the data were pooled.

Effect of microinjection of phosducin and βγ subunit on sperm-induced egg activation

βγ, as well as α subunits of heterotrimeric G proteins can function downstream to modulate specific effectors (e.g., adenylyl cyclase, phospholipase A2, PLC-β) or ion channels (e.g., K+ channels, Ca2+ channels) (for review see Hepler and Gilman, 1992). Recently, it has been shown that, in starfish oocytes, microinjection of βγ subunits induces oocyte maturation in the absence of 1-methyladenine, which is the physiological inducer of oocyte maturation and these matured oocytes are fully capable of being fertilized (Jaffe et al., 1993). Moreover, microinjection of phosducin, which is a protein of Mr=33,000 that is present in the retinal rods and has a high affinity for free transducin βγ subunits (βγ) (Lee et al., 1987), inhibits the 1-methyladenine stimulation of oocyte maturation.

In order to evaluate if βγ subunits play a role in mouse egg activation, we microinjected βγ into metaphase II-arrested eggs. βγ (5 µM) did not induce modifications of ZP or PN formation in the absence of sperm. Moreover, insemination of βγ-injected eggs displayed similar extents of the ZP2 to ZP2f conversion (>80%), polar body emission (>90%), or PN formation (>90%) when compared to the buffer-injected and inseminated eggs.

To test whether βγ subunits generated in response to sperm-egg interaction lead to signaling events that are involved in egg activation, metaphase II-arrested mouse eggs were microinjected with phosducin (5 µM final concentration) and then inseminated. No significant inhibitory effect of phosducin on the extent of ZP2 to ZP2f conversion was observed when compared to buffer-injected eggs (Fig. 3). Nevertheless, this concentration of phosducin significantly decreased the extent of second polar body emission (P<0.01) and PN formation (P<0.05) when compared to buffer-injected eggs (Fig. 3). The inhibitory effect of phosducin on polar body emission and PN formation occurred in a concentration-dependent manner, and phosducin did not appear to inhibit significantly the extent of the ZP2 to ZP2f conversion at any of the concentrations tested (Fig. 4).

Since phosducin inhibited late events of sperm-induced egg activation, we assessed if phosducin was also affecting cell cycle resumption. Unfertilized metaphase II-arrested eggs display high levels of cdc2 kinase activity, as evidenced by high levels of histone H1 phosphorylation (Rime and Ozon, 1990). Within the first hour following fertilization, the cdc2 kinase activity decreases and this change in activity can be monitored in vitro by the ability of cdc2 to phosphorylate exogenously added histone H1 protein in an H1 kinase assay.

Eggs were microinjected with phosducin (5 µM) or buffer and the ZPs were then removed to synchronize the timing of fertilization following insemination. Individual eggs were collected before insemination, as well as 1, 2 and 3 hours postinsemination, and the in vitro H1 kinase assay was performed. H1 kinase activity decreased more than 50% in
buffer-injected eggs after 1 hour of insemination, while in the phosducin-injected eggs the activity was significantly higher (Fig. 5). Between 1 and 3 hours postinsemination the histone H1 kinase activity decreased in the phosducin-injected eggs but was always higher than buffer-injected eggs. This decrease in H1 kinase activity in the phosducin-injected and inseminated eggs shown in Fig. 5 was due to the fact that although after 3 hours none of the phosducin-injected eggs had a PN, which normally appears starting 5 hours postfertilization, 50% of these eggs would eventually form a PN. Thus the decrease in H1 kinase activity reflected those eggs that were fertilized and would ultimately form a PN. This was confirmed by the results of the following experiment. Metaphase II-arrested eggs were injected with phosducin and then inseminated. Following 10 hours of culture, the eggs were separated into two groups that either had or had not formed a PN and H1 kinase activity was assessed in individual eggs in each group. H1 kinase activity remained high in those eggs that had not formed a PN, whereas in those that had formed a PN the enzyme activity decreased and was similar to that found in inseminated eggs that were buffer-injected (data not shown).

In order to determine whether these inhibitory effects of phosducin on egg activation were due to the ability of phosducin to recognize specifically and bind to free βγ subunits that are generated from GTP-bound Rho proteins (Rho A, B and C), whose functions have, in part, been elucidated by the use of the C3 transferase from *Clostridium botulinum* (C3-CB). For example, the C3-CB toxin selectively ADP-ribosylates Rho proteins and this covalent modification results in the loss of actin stress fibers and alterations in the morphology of many cell types (Hall, 1992; Ridley and Hall, 1992). Following fertilization, eggs undergo rapid changes in cytoskeletal organization, with a remarkable increase in actin polymerization (Maro et al., 1984), especially in the region of the metaphase spindle (see Fig. 8E,F). This set of changes culminates in the emission of the second polar body, which involves an actin-based system in the contractile ring, and is an event that signals the completion of the second meiotic division.

