**βγ subunits of heterotrimeric G-proteins contribute to Ca\(^{2+}\) release at fertilization in the sea urchin**

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**Summary**

A cytoplasmic Ca\(^{2+}\) transient is required for egg activation at fertilization in all animals. The pathway leading to release of Ca\(^{2+}\) from the endoplasmic reticulum in echinoderms includes activation of a SRC homolog, followed by phospholipase C\(_{γ}\) activation, and formation of inositol trisphosphate. However, the upstream activators or modulators of this signaling pathway are not known. We recently identified four Gα subunits of heterotrimeric G-proteins present in the sea urchin egg, and here we find that activation of G-proteins of the G\(αs\) and G\(αq\) type, but not G\(αi\) or G\(α12\) type, is required for normal Ca\(^{2+}\) dynamics at fertilization. The effects of these G-proteins are mediated by the G\(βγ\) subunits, occur upstream of the cytoplasmic Ca\(^{2+}\) release, and influence both the amplitude of Ca\(^{2+}\) release and the duration of the lag phase. We propose integration of the G-protein input into the framework of signaling at sea urchin fertilization.

Key words: Fertilization, Egg activation, Heterotrimeric G-proteins, Sea urchin, Ca\(^{2+}\)

**Introduction**

Fusion of egg and sperm during fertilization causes rapid and widespread changes in the egg. Egg responses, collectively termed ‘egg activation’, may differ between organisms, but exhibit common features such as significant cytoplasmic Ca\(^{2+}\) release, increased cytoplasmic pH, cortical granule exocytosis (CGE), initiation of protein synthesis, and DNA replication. Exocytosis of cortical granules is necessary for the formation of a permanent block to polyspermy, and is a consequence of cytoplasmic Ca\(^{2+}\) release. In all deuterostome eggs studied to date, Ca\(^{2+}\) release from the endoplasmic reticulum (ER) is triggered by inositol (1,4,5)-trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]\) production (reviewed by Runft et al., 2002), whereas eggs of certain protostome species, such as bivalve mollusks, exhibit influx of external Ca\(^{2+}\) through voltage-gated channels at fertilization (Deguchi and Morisawa, 2003). The physiology of egg activation has been extensively documented, although the signaling mechanisms leading to these responses are less well understood.

How is the signal for Ca\(^{2+}\) release \([\text{Ins}(1,4,5)\text{P}_3]\) produced in the deuterostome egg? The phospholipase C (PLC) family of enzymes hydrolyzes a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, to generate \(\text{Ins}(1,4,5)\text{P}_3\) and diacylglycerol. PLC enzymes are usually cytoplasmic but, upon egg activation, are recruited to the plasma membrane where their substrate resides. The PLC family consists of five subgroups – β, γ, δ, ε and ζ – and molecular mechanisms of activation and membrane recruitment differ between the subfamilies (Rhee, 2001; Saunders et al., 2002). PLCβ can be activated by heterotrimeric G-proteins, and PLCγ by tyrosine kinases; less is known about δ, ε and ζ isoforms, although the ζ isoform is sperm specific. Signaling mechanisms leading to the production of \(\text{Ins}(1,4,5)\text{P}_3\) at fertilization are under intense investigation in many deuterostome organisms, and pathways for both G-protein and tyrosine-kinase-mediated Ca\(^{2+}\) release have been found in the eggs of both vertebrate and invertebrate organisms (reviewed by Runft et al., 2002).

Evidence for heterotrimeric G-protein signaling at fertilization of invertebrate deuterostomes appears contradictory. Initial experiments in sea urchins showed that GTP\(_S\) (causing constitutive G-protein activity) induced CGE, an indicator of cytoplasmic Ca\(^{2+}\) release (Turner et al., 1986), whereas GDP\(_S\) (blocking G-protein activity) blocked CGE in response to sperm (Turner et al., 1986). These effects appeared to be upstream of Ca\(^{2+}\) release as buffering intracellular Ca\(^{2+}\) concentration at 0.1 µM prevented GTP\(_S\)-mediated activation, whereas injecting \(\text{Ins}(1,4,5)\text{P}_3\) into GDP\(_S\)-injected eggs rescued CGE (Turner et al., 1986). By contrast, inhibition of G-proteins by injection of GDP\(_S\) failed to block the raise in Ca\(^{2+}\) concentration during fertilization, and even caused Ca\(^{2+}\) transients on its own (Crossley et al., 1991). However, it must be kept in mind that experiments utilizing GTP\(_S\) or GDP\(_S\) do not address the role of specific G-proteins, and will activate or inhibit all GTPases present in the cell (heterotrimeric, as well as small GTPases such as Ras and Rab, and large GTPases such as dynamin). Given the documented feedback and cross-talk of various cell-signaling pathways, these experiments are now hard to interpret.

