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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 $G\alpha_q$ 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 $\beta\gamma_t$ 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 Ras^{val12}, 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 (Ras^T) did not affect events of sperm-induced egg activation. In contrast, microinjection of Ras^T 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 (M_r=39,000-46,000), β (M_r=35,000-37,000) and γ (M_r=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., adenylyl cyclase, phospholipase A_2, phospholipase C (PLC)-β, or ion channels (e.g., K^+ channels, Ca^{2+} 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 Ca^{2+} to variable extents and the likely exocytosis of CGs (Cran et al., l988; Miyazaki, 1988; Swann et al., l989; Swann, 1992). Conversely, microinjection of hamster eggs with the G protein antagonist, GDPβS, blocks the release of intracellular Ca^{2+} 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 (PIP_2) with the generation of inositol 1,4,5-trisphosphate (IP_3) 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 IP_3 (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 PIP_2 may be involved in mouse egg activation since microinjection or treatment of mouse eggs with IP_3 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 IP_3 receptor (18A10), which does not interfere with IP_3 binding but blocks IP_3-induced Ca^{2+} release, inhibits the fertilization-associated increase in intracellular Ca^{2+} 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 (M_r=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), superoxide generation (Rac and Rap) 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 $G\alpha_i$ family of heterotrimeric G proteins with pertussis toxin (PT) or blocking the activity of $G\alpha_q$ 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 $ZP2_f$ conversion. In contrast, microinjection of $\beta\gamma_t$ 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, Ras^{val12}, 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 (Ras^T) inhibits the events of sperm-induced egg activation. In contrast, however, microinjection of Ras^T 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 Ras^{val12}, Ras^{gly12} and Ras^T [in 25 mM Hepes, 1 mM EDTA, 1 mM DL-dithiothreitol (DTT), pH 7.4] were a gift from Dr Jackson Gibbs (Merck, Sharp, Dohme). C3 transferase from *Clostridium botulinum* (C3-CB) (in water) was generously donated by Dr Klaus Aktories (Pharmakologisches Institut des Universitätsklinikums Essen Hufelandstr, FRG). Z811 and X384 antibodies directed against a peptide sequence representing the common C terminus of both $G_{\alpha q}$ and $G_{\alpha 11}$ were a gift from Dr Paul Sternweis (Univ. Texas, Southwestern Medical Center, Dallas, Texas). These antibodies were purified on a protein G agarose column (Gibco). Phosducin (10 mM Hepes, 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 $\beta\gamma_t$-subunit (10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 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 $NaHCO_3$, 20 mM Hepes, 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-isobutyryl-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% CO_2/5% O_2/90% N_2 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% CO_2/5%O_2/90% N_2. Ten eggs were transferred into 10 μl drops of Whitten's medium containing capacitated sperm (2.5×10^4/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 $ZP2_f$ 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^{val12} 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 botulinum* (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 [^{32}P]ADP-ribosylation of Lubrol PX egg extracts (Ridley and Hall, 1992). Briefly, in vitro [^{32}P]ADP-ribosylation was initiated by the addition of 40 μl of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.3 mM GTP with 0.5 mCi [^{32}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 2× 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 [^{32}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 (Immunopure NHS-LC Biotin, Pierce in 100 mM $NaHCO_3$, 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-glycerolphosphate, 100 μM $NaVO_4$, 15 mM *p*-nitrophenylphosphate, 10 mM EGTA, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml PMSF, 100 nM 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 [γ-^{32}P]ATP (3,000 Ci/mmol) was added to a final concentration of 100 μCi/ml in a final volume of 10 μl. The reaction was allowed to proceed for 30 minutes at 30°C and then stopped by adding 2× sample buffer (Laemmli, 1970). The extracts were subjected to SDS-PAGE (12% gel). Gels were stained with Coomassie 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 $ZP2_f$ 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 $ZP2_f$ 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), 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 $ZP2_f$ conversion (Endo et al., 1987a; Ducibella et al., 1993) and appears to act distally to G protein