To evaluate the involvement of the Rho subfamily on sperm-induced egg activation, we first determined whether this protein was present in the egg by monitoring its ability to serve as a substrate for C3-CB-catalyzed ADP-ribosylation. 50

![Graph](image_url)

**Fig. 5.** Effect of microinjected phosducin on cdc2 kinase activity of metaphase II-arrested eggs prior to and following insemination. Eggs were microinjected with buffer (open bars) or phosducin (5 μM final concentration, solid bars) and then ZP-free eggs were inseminated as described under Materials and Methods. Eggs were collected before insemination (time 0) or 1, 2 and 3 hours after insemination in the appropriate buffer and the H1 kinase assay was performed as described under Materials and Methods. Following electrophoresis and autoradiography, the gels were subjected to densitometric analysis. Values were standardized to the phosphorylation levels obtained in uninjected metaphase II-arrested eggs. The data are expressed as the mean ± SEM.

![Table](image_url)

**Table 1. Effect of phosducin or phosducin/βγ microinjection on polar body emission and pronucleus formation of inseminated eggs**

<table>
<thead>
<tr>
<th>Substance Microinjected</th>
<th>No. embryos with PB/total no. eggs injected</th>
<th>No. embryos with PN/total no. eggs injected</th>
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<tbody>
<tr>
<td>Buffer</td>
<td>22/23</td>
<td>21/23</td>
</tr>
<tr>
<td>Phosducin</td>
<td>17/24</td>
<td>12/24</td>
</tr>
<tr>
<td>Phosducin + βγ</td>
<td>26/26</td>
<td>24/26</td>
</tr>
</tbody>
</table>

The difference between the phosducin and phosducin + βγ microinjected samples is significant (P < 0.01, χ²). The final concentration of phosducin or the phosducin + βγ complex was 5 μM.

In other experiments, the sperm chromatin inside the egg was stained with DAPI to ascertain whether the inhibitory effect of phosducin on second polar body emission and PN formation reflected a decrease in sperm penetration. Greater than 90% of the phosducin-injected eggs displayed sperm chromatin in the egg cytoplasm (data not shown), suggesting that initial interactions between the sperm and egg plasma membranes and incorporation of the sperm into the egg cytoplasm were not affected by phosducin.

Taken together, these data suggest that the inhibitory effects of phosducin on egg activation were specifically due to its ability to complex with free βγ subunits that are generated following sperm interaction with the egg plasma membrane and that are required for egg activation.

**Involvement of monomeric G proteins in sperm-induced egg activation**

The inhibitory effects of GDPβS and phosducin on the events of sperm-induced egg activation strongly suggest that heterotrimeric G proteins are involved in sperm-induced egg activation. However, the effects of GDPβS could also occur as a consequence of its ability to inhibit monomeric G proteins, since their activity is also regulated by GDP-GTP exchange. For this reason, we evaluated the involvement of two monomeric G protein subfamilies, Rho and Ras, on sperm-induced egg activation.

The Rho subfamily includes a number of closely related proteins (Rho A, B and C), whose functions have, in part, been elucidated by the use of the C3 transferase from *Clostridium botulinum* (C3-CB). For example, the C3-CB toxin selectively ADP-ribosylates Rho proteins and this covalent modification results in the loss of actin stress fibers and alterations in the morphology of many cell types (Hall, 1992; Ridley and Hall, 1992). Following fertilization, eggs undergo rapid changes in cytoskeletal organization, with a remarkable increase in actin polymerization (Maro et al., 1984), especially in the region of the metaphase spindle (see Fig. 8E,F). This set of changes culminates in the emission of the second polar body, which involves an actin-based system in the contractile ring, and is an event that signals the completion of the second meiotic division.

To evaluate the involvement of the Rho subfamily on sperm-induced egg activation, we first determined whether this protein was present in the egg by monitoring its ability to serve as a substrate for C3-CB-catalyzed ADP-ribosylation. 50
metaphase II-arrested eggs were microinjected with 100 ng/ml of C3-CB toxin and another 50 were left intact as controls. After 4 hours the eggs were collected, extracts prepared and in vitro [32P]ADP-ribosylation was performed. Eggs microinjected with the C3-CB toxin displayed a 79% decrease in the in vitro [32P]ADP-ribosylation of a substrate of Mr = 21,000 when compared to the uninjected group of eggs (Fig. 6); the mobility of this ADP-ribosylated species corresponded to that previously described for the Rho family (Hall, 1992). This result showed that the in situ ADP-ribosylation following microinjection of C3-CB effectively reduced the efficiency of the subsequent in vitro [32P]ADP-ribosylation of the egg extract. These results demonstrate that mouse eggs contain Rho proteins and that microinjection of eggs with C3-CB results in ADP ribosylation and functional inactivation of this class of monomeric G proteins.