Other studies utilized more-specific G-protein-targeting reagents to dissect signaling at fertilization in invertebrates. Cholera toxin, which activates G\(αs\)–mediated signaling by ADP-ribosylation of the G\(αs\) protein, induced CGE in sea urchin eggs (Turner et al., 1987). By contrast, pertussis toxin, which ADP-ribosylates and thus inhibits G\(αi\) protein, produced inconsistent effects on CGE, being inhibitory only on occasion (Turner et al., 1987). Activation of the mammalian...
serotonin receptor expressed exogenously in starfish eggs led to CGE, supporting the presence of a functional G-protein signal transduction pathway that could also be active at fertilization (Shilling et al., 1994). Thus, certain heterotrimeric G-proteins are able to activate echinoderm eggs. However, without particular knowledge of the G-proteins present in the egg and reagents specific to each, it is not clear if heterotrimeric G-proteins actually do function in egg activation.

Because Ca\(^{2+}\) release at fertilization is mediated by Ins(1,4,5)\(P_3\) production, more-specific experiments were directed at elucidation of which PLC isoform is active at fertilization. PLC\(\gamma\) (normally activated by tyrosine kinases) can be inhibited by introduction of PLC\(\gamma\) Src-homology 2 (SH2) domains competing for the activator (Src-family kinase) from the PLC. Injection of starfish, sea urchin or ascidian eggs with PLC\(\gamma\) SH2 domains inhibits Ca\(^{2+}\) release at fertilization, suggesting that this PLC isoform functions in a Ca\(^{2+}\) release pathway (Carroll et al., 1999; Carroll et al., 1997; Runft et al., 2002; Shearer et al., 1999). However, PLC\(\gamma\) SH2 domains do not inhibit Ca\(^{2+}\) release initiated by the injection of cholera toxin, suggesting that the mechanism of Ca\(^{2+}\) release might be different in this case (Carroll et al., 1999). A candidate Src-family kinase, SFK1, was recently identified in the sea urchin (Giusti et al., 2003).

The most common argument against the involvement of the G-protein-mediated signaling in Ca\(^{2+}\) release at fertilization is that PLC\(\gamma\) is responsible for Ins(1,4,5)\(P_3\) production at fertilization. PLC\(\gamma\) activity is typically stimulated by tyrosine kinases and not directly by G-proteins, suggesting that G-proteins do not contribute to the signaling for Ca\(^{2+}\) release (Runft et al., 2002). However, recent progress in elucidating signal transduction networks makes this premise worth re-evaluating. Studies in mammalian cell cultures suggest that the tyrosine kinase and heterotrimeric G-protein signaling pathways cross-talk considerably. In fact, activation of Src homologs has been reported in many cases to be dependent on activation of heterotrimeric G-proteins (reviewed by Hall et al., 1999; Luttrell and Lefkowitz, 2002). Moreover, the contribution of G-proteins to regulated exocytosis occurs by stimulating Ca\(^{2+}\) release from the ER (Zeng et al., 2003) or even downstream of Ca\(^{2+}\) release (Blackmer et al., 2001; Kreft et al., 1999). These reports stimulated us to re-investigate the role of G-proteins in signaling at fertilization. We have identified which G-proteins are present in the sea urchin egg, and have used specific reagents to interfere with their function. These results suggest a re-integration of G-proteins into the bigger picture of fertilization signaling.

Materials and Methods

Reagents

All chemicals were purchased from Sigma, unless otherwise noted.

Animals

Adult *Lytechinus variegatus* were obtained from Duke University Marine Laboratory (Beaufort, NC) or collected in Tampa, FL, and handled as described (Voronina and Wessel, 2004).

Immunological approaches

Immunogens for specific G-protein antibodies were designed from the C-terminal peptides of each sea urchin G-protein alpha subunit, and antibodies were generated as described (Voronina and Wessel, 2004). The sequences were obtained from full-length cDNA cloning of each Gs subunit from two species, *Strongylocentrotus purpuratus* (Sp) and *L. variegatus* (Lv); the C-terminal peptide sequences for each Gs subunit were identical between the two species. Antibodies to G\(\alpha_q\) used for immunoblots were subsequently affinity purified by linking the peptide immunogen to agarose beads (Pierce SulfoLink Kit) with sequential binding and eluting of the antibody as per the manufacturer’s protocol. Additional antisera to sea urchin G\(\alpha_q\) C-terminal peptide (CRDIIQRMHLPQYEL) were generated in two rabbits by Sigma Genosys. These sera showed results consistent with previously described commercial ones (Voronina and Wessel, 2004).

Protein samples of total egg lysates and cell-surface complex were made as described (Kinsey, 1986) and were subjected to SDS-PAGE followed by immunoblotting as described (Voronina and Wessel, 2004). Cell-surface complex proteins were resolved along with total egg lysate proteins (in a ratio of 1:1). Labeling specificity was ascertained by peptide blocking experiments (Voronina and Wessel, 2004) (data not shown), the diluted antisera were incubated for 1 hour with 300 \(\mu\)M antigenic peptides and then cleared by centrifugation.