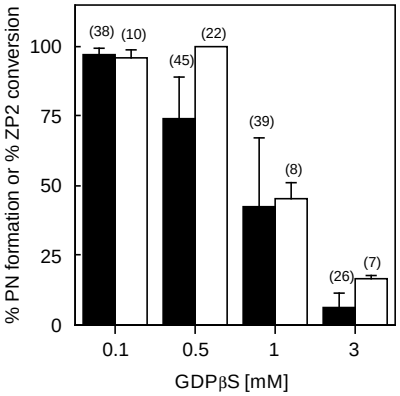

Fig. 1. Effect of GDPβS microinjection on ZP2 to $ZP2_f$ conversion and pronucleus formation following insemination of metaphase II-arrested eggs. Eggs were microinjected with GDPβS and then inseminated 2 hours later as described under Material and Methods. The extent of PN formation was evaluated ~18 hours postinsemination and then individual ZP were isolated and the ZP2 to $ZP2_f$ conversion was analyzed. The values represent percentage of PN formation or ZP2 to $ZP2_f$ conversion standardized to the values obtained in buffer-injected eggs. The data are expressed as the mean ± SEM, and the numbers in parentheses in this and subsequent figures represent the number of individual eggs analyzed. Solid bars, % ZP2 to $ZP2_f$ conversion; open bars, % PN formation.

activation (Williams et al., 1992). TPA treatment of these GDPβS-injected eggs resulted in an extent of ZP2 to $ZP2_f$ 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-ribosylation 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 G_s family of G proteins following cholera-toxin catalyzed ADP-ribosylation does not activate mouse eggs (Williams et al., 1992), we determined whether inactivation of the heterotrimeric G_i 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-ribosylation and functional inactivation of >95% of the endogenous $G\alpha_i$ substrate (Moore et al., 1993). Metaphase II-arrested eggs were subjected to in vitro fertilization and the extent of the ZP2 to $ZP2_f$ 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 $ZP2_f$ 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.