To evaluate the effects of C3-CB-catalyzed ADP-ribosylation on sperm-induced egg activation, eggs were microinjected with either the C3-CB (100 ng/ml) or water and then inseminated. No effects of C3-CB on the extent of ZP2 to ZP2f conversion and PN formation were observed (Fig. 7). C3-CB microinjection did result, however, in a substantial decrease in the extent of second polar body emission and cleavage to the 2-cell stage (Fig. 7). Consistent with these results was the observation that treatment of eggs with cytochalasin D (1 µg/ml) during and after insemination had no inhibitory effect on the extent of the ZP2 to ZP2f conversion (>70%) and PN formation (>90%) but did inhibit second polar body emission (<3%) and cleavage to 2-cell stage (0%).

The inhibitory effect of each of these treatments on second polar body emission and cleavage to the 2-cell stage was likely due to the ability of these agents to disrupt microfilaments. Localization of actin filaments with phalloidin in inseminated eggs following C3-CB microinjection (Fig. 8C,G) or cytochalasin D treatment (Fig. 8D,H) displayed a notable decrease in the global amount of actin staining when compared to the water-injected and inseminated (Fig. 8F,B) or unfertilized eggs (Fig. 8A,E).

Taken together, these data demonstrate that Rho is involved in cytoskeletal changes required for the emission of second polar body and cleavage but is not required for CG exocytosis or PN formation.

Since Ras has been implicated in the regulation of cell cycle events (Bokoch and Der, 1993), we examined if microinjection of eggs with a constitutively activated form of Ras, Rasval12, could mimic events of egg activation in the absence of sperm. Microinjection of Rasval12 (50 µg/ml) into metaphase II-arrested eggs did not mimic any events of egg activation in unoinseminated eggs, e.g., ZP2 to ZP2f conversion, polar body emission. In addition, the time course of second polar body emission and PN formation following insemination of these injected eggs was similar to that of eggs injected with a non-oncogenic form of Ras, Rasval12 (Fig. 9A,B).1

Targeting of Ras to the plasma membrane by isoprenylation is essential for Ras function (Willingham et al., 1980; Ulsh and Shih, 1984; Grand et al., 1987). The lack of an effect of Rasval12 to promote egg activation in the absence of sperm was not due to the inability of the injected protein to localize to the plasma membrane. Immunocytochemical localization of the endogenous Ras and microinjected Rasval12 was performed by confocal microscopy and revealed that the endogenous Ras protein was detected at the plasma membrane (Fig. 10B) and the signal increased several fold after microinjection of Rasval12 (50 µg/ml) (Fig. 10C).

To evaluate the involvement of Ras in the sperm-induced egg activation, two approaches were used. The first was to microinject an anti-Ras Y13-259 antibody and the second was to microinject a dominant negative form of Ras, RasT. Both the Y13-259 antibody and RasT have been previously shown to inhibit the biological activity of endogenous Ras in intact cells (Deshpande and Kung, 1987; Gibbs et al., 1989).

1 It should be noted that this preparation of Rasval12 was active as shown by its ability to accelerate both insulin- and progesterone-induced oocyte maturation in Xenopus oocytes.

These experiments were performed by Susan Sadler, University of Colorado, Denver.
Microinjection of the Y13-259 antibody (final concentration of 500 µg/ml) or RasT (final concentration of 600 µg/ml) followed by insemination did not change the extent of ZP2 to ZP2f conversion and PN formation when compared to buffer-injected and inseminated eggs; in each instance, the extent of the ZP2 to ZP2f conversion and PN formation was >80% and 90%, respectively. Moreover, when the time of fertilization was synchronized by insemination of ZP-free eggs, RasT did not affect the time course of second polar body emission and PN formation when compared to buffer-injected eggs (Fig. 9C,D). In contrast, 97% of the buffer-injected eggs reached the 2-cell stage while just 51% of the RasT-injected eggs reached this stage of development (Table 2). These data demonstrate that although Ras is not involved in the events of sperm-induced egg activation, it may play a role later in progression from the G2 to M phase of the cell cycle.