Immunofluorescent localization was performed on thick paraffin sections of eggs and embryos that were fixed and processed as previously described (Laidlaw and Wessel, 1994; Voronina and Wessel, 2004). Fertilized eggs were fixed 10 minutes after fertilization; the event of fertilization was confirmed by 100% fertilization envelope (FE) formation in the batch of eggs. For double labeling with hyalin, the sections were incubated with a 2B7 monoclonal antibody against hyalin (1:1) (Wessel et al., 1998) followed by anti-mouse rhodamine-conjugated Fabs (1:20; Jackson Research Laboratories). The G-proteins were then labeled with appropriate primary antibodies as described, followed by FITC-labeled secondary anti-rabbit Fabs (1:20; Jackson Research Laboratories). Signals were visualized and recorded by laser-scanning microscopy with a Zeiss LSM 410 confocal microscope with appropriate filters.

Microinjections

Egg injections were performed as described (http://155.37.3.143/panda/injection/index.html) (Jaffe, 1999; Kiehart, 1982; Voronina et al., 2003). Unfertilized eggs were placed in a Kiehart chamber in artificial seawater (ASW), and injected with appropriate solutions that never exceeded 5% of the cell volume. The injection volume was kept to 5\%.

Protein samples of total egg lysates and cell-surface complex were made as described (Laidlaw and Wessel, 1994; Voronina and Wessel, 2004). Fertilized eggs were fixed 10 minutes after fertilization; the event of fertilization was confirmed by 100% fertilization envelope (FE) formation in the batch of eggs. For double labeling with hyalin, the sections were incubated with a 2B7 monoclonal antibody against hyalin (1:1) (Wessel et al., 1998) followed by anti-mouse rhodamine-conjugated Fabs (1:20; Jackson Research Laboratories). The G-proteins were then labeled with appropriate primary antibodies as described, followed by FITC-labeled secondary anti-rabbit Fabs (1:20; Jackson Research Laboratories). Signals were visualized and recorded by laser-scanning microscopy with a Zeiss LSM 410 confocal microscope with appropriate filters.
described above for anti-Gαq IgG. For microinjections, Fab s were exchanged into the injection buffer and concentrated as above.

Mastoparan (Mas7; Biomol) was resuspended for microinjection in deionized water at 10 mM and injected to a final concentration of 10 μM. Pertussis toxin (A-subunit; Biomol) was resuspended in deionized water, and activated prior to injection by incubation at 35°C with 50 mM DTT, as previously described (Shilling et al., 1989). The calculated concentration of introduced toxin in the egg cytoplasm was 0.2 μg/ml.

FE formation was scored after challenging injected eggs with sperm. The percentage of eggs exhibiting normal FE was determined for each treatment in two or three independent experiments involving 10-12 cells each. The eggs counted as ‘abnormal’ conforming to either of the following criteria: no FE at all, partial FE (as seen in Fig. 2F), or low FE (with clearance of the envelope from the surface of the cell half or less than that in the non-injected eggs of the same batch). For each egg, the event of fertilization was confirmed by identifying the sperm pronucleus by DNA staining (0.2 μg/ml Hoechst 33258; Molecular Probes).

Quantification of CGE
FM1-43 recordings were performed with the Zeiss LSM 410 confocal microscope. The eggs were first equilibrated in 2 μM FM1-43 solution in ASW for 5 minutes in a Kiehart chamber, and the contents were then replaced with a dilute sperm suspension in ASW/FM1-43 (predilution of sperm in FM1-43 is necessary to prevent exhaustion of the label by binding to sperm). The egg to be recorded was centered, and brought in focus under the 40× objective (C-apochromat 40×, NA 1.2), zoom 1.5×. Time-lapse recording was performed with the following parameters: FITC filter set (excitation: LP 460, emission LP 515; 488 laser line), 1-second scan speed, 2× line averaging, 10-second interval between scans. This way of recording provided the greatest dynamic range of the increase in membrane-associated fluorescence leading to the Ca2+ wave (Carroll et al., 1997).

Results

Four members of the Gα protein family are present in eggs
We have cloned four Gα subunits – Gαi, Gαq, Gαs and Gα12 – from two species of sea urchin, produced and characterized antibodies against these proteins, and found them expressed in sea urchin oocytes and eggs (Voronina and Wessel, 2004). Here, we document the localization of Gα proteins with antibodies directed against subunit-specific C-terminal peptides of sea urchin Gα subunits (Fig. 1A-D) double labeled with an antibody to the cortical granule content protein hyalin (Wessel et al., 1998). Three of the G-proteins, Gαi, Gαq and Gαs, are associated with the cell cortex in eggs, whereas Gα12 is distributed throughout the cytoplasm.