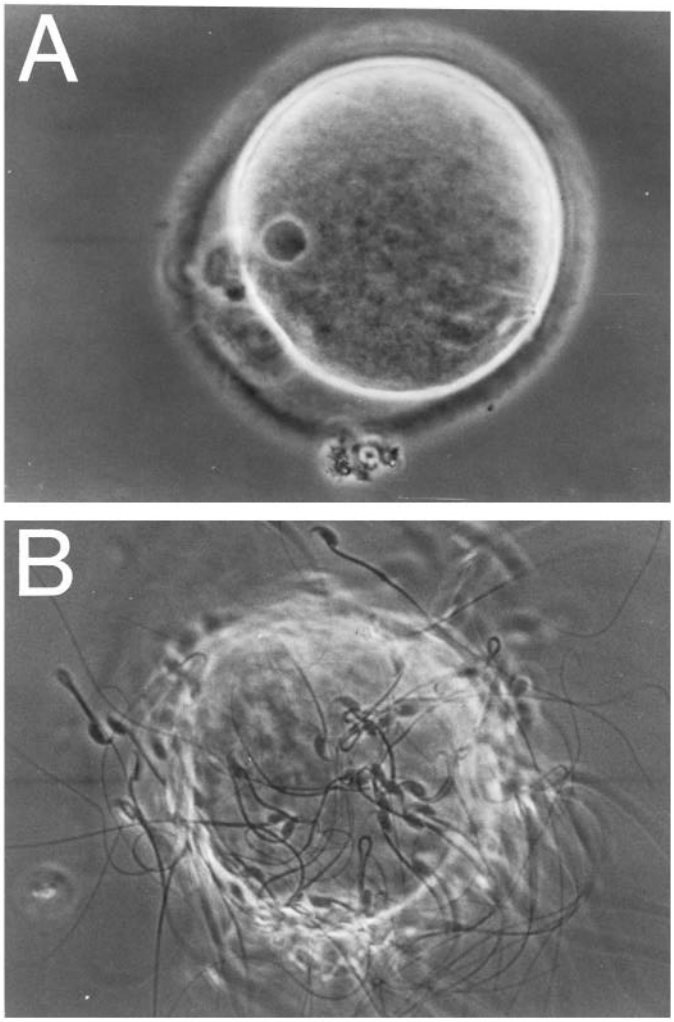

Fig. 2. Photomicrograph of buffer- or GDPβS-microinjected eggs followed by insemination. (A) Buffer microinjected embryo. (B) GDPβS (3 mM)-microinjected embryo. Eggs were microinjected and inseminated as described in the legend to figure 1. In B, although the ZP is out of focus the sperm are present within the perivitelline space.

Effect of microinjection of antibodies directed against the $G\alpha_q$ subunit on sperm-induced egg activation

G_q 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 PIP_2 to produce DAG and IP_3, 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 G_q was involved in the sperm-induced egg activation.

Two antibodies directed to $G\alpha_q/G_{\alpha 11}$ were microinjected into mouse eggs and their effects on sperm-induced egg acti-

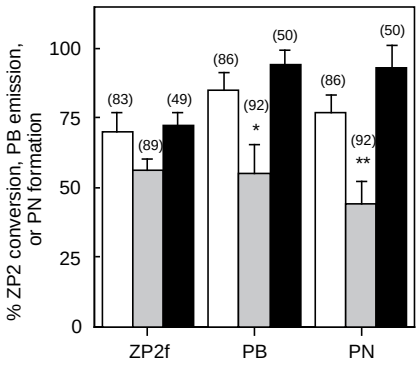

Fig. 3. Effect of microinjected phosducin on the extent of the ZP2 to $ZP2_f$ conversion, second polar body emission and pronucleus formation. Eggs were microinjected with buffer (open bars), 5 μM phosducin (stippled bars), or 5 μM phosducin inactivated at 90°C for 10 minutes (solid bars) and then inseminated 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).

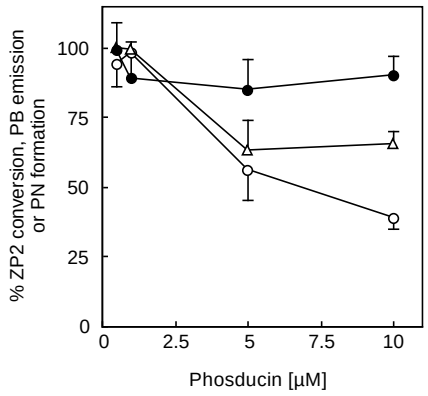

Fig. 4. Relationship between the final concentration of microinjected phosducin and the effects on ZP2 to $ZP2_f$ conversion (●), second polar body emission (Δ) and pronucleus formation (O) following insemination. Eggs were microinjected and inseminated as described under Materials and Methods. Polar 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.

vation were assessed. The Z811 and X384 antibodies recognize native $G\alpha_q/G_{\alpha 11}$ and block $G\alpha_q$ activation of PLC-β1 in cell membranes and $G\alpha_q/G_{\alpha 11}$-induced activation of K^+ currents in perfused membrane patches (Gutowski et al., 1991; Wilk-Blaszczak et al., 1994). After microinjection of the eggs with each of the antibodies (700 μg/ml final concentration; this concentration is higher than that used in the studies of Gutowski et al., 1991 and Wild-Blaszczak et al., l994) and insemination, the extent of the ZP2 to $ZP2_f$ conversion (>75%), and the time course and extent of the second polar body emission (>90 %) and PN formation (>90%) were no different from those observed for the control IgG microinjected eggs. The lack of inhibition was unlikely to be due to an inability of the antibodies to interact with $G\alpha_q$, as both antibodies were demonstrated to bind specifically to the plasma membrane following microinjection, as determined using immunocytochemical detection (data not shown).