DISCUSSION

Taken together, our data suggest a role for both heterotrimeric and monomeric G proteins in sperm-induced egg activation. Although we have not been able to date to determine which α subunit(s) is involved, the phosducin sensitivity and PT insensitivity of certain aspects of sperm-induced egg activation suggests a role for heterotrimeric G proteins of the PT-insensitive class. Monomeric G proteins of the Rho family are also implicated in events of egg activation, e.g., emission of the second polar body. Members of the Ras family, however, do appear not to be involved in these events, but may be required for cleavage to the 2-cell stage.

Fig. 8. Photomicrographs of metaphase II-arrested eggs or 1-cell embryos microinjected with water or C3-CB transferase from Clostridium botulinum. A-D are phase-contrast photomicrographs and E-H are fluorescence photomicrographs. (A,E) metaphase II-arrested eggs; (B,F) 1-cell embryo microinjected with water; (C,G) 1-cell embryo microinjected with C3-CB; (D,H), 1-cell embryo treated with cytochalasin D. Direct fluorescence detection of phallolidin was performed as described under Materials and Methods. In F and G the arrowheads point to regions of either sperm incorporation or spindle formation.

Fig. 9. Effect of Rasval12 or RasT on the time course of polar body emission and pronucleus formation. Eggs were microinjected and then ZP-free eggs were inseminated as described under Materials and Methods. (A,B) Rasval12-injected eggs; (C,D) RasT-injected eggs. A minimum of 15 eggs were assessed per each time point. The experiments were performed three times with similar results. (A,C) The time course of polar body emission; (B,D) time course of PN formation. (□) uninjected eggs; (○) Rasval12 (50 µg/ml final concentration); (●) RasThr12 (50 µg/ml final concentration); (△) Buffer; (▲) RasT (600 µg/ml final concentration).
antibodies recognize only the Gαq (Blaszczak et al., 1994). It should be noted, however, that these titrations higher than those used to block effectively the activity of X384 antibodies on sperm-induced egg activation at concentrations 50% inhibition of PN formation following receptor mRNA microinjection and treated with acetylcholine results in a 50% inhibition of PN formation (Moore et al., 1993). This apparent contradiction may lie in inherent differences in the experimental models utilized in these two reports. For example, the overexpression of the hm1R may lead to promiscuous G protein-receptor coupling (e.g., through PT-sensitive and-insensitive G proteins) due to nonphysiological concentrations of receptors and/or G proteins, as well as mass action considerations.

Table 2. Effect of RasT on cleavage to the 2-cell stage

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. 2-cell embryos/total eggs injected</th>
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<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>I</td>
<td>15/15</td>
</tr>
<tr>
<td>II</td>
<td>22/24</td>
</tr>
<tr>
<td>III</td>
<td>30/30</td>
</tr>
<tr>
<td>Total</td>
<td>67/69</td>
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The difference between the buffer and RasT microinjected samples is significant (P<0.001, χ^2).

In the mouse egg, Gαs, Gα11, Gα12, Gα13 and Gαq are present (Jones and Schultz, 1990; Williams et al., 1992). Gαi2 is not involved in egg activation, since cholera toxin (CT)-catalyzed ADP-ribosylation of Gαi2 does not activate metaphase II-arrested eggs (Williams et al., 1992). Moreover, it does not appear that a PT-sensitive G protein is involved in sperm-induced egg activation. At first, this observation may appear contradictory to our previous observation that PT treatment of eggs expressing the human m1 muscarinic receptor (hm1R) following receptor mRNA microinjection and treated with acetylcholine results in a 50% inhibition of PN formation (Moore et al., 1993). This apparent contradiction may lie in inherent differences in the experimental models utilized in these two reports. For example, the overexpression of the hm1R may lead to promiscuous G protein-receptor coupling (e.g., through PT-sensitive and-insensitive G proteins) due to nonphysiological concentrations of receptors and/or G proteins, as well as mass action considerations.

The CT- and PT insensitivity of sperm-induced egg activation leaves Gαq as a good candidate, especially in light of the fact that Gαq can activate PLC-β1 and the products of PIP2 hydrolysis, IP3 and DAG, can elicit events of egg activation. We did not observe, however, any inhibitory affect of Z811 or X384 antibodies on sperm-induced egg activation at concentrations higher than those used to block effectively the activity of Gαq in somatic cells (Gutowski et al., 1991; Wilk-Blaszczyk et al., 1994). It should be noted, however, that these antibodies recognize only the Gαq and Gα11 members of Gαi, which also comprises other members (α12, α13, α14, α15 and α16). The complement of αi family members expressed in the mouse egg is not known. It is also possible that the lack of an inhibitory effect of these antibodies is due to an incomplete block of functional Gαq activity.