To test biochemically if Gαi, Gαq and Gαs proteins are enriched in the egg cortex, we performed fractionation of eggs, followed by western blotting of the isolated cell-surface complex (CSC, containing plasma membrane with docked cortical granules), in comparison with the total egg lysate (Fig. 1E). The quality of fractionation was assessed by blotting for the known cortical granule components enriched in the CSC (MGB) (Haley and Wessel, 2004) and for yolk protein absent from the CSC (YP30) (Wessel et al., 2000). Gαi, Gαs and Gαq are all detected in CSC, and are considerably enriched in this fraction compared with whole egg lysate (note that egg samples are loaded at 10× the mass of the CSC samples). In agreement with the immunolocalization data, Gα12 is not enriched in the CSC. Documentation of anti-Gα subunit immunospecificity includes competitive ablation of the signal
(both by western blotting and immunolocalization) by the immunogenic peptides (Voronina and Wessel, 2004) (data not shown). Higher molecular weight bands detected by anti-Gα antisera represent nonspecific binding of the antibodies to the abundant content proteins of cortical granules, and are not ablated by peptide competition. These epitopes are not accessible to antibodies subsequently microinjected into the cytoplasm. The presence of Gαi and Gαs at the cell surface of mature sea urchin eggs is consistent with a previous report detecting ADP-ribosylated substrates of pertussis toxin (Gαi) and cholera toxin (Gαs) in CSC (Turner et al., 1987). Gαq and Gαs integrate into the plasma membrane upon exocytosis of the cortical granules at fertilization, whereas Gαi moves to endocytic vesicles (data not shown). We pursued a functional analysis of the G-proteins localized to the cell surface (Gαs, Gαq and Gαi), since they are poised to receive signal input at fertilization.

Interference with specific G-protein signaling inhibits CGE

To test G-protein function at fertilization, we injected commonly accepted and widely used specific inhibitory reagents. These include antibodies and peptides that prevent interaction of a particular Gα subunit with its cognate receptor (Gilchrist et al., 1998; Manning, 1999; Rasenick et al., 1994), or pertussis toxin, which inhibits Gαi-mediated signaling by irreversible ADP-ribosylation of the protein (Manning, 1999). These types of reagents have shown great efficacy in a diverse array of cells. Furthermore, they offer a significant advantage of affecting a specific target.

We used FE formation as an initial screen for G-protein function, since CGE is a consequence of Ca2+ signaling and formation of FE reports CGE. For these experiments, we determined the fraction of the egg population that exhibited normal FE elevation upon fertilization following treatment with the G-protein inhibitors (Fig. 2). Inhibition of Gαs by injection of anti-Gαs antibodies interfered most with CGE (90% of eggs with aberrant CGE), closely followed by inhibition of Gαq (73% of eggs with aberrant CGE), whereas interfering with Gαi had the least effect (25% of eggs with aberrant CGE; Fig. 2A-C). These effects are specific since denatured antibodies did not have any effect on fertilization, and nonspecific IgGs from preimmune antisera did not alter CGE either (Fig. 2A-C). For microinjections, we chose 100 µg/ml final concentration of antibodies in the cytoplasm, which is at the lower range of published effective inhibitory concentrations (Moore et al., 1994; Runft et al., 1999; Williams et al., 1998). The effectiveness of anti-Gαq and anti-Gαi at these low concentrations gives us greater confidence in the specificity of these reagents.

A common concern in using whole antibodies in inhibition studies is that they are multivalent, and could lead to artifactual inhibition of signaling by crosslinking the antigens, as opposed to Fab s that only block the accessibility of the antigen. Therefore, we generated monovalent Fab fragments of the anti-Gαq antibodies, and affinity purified these Fab fragments. Injection of 4 µg/ml of affinity-purified anti-Gαq Fabs prevented FE formation in 80% of treated eggs on average, similar to the effect produced by introduction of whole anti-Gαq IgGs (Fig. 2B).

As an independent test of Gα function at fertilization, we also interfered with G-protein signaling by injecting alternative reagents. A C-terminal competitor peptide for Gαq recapitulated the antibody-induced phenotype inhibiting CGE in 55% of cases on average. Consistent with the phenotypes produced by anti-Gαi antibodies, inhibition of Gαi by microinjection of competitor peptide or pertussis toxin seldom interfered with fertilization (83% and 70% normal fertilization on average in the case of peptide and pertussis toxin respectively).

Is the activation of G-proteins sufficient to cause CGE? Earlier reports indicate that activation of Gαs signaling by treatment with cholera toxin induces CGE by inducing Ca2+ release (Turner et al., 1987), apparently utilizing a PLCγ-independent signaling pathway (Carroll et al., 1999). In our hands, artificial activation of Gαi-mediated signaling by injection of mastoparan failed to induce CGE. Furthermore, mastoparan did not interfere with CGE upon insemination (Fig. 2C). Thus, our overall results suggest that Gαs and Gαq play...
the dominant role in signal generation for egg activation, making them the focus of our subsequent studies.