Effect of microinjection of phosducin and $\beta\gamma_t$ 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 A_2, PLC-β) or ion channels (e.g., K^+ channels, Ca^{2+} 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 M_r=33,000 that is present in the retinal rods and has a high affinity for free transducin βγ subunits ($\beta\gamma_t$) (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 $\beta\gamma_t$ into metaphase II-arrested eggs. $\beta\gamma_t$ (5 μM) did not induce modifications of ZP or PN formation in the absence of sperm. Moreover, insemination of $\beta\gamma_t$-injected eggs displayed similar extents of the ZP2 to $ZP2_f$ 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 $ZP2_f$ 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 $ZP2_f$ 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

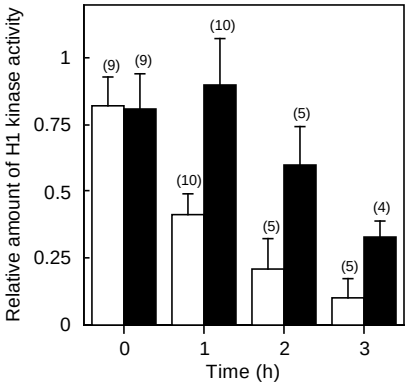

Fig. 5. Effect of microinjected phosducin on cdc2 kinase activity of metaphase II-arrested eggs prior to and following insemination. Eggs were microinjected with buffer (open bars) or phosducin (5 μM final concentration, solid bars) and then ZP-free eggs were inseminated as described under Materials 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.

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 in the egg, we performed two control experiments. First, phosducin was inactivated by heat treatment at 90°C for 10 minutes. Following microinjection of the heat-inactivated phosducin and insemination, the extent of polar body emission and PN were no different when compared to buffer-injected eggs (Fig. 3). Second, prior to microinjection phosducin was incubated with an equimolar concentration of $\beta\gamma_t$ for 12 hours in order to form a phosducin-$\beta\gamma_t$ complex. Following microinjection of this complex and insemination, the inhibitory effect of phosducin on polar body emission and PN formation was lost (Table 1).

Table 1. Effect of phosducin or phosducin/$\beta\gamma_t$ microinjection on polar body emission and pronucleus formation of inseminated eggs

Substance Microinjected	No. embryos with PB/ total no. eggs injected	No. embryos with PN/ total no. eggs injected
Buffer	22/23	21/23
Phosducin	17/24	12/24
Phosducin + $\beta\gamma_t$	26/26	24/26

The difference between the phosducin and phosducin + $\beta\gamma_t$ microinjected samples is significant ($P< 0.01$, χ^2). The final concentration of phosducin or the phosducin + $\beta\gamma_t$ 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

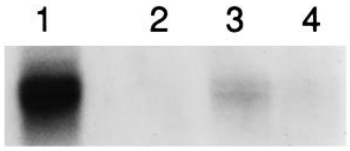

Fig. 6. Autoradiogram of in vitro ADP-ribosylated egg proteins by C3-CB transferase. Following microinjection the eggs were washed and egg extracts were prepared for C3-CB-catalyzed [^{32}P]ADP-ribosylation as described under Material and Methods. Following electrophoresis and autoradiography, radiolabeled bands were cut out of the gel and the radioactivity was quantified by liquid scintillation counting. Shown is a region of the autoradiogram and no other specifically radiolabeled bands were detected. Lane 1, brain extract subjected to [^{32}P]ADP-ribosylation in the presence of C3-CB; lane 2, egg extract incubated with the ADP-ribosylation reagents in the absence of C3-CB; lane 3, egg extract subjected to [^{32}P]ADP-ribosylation in the presence of C3-CB; lane 4, eggs microinjected with C3-CB prior to extract preparation and subsequent [^{32}P]ADP-ribosylation in the presence of C3-CB.