We also examined the role of βγ in egg activation, since βγ dimers, as well as α subunits can couple cell-surface receptors to various intracellular effectors (for review see Clapham and Neer, 1993). Microinjection of βγ subunit neither activates mouse metaphase II-arrested eggs in the absence of sperm nor inhibits sperm-induced egg activation. Several reasons could account for this lack of effect. Since there have been four β and seven different γ subunits identified to date and different combinations of these subunits display preferential effector specificity (Kleuss et al., 1992; Schmidt et al., 1992), it is possible that βγ (βγi) microinjected in these studies is not an appropriate subunit combination utilized during sperm-induced egg activation. Alternatively, fertilization may result in the activation of multiple signaling pathways, some of which are βγ dependent and independent, but all of which are necessary for complete egg activation. The lack of an effect of βγ is also consistent with its modulating the activity of effectors that are regulated by free α subunits. For example, βγ modulates the activity of adenylyl cyclase type II only in the presence of αi (Tang and Gilman, 1991); βγ alone has no modulatory effect on the activity of adenylyl cyclase type II. Consistent with these interpretations is the observation that microinjected phosducin only partially inhibited the extent of emission of the second polar body and PN formation (see below).

Although microinjected βγ has no effect on egg activation in the absence of sperm, βγ subunits released at the time of fertilization may be involved in egg activation, since microinjected phosducin inhibits some events of sperm-induced egg activation. It should be noted that whereas the ZP2 to ZP2 conversion, which is a consequence of CG exocytosis, is unaffected, cell cycle resumption is partially inhibited. This lack of an inhibitory effect on the ZP conversion may reflect that βγ-regulated pathways are not involved in this particular event. Alternatively, βγ-regulated pathways may be involved but are compensated by other signaling pathways, since it appears that requirements for CG exocytosis are less stringent than those for resumption of the cell cycle (Williams et al., 1992; Moore et al., 1993). Consistent with this idea is the fact that CG exocytosis appears less sensitive to modulation of intracellular Ca^2+ concentrations than is second polar body emission (Kline et al., 1994)
and Kline, 1992). Still a third possibility is that, unlike CG 
exocytosis that would require a single round of G protein activ-
cation, continual cycles of G protein activation are needed to
initiate events that lead to cell cycle resumption. In this case,
microinjected phosducin would not inhibit the first round of
αβγ dissociation, but would inhibit subsequent reassociation and
continual G protein activation. For example, in the retina,
phosducin interferes with cGMP hydrolysis by trapping and
decreasing the amount of free βγ available for binding with αG
and therefore inhibits new cycles of G activation (Lee et al.,

Following fertilization, the egg undergoes both dramatic
changes in cytoskeleton organization and resumption of the
cell cycle, and members of the Rho and Ras family of
monomeric G proteins, respectively, are implicated in these
processes in somatic cells. Consistent with the effect of C3-
CB-catalyzed ADP ribosylation of Rho proteins on the loss of
actin stress fibers in somatic cells is our observation that this
toxin also results in the loss of microfilaments in the egg and
the concomitant inhibition of both second polar body emission
and cleavage to the 2-cell stage. The mechanism by which Rho
regulates cytoskeleton organization is poorly understood (Hall,

Since Ras has not been implicated in the metaphase to
anaphase transition, it is not surprising that the constitutively
active form of Ras, Rasαval12 has no effect on inducing egg acti-
vation in the absence of sperm. Ras is implicated, however, in
cell cycle events prior to M phase. For example, a dominant
negative form of Ras, RasT, blocks a new round of DNA
synthesis and cell division in somatic cells (Stacey et al., 1991).
Consistent with the role of Ras in the cell cycle, we find that
RasT does not inhibit the sperm-induced metaphase to
anaphase transition but does inhibit cleavage to the 2-cell
stage. This inhibition of cleavage, however, is unlikely due to
Ras-mediated inhibition of DNA synthesis, since RasT does not
result in any readily discernible difference in BrdU incorpora-
tion (Moore, Kopf, Schultz, unpublished observations). Thus,
Ras may be involved in the G2/M transition of the first cell
cycle of the fertilized mammalian egg.

The results presented here are consistent with the hypoth-
thesis that the activation of heterotrimeric and monomeric G
proteins is involved in signaling mechanisms that mediate the
different aspects of sperm-induced egg activation. It appears
that heterotrimeric G proteins are involved in events that are
more immediately distal to sperm-egg plasma membrane inter-
action. Future experiments will address the role of the identity
of the PT-insensitive α subunit(s), as well as the type of βγ
subunits that are involved in sperm-induced egg activation.

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