Direct analysis of CGE

To relate the qualitative morphological observation of interference with FE formation to the quantitative changes in CGE, we adapted an assay quantifying CGE at fertilization (Carroll and Jaffe, 1995; Terasaki, 1995). We used FM1-43, a membrane impermeable lipophilic dye that fluoresces only upon insertion into a hydrophobic environment. Sea urchin eggs were then treated with various reagents to inhibit G-protein signaling and allowed to fertilize. The extent of FM1-43 incorporation into the plasma membrane was quantified by fluorescence microscopy.

Is G-protein activity upstream or downstream of Ca2+ release?

The observed effects of G-protein inhibitors on CGE could be due to a direct effect of the G-proteins on the secretion machinery, or to an indirect effect on Ca2+ dynamics. To distinguish between these possibilities, we measured Ca2+ levels in the cells injected with inhibitors of G-protein signaling. For this purpose, we used recordings of eggs injected with Oregon Green 488 BAPTA-1 (OGB-1) dextran, which is a fluorescent Ca2+ sensor. The OGB-1 dextran is co-injected into the cell with Texas Red (TR) dextran, which is insensitive to Ca2+ levels, and fluorescence of the cell in both channels is recorded, such that a ratio of OGB-1 fluorescence to TR fluorescence as they normalize for potential variation in injected dye quantities, or photobleaching during fluorescence recordings (Stricker and Whitaker, 1999). The recording parameters in these experiments required 10-second intervals between frames, precluding us from reliably detecting the
activating potential at sperm-egg fusion and thus measuring the latent period between fertilization and cytoplasmic Ca\textsuperscript{2+} transient (see, however, Fig. 6 below).

We focused on the phenotypes of G\textalpha\textsubscript{s}- and G\textalpha\textsubscript{q}-inhibited eggs since they appear most relevant to egg activation. Control eggs injected with the Ca\textsuperscript{2+}-imaging mix exhibit the following characteristics: ~1.6 times increase in relative fluorescent intensity in the time span of 40-60 seconds, and a slower re-uptake of Ca\textsuperscript{2+} during the following 4-5 minutes. By contrast, the eggs injected with 100 \(\mu\)g/ml of anti-G\textalpha\textsubscript{s} or with 50 \(\mu\)M of G\textalpha\textsubscript{q} competitor peptide showed a significantly reduced Ca\textsuperscript{2+} release, exhibiting a decreased rate of Ca\textsuperscript{2+} release and reaching a peak of approximately 60% and 68% of the control eggs respectively (Fig. 4, Table 1). The experimental eggs also at times displayed abnormal propagation of the Ca\textsuperscript{2+} wave as compared with the control (Fig. 4D).

As a separate test of whether the observed defects of G-protein inhibition are upstream or downstream of the Ca\textsuperscript{2+} release, we asked if the effects of G-protein inhibition could be rescued by providing the eggs with Ca\textsuperscript{2+}. Such treatment would rescue exocytosis in the case of G-proteins being upstream of Ca\textsuperscript{2+} release, but not in the case of G-proteins having a direct influence on the vesicle fusion machinery. Injection of 28 nM Ins(1,4,5)P\textsubscript{3} (Turner et al., 1986) into the sea urchin eggs pre-injected with anti-G\textalpha\textsubscript{q}Fabs was sufficient to induce CGE in 100% of cases (\(n=9\); Fig. 2B). This argues that the effect of G-protein inhibition is upstream of Ca\textsuperscript{2+} release.

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The Gβγ complex is a primary mediator of G-protein signaling at fertilization

Inhibition of the signaling through two different Gα subunits, Gαs and Gαq, produces the same result of interference with CGE. This phenomenon could be explained by both Gαs and Gαq acting on a common downstream effector. An alternative hypothesis is that activation of members of different heterotrimeric G-protein complexes generates one common consequence of liberating Gβγ subunits, which are the signaling mediators (Fig. 5B). Suppression of signaling