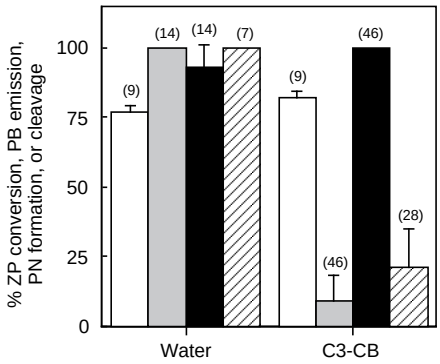

Fig. 7. Effect of *Clostridium botulinum* C3 transferase (C3-CB)-catalyzed ADP-ribosylation on ZP2 to ZP2$_f$ conversion (open bars), polar body emission (stippled bars), pronucleus formation (solid bars) and cleavage to the 2-cell stage (hatched bars). Eggs were microinjected with water or C3-CB and then inseminated as described under Materials and Methods. Polar body emission was evaluated ~7 hours, PN formation and ZP2 to ZP2$_f$ was assayed after ~18 hours and cleavage to 2-cell stage was determined ~24 hours following insemination, respectively. The data are expressed as the mean ± SEM.

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 [^{32}P]ADP-ribosylation was performed. Eggs microinjected with the C3-CB toxin displayed a 79% decrease in the in vitro [^{32}P]ADP-ribosylation of a substrate of M_r=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 [^{32}P]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 the ZP2 to ZP2$_f$ 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 ZP2$_f$ 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. 8B,F) 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, Ras^{val12}, could mimic events of egg activation in the absence of sperm. Microinjection of Ras^{val12} (50 μg/ml) into metaphase II-arrested eggs did not mimic any events of egg activation in uninseminated eggs, e.g., ZP2 to ZP2$_f$ 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, Ras^{gly12} (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 Ras^{val12} 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 Ras^{val12} 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 Ras^{val12} (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, Ras^T. Both the Y13-259 antibody and Ras^T 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 Ras^{val12} 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.