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group (n)</th>
<th>Peak Ca2+, normalized fluorescence ratio (NFR)</th>
<th>Rate of rise, NFR units/second</th>
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</thead>
<tbody>
<tr>
<td>Anti-Gαs</td>
<td>Anti-Gαs (6)</td>
<td>1.29±0.03†</td>
<td>0.010±0.002†</td>
</tr>
<tr>
<td>Denatured anti-Gαs (3)</td>
<td>1.48±0.02</td>
<td>0.019±0.003</td>
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<tr>
<td>Gαq peptide</td>
<td>Peptide (8)</td>
<td>1.55±0.05†</td>
<td>0.015±0.004†</td>
</tr>
<tr>
<td></td>
<td>Sham (4)</td>
<td>1.76±0.12</td>
<td>0.025±0.006</td>
</tr>
<tr>
<td>βγ sequestration</td>
<td>βARK (6)</td>
<td>1.33±0.03†</td>
<td>0.010±0.002†</td>
</tr>
<tr>
<td></td>
<td>Phosducin (8)</td>
<td>1.23±0.10†</td>
<td>0.008±0.003†</td>
</tr>
<tr>
<td></td>
<td>Sham (4)</td>
<td>1.44±0.04</td>
<td>0.016±0.003</td>
</tr>
</tbody>
</table>

Summary of the parameters of Ca2+ transient dynamics presented on Figs 4 and 6. Eggs were treated and Ca2+-dependent fluorescent ratios were determined as described in the Materials and Methods and figure legends. Peak Ca2+ is expressed as a ratio of OGB-1/TR fluorescence, normalized to the ratio value at time 0; the presented value is an average of the indicated number of recordings. Rate of rise in Ca2+ concentration (units of normalized fluorescent ratio per second) was determined for each egg as a slope of linear regression through the 10, 20 and 30 seconds data points of respective data sets; the presented value is an average of the indicated number of recordings (n).†Significantly different from control, P<0.001.
‡Significantly different from control, P<0.01.

Fig. 5. (A) Sequestration of βγ subunits in the egg achieved by using two alternative reagents (100 µg/ml phosducin or 200 µg/ml βARKct, unless otherwise indicated) leads to abnormal fertilization envelope (FE) formation. Percentage of eggs forming normal FEs was determined for each treatment. Plotted are the average values of three or four independent experiments (10 eggs analyzed per each experiment; error bars, 1±s.d.). (B) βγ subunits might mediate signaling initiated by disparate Gα subunit activation. (C) High concentrations of injected phosducin prevent FE formation altogether, whereas lower ones cause low or partial FE elevation (10-20 eggs analyzed per each injected concentration). (D) Sequestration of βγ subunits leads to lower amount of membrane added to cell surface at fertilization. Each data point represents an average of 3 or 4 recordings (error bars, 1±s.d.).
through one particular Gα would decrease the overall abundance of released Gβγ subunits, and result in the decrease or suppression of downstream signaling.

To test the hypothesis that Gβγ complexes mediate G-protein-dependent signaling at fertilization, we overexpressed, purified and introduced Gβγ-sequestering proteins into sea urchin eggs and then challenged the eggs with sperm. The Gβγ inhibitors we used are the C-terminal domain of β-adrenergic receptor kinase, βARK1, or GRK2, (Blackmer et al., 2001), and phosducin (Thulin et al., 2001). Both these reagents serve as scavengers and bind to free Gβγ subunits, thereby preventing their interaction with normal cellular partners in a dominant fashion. The phosducin construct we used contains multiple substitutions of alanine for serine, rendering it insensitive to regulatory phosphorylation that could otherwise reduce its affinity for Gβγ complexes (Thulin et al., 2001). Introduction of either of these proteins into sea urchin eggs interfered with FE formation upon sperm addition (Fig. 5A). We tested several concentrations of injected phosducin, finding that the effects are concentration dependent (Fig. 5C); the difference between the effects of the 15 µg/ml and 113 µg/ml phosducin is statistically significant (P<0.001) by the Wilcoxin Rank Sum test. Phosducin injected at 45 µg/ml was the minimal concentration causing complete inhibition of CGE in at least some eggs. However, even as little as 15 µg/ml phosducin in the egg resulted in interference with CGE as determined by formation of low or incomplete FEs (Fig. 5C). The βARK-mediated effect was also dependent on the amount of protein introduced into the egg (data not shown). These effects were specific, as they were abolished by denaturing phosducin or βARK prior to injection into the eggs (Fig. 5A). Recording of the dynamics of cortical granule secretion by FM1-43 in these injected eggs confirmed that exocytosis is strongly reduced (Fig. 5D). We conclude from these data that the Gβγ subunits