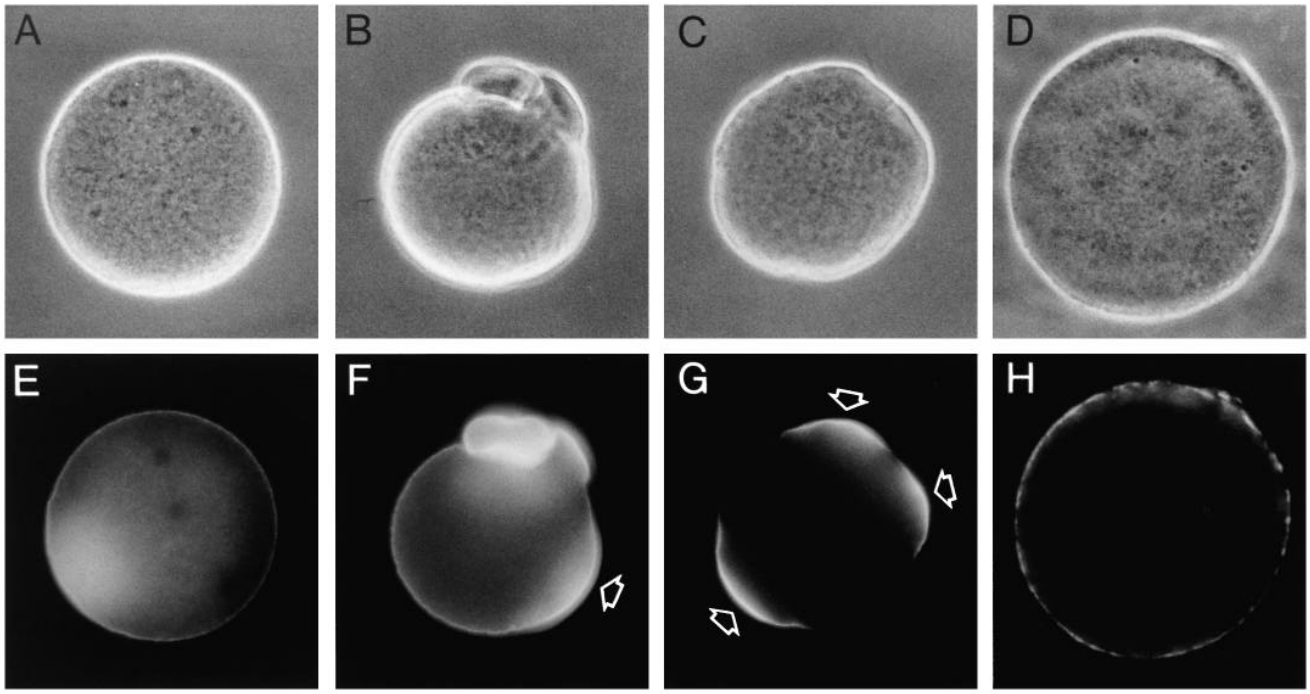

Fig. 8. Photomicrographs of metaphase II-arrested eggs or 1-cell embryos microinjected with water or C3-CB transferase from *Clostridium botulinum*. A-D are phase-contrast photomicrographs and E-H are fluorescence photomicrographs. (A,E) metaphase II-arrested eggs; (B,F) 1-cell embryo microinjected with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

Microinjection of the Y13-259 antibody (final concentration of 500 μg/ml) or Ras^T (final concentration of 600 μg/ml) followed by insemination did not change the extent of ZP2 to $ZP2_f$ conversion and PN formation when compared to buffer-injected and inseminated eggs; in each instance, the extent of the ZP2 to $ZP2_f$ conversion and PN formation was >80% and 90%, respectively. Moreover, when the time of fertilization was synchronized by insemination of ZP-free eggs, Ras^T 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 Ras^T-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 G_2 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.

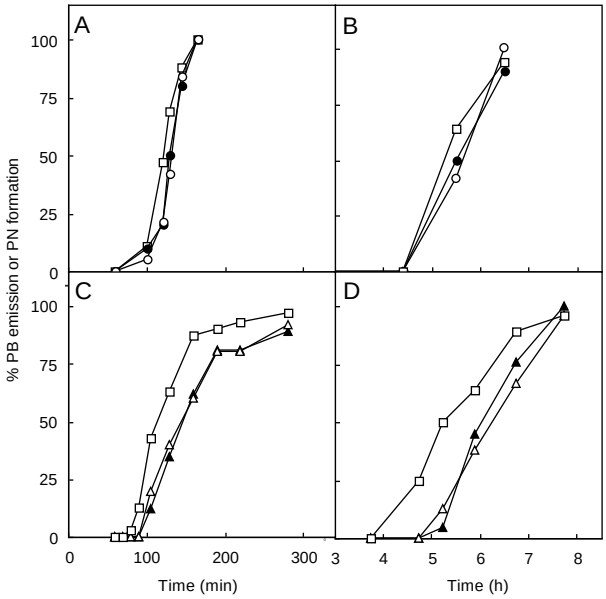

Fig. 9. Effect of Ras^{val12} or Ras^T on the time course of polar body emission and pronucleus formation. Eggs were microinjected and then ZP-free eggs were inseminated as described under Materials and Methods. (A,B) Ras^{val12}-injected eggs; (C,D) Ras^T-injected eggs. A minimum of 15 eggs were assessed per each time point. The experiments were performed three time with similar results. (A,C) The time course of polar body emission; (B,D) time course of PN formation. (□) uninjected eggs; (○) Ras^{val12} (50 μg/ml final concentration); (●) Ras^{gly12} (50 μg/ml final concentration); (△) Buffer; (▲) Ras^T (600 μg/ml final concentration).