**Fig. 6.** (A) Injection of phosducin or βARKct prevents normal Ca²⁺ release at fertilization. Each data point represents an average of 6 or 8 recordings (error bars, 1±s.d.). (B) Delay of Ca²⁺ release during fertilization of sea urchin eggs injected with 80 µg/ml phosducin. Fluorescence intensity over the individual eggs (injected with the indicated reagents) is shown over time, with the number of recordings in parentheses. Asterisks over traces indicate gamete fusion. Phosducin-injected eggs exhibited either a dramatically delayed and attenuated Ca²⁺ transient (top trace) or a mildly delayed and attenuated Ca²⁺ transient (bottom trace). The average delays for each group are summarized in below; *, values are significantly different from the denatured control by non-parametric Wilcoxin Rank Sum test, P<0.02. (C) Injection of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃] overrides phosducin effects and causes cortical granule exocytosis (CGE). Two eggs were injected with 80 µg/ml phosducin with Rhodamin dextran (red fluorescence). After 1 hour incubation, the left egg was injected with 28 nM Ins(1,4,5)P₃ with Alexa Fluor488 dextran (green fluorescence; orange when overlaid with red), which caused CGE and formation of fertilization envelope (FE). Arrowheads indicate oil droplets resulting from microinjection. (D) Response of phosducin-injected eggs to suboptimal amounts of Ins(1,4,5)P₃ (10 nM final concentration) is indistinguishable from that of control eggs. Shown are the results of a representative experiment; 6-15 eggs were analyzed per group. One hour after microinjection of the control or test reagent, the eggs were challenged with 10 nM Ins(1,4,5)P₃, and the time to initiation of CGE was monitored (white diamonds: the average time for the group; error bars, 1±s.d.). Black bars represent the percentage of eggs that were able to initiate CGE within 1 minute of Ins(1,4,5)P₃ microinjection.
are the primary mediators of the G\(\alpha s\) and G\(\alpha q\) signaling at fertilization.

**G\(\beta\gamma\) signaling is upstream of Ca\(^{2+}\) release**

Injection of phosducin or \(\beta\alpha{\text{ARK}}\) also significantly \((P<0.01)\) decreased Ca\(^{2+}\) release at fertilization in the treated eggs (46% and 69% of control on average; Fig. 6A). Phosducin appeared to be a more efficient inhibitor than \(\beta\alpha{\text{ARK}}\); it was inhibitory at lower concentrations, and the effects were more pronounced (Fig. 5D, Fig. 6A). These data complement the assays of CGE by FE formation and support the hypothesis of differential sensitivity of cortical granule subpopulations to available free Ca\(^{2+}\) (Blank et al., 1998; Matese and McClay, 1998; Terasaki, 1995). Variability in the threshold Ca\(^{2+}\) concentration that activates the fusion machinery of cortical granules is one possible explanation for the observed phenomenon.

The effect of G-protein signaling of the Ca\(^{2+}\) release could be in the initiation and/or the amplification step of the pathway. To test each of these possibilities, we measured the lag phase of the Ca\(^{2+}\) transient (the delay between gamete fusion and the initiation of Ca\(^{2+}\) release), using high-speed single-channel time-lapse recordings of OGB-1 dextran fluorescence in the eggs at fertilization. This assay is less quantitative than ratiometric analysis but provides better time resolution. The Ca\(^{2+}\) action potential functions as a fast electrical block to polyspermy and occurs simultaneously with sperm-egg fusion (McCulloh and Chambers, 1992). The action potential is detectable by this assay and serves as an indicator of gamete fusion. We find that inhibition of G\(\beta\gamma\) signaling by injection of 80 \(\mu\)g/ml phosducin significantly \((P<0.02)\) increases the delay between gamete fusion and the initiation of the Ca\(^{2+}\) wave to an average of 31 seconds (Fig. 6B), compared with either 7 seconds in the untreated eggs or 17 seconds in the eggs injected with denatured protein. We conclude that G\(\beta\gamma\) signaling is a necessary signaling pathway contributing to the initiation and amplification of the cytoplasmic Ca\(^{2+}\) transient.

Additionally, we tested whether CGE could be rescued by supplementing cells treated with G\(\beta\gamma\) scavengers with Ca\(^{2+}\). Eggs were injected with 130 \(\mu\)g/ml phosducin and then exposed to the Ca\(^{2+}\) ionophore A23187 in artificial seawater (normally containing 10 mM Ca\(^{2+}\)). In these experiments, 100% of phosducin-injected eggs \((n=4)\) were able to exocytose cortical granules and form full FEs as well as the control cells (data not shown). As a more rigorous and physiologically relevant test, we asked if CGE in phosducin-injected eggs could be rescued by Ins\(1,4,5\)P\(_3\). Providing phosducin-injected eggs with Ins\(1,4,5\)P\(_3\) (28 nM) also induced CGE as assessed by normal FE formation in 100% of cases \((n=5;\) Fig. 6C). The Ins\(1,4,5\)P\(_3\) added (28 nM) is less than that normally experienced by a sea urchin egg at fertilization (67 nM increase by 30 seconds post-fertilization) (Lee and Shen, 1998). The ability of low Ins\(1,4,5\)P\(_3\) levels to cause CGE suggests that the effect of phosducin is a result of interfering with a signal transduction pathway leading to Ca\(^{2+}\) release, and not due to the interference with the cortical granule fusion machinery.