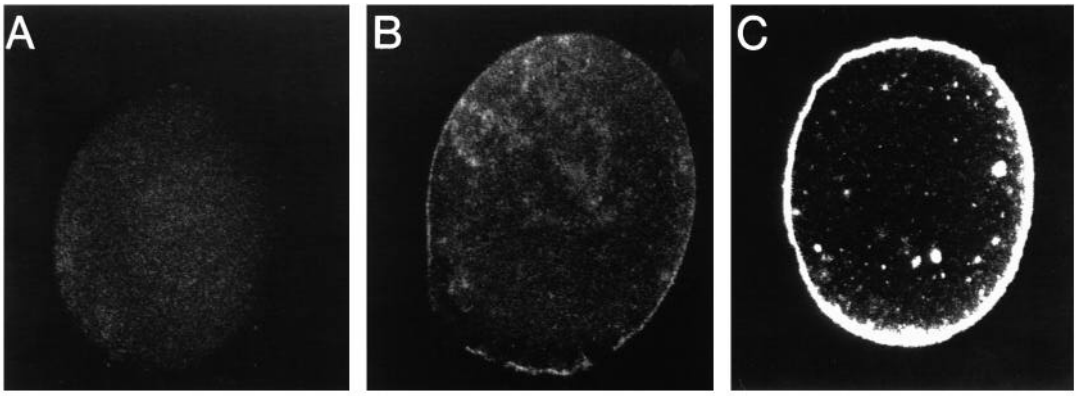

Fig. 10. Localization of endogenous Ras and microinjected Ras^{val12} in metaphase II-arrested eggs by confocal microscopy. (A) Control in which primary antibody was omitted and only the secondary antibody used. (B) Endogenous Ras detected with antibody Y13-259. Note the enhanced fluorescence at the plasma membrane when compared to panel A. (C) Egg injected with Ras^{val12} that was then detected with antibody Y13-259. Note the enhanced fluorescence at the plasma membrane when compared to that observed in B.

Table 2. Effect of Ras^T on cleavage to the 2-cell stage

	No. 2-cell embryos/total eggs injected	
Experiment	Buffer	Ras^T
I	15/15	5/11
II	22/24	9/22
III	30/30	14/21
Total	67/69	28/54

The difference between the buffer and Ras^T microinjected samples is significant ($P<0.001$, χ^2).

In the mouse egg, $G\alpha_s$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_q$ are present (Jones and Schultz, 1990; Williams et al., 1992). $G\alpha_s$ is not involved in egg activation, since cholera toxin (CT)-catalyzed ADP-ribosylation of G_s 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 $G\alpha_q$ as a good candidate, especially in light of the fact that $G\alpha_q$ can activate PLC-β1 and the products of PIP_2 hydrolysis, IP_3 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 $G\alpha_q/G_{\alpha 11}$ in somatic cells (Gutowski et al., 1991; Wilk-Blaszczak et al., l994). It should be noted, however, that these antibodies recognize only the $G\alpha_q$ and $G\alpha_{11}$ members of Gq, which also comprises other members ($\alpha 12$, $\alpha 13$, $\alpha 14$, $\alpha 15$ and α_{16}). The complement of α_q 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 G_q 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 $\beta\gamma_t$ 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 $\beta\gamma_t$ ($\beta_1\gamma_1$) 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 that 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 α_s (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 $ZP2_f$ 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

and Kline, 1992). Still a third possibility is that, unlike CG exocytosis that would require a single round of G protein activation, 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 $\alpha\beta\gamma$ 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 $\beta\gamma_t$ available for binding with α_τ and therefore inhibits new cycles of G_t activation (Lee et al., 1992).

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, 1992).

Since Ras has not been implicated in the metaphase to anaphase transition, it is not surprising that the constitutively active form of Ras, Ras^{val12} has no effect on inducing egg activation 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, Ras^T, 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 Ras^T 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 Ras^T does not result in any readily discernible difference in BrdU incorporation (Moore, Kopf, Schultz, unpublished observations). Thus, Ras may be involved in the G_2 to M transition of the first cell cycle of the fertilized mammalian egg.

The results presented here are consistent with the hypothesis 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 interaction. Future experiments will address the role of the identity of the PT-insensitive α subunit(s), as well as the type of $\beta\gamma$ subunits that are involved in sperm-induced egg activation.

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