We further tested this conclusion by assessing the response of phosducin-treated eggs to suboptimal concentrations of Ins\(1,4,5\)P\(_3\). Although sub-physiological doses of Ins\(1,4,5\)P\(_3\) can trigger CGE, sub-threshold levels of Ins\(1,4,5\)P\(_3\) would produce a condition sensitized to the effects downstream of Ins\(1,4,5\)P\(_3\) production. This allowed us to ask rigorously whether injected phosducin has effects downstream of Ins\(1,4,5\)P\(_3\) production. We determined the minimal concentration of Ins\(1,4,5\)P\(_3\) that was sufficient to induce FE formation when injected into the center of the cell (indicating near-normal Ca\(^{2+}\) transient and CGE) in 80% of non-treated eggs within 1 minute of injection (10 nM). This amount of Ins\(1,4,5\)P\(_3\) was then supplied to phosducin or control treated eggs to determine if CGE would still occur (Fig. 6D). Phosducin-injected eggs were indistinguishable from controls injected with the denatured protein by either the delay of response, or the percentage of eggs able to initiate CGE within 1 minute of injection. Thus, we conclude that the major contribution of the G\(\beta\gamma\) signaling at fertilization is upstream of the Ca\(^{2+}\) release and does not influence the exocytosis machinery directly or Ins\(1,4,5\)P\(_3\) responsiveness.

**Discussion**

Study of sea urchin eggs has led to the identification of key elements of the Ca\(^{2+}\) transient at fertilization. Recently, this research has determined several molecular participants in this process: SFK1 kinase activates PLC\(\gamma\), which produces Ins\(1,4,5\)P\(_3\) (reviewed by Runft et al., 2002). However, the upstream signaling events of egg activation are not clear, and the cellular factors impinging on the activity of SFK1 and PLC\(\gamma\) in the egg are not well understood (Sato et al., 2004). The participation of heterotrimeric G-proteins in egg activation has been controversial, largely because of a lack of specific G-protein reagents. The results presented here help resolve this controversy by identifying which G\(\alpha\) subunits are present in the egg, and by using inhibitory reagents specific for identified G\(\alpha\) subunits to address their specific function, if any, in fertilization.

We bridge several levels of effector activity manifest by the egg during its activation in this study, and find that signaling mediated by two of the heterotrimeric G-proteins in the egg, G\(\alpha s\) and G\(\alpha q\), is necessary for normal CGE during sea urchin fertilization. The release of free G\(\beta\gamma\) subunits produced by G-protein activation contributes to the timing and amplification of the cytoplasmic Ca\(^{2+}\) transient at sea urchin fertilization. We propose integration of G\(\beta\gamma\)-mediated signaling into the current model of egg activation, potentially through contribution to activation of Src homolog (Giusti et al., 2003). Such activation of Src through G\(\beta\gamma\) released by G\(\alpha i\) and G\(\alpha q\) activation has been previously reported in mammalian tissue culture cells (Canet-Aviles et al., 2002; Iigishi and Gutkind, 1998; Luttrell et al., 1997). Our model re-integrates a G-protein contribution with Src signaling in echinoderm fertilization instead of positioning them as contradictory and mutually exclusive mechanisms. Another mechanism allowed by our evidence is the recently proposed gating of the Ins\(1,4,5\)P\(_3\) receptor Ca\(^{2+}\) channels directly by the free G\(\beta\gamma\) subunits (Zeng et al., 2003).

How do our findings on specific G-protein involvement in the signaling at echinoderm fertilization fit with previous results? Earlier reports showing a variable effect of pertussis toxin on FE formation in sea urchin can now be explained by slower kinetics of exocytosis resulting from G\(\alpha i\) inhibition (Turner et al., 1987). A recent report suggests that mastoparan, a G-protein-activating peptide, induces Ca\(^{2+}\)-independent CGE in sea urchin eggs, whereas the inactive mastoparan analog,
MAS-17, is without effect (Lopez-Godinez et al., 2003). However, we did not detect induction of CGE by microinjecting mastoparan. The eggs in the Lopez-Godinez et al. study were bathed in solutions with a high concentration of mastoparan, taking advantage of the potential ability of the peptide to cross the plasma membrane. However, it is unclear how much mastoparan actually enters the cell. The concentration we tested in our study is 10 µM, which is ten times lower than the smallest effective concentration in the study by Lopez-Godinez et al. (2003); in our hands, mastoparan concentrations higher than 25 µM were toxic upon injection. Furthermore, mastoparan is known to produce nonspecific effects at high concentrations, which include plasma membrane pore formation (Suh et al., 1996), or activation of phospholipase D2 (Chahdi et al., 2003). A commonly accepted control for the specificity of mastoparan or activation of phospholipase D2 includes plasma membrane pore formation (Suh et al., 1996), to produce nonspecific effects at high concentrations, which in our hands, mastoparan concentrations higher than 25 µM were toxic upon injection. Furthermore, mastoparan is known to produce nonspecific effects at high concentrations, which include plasma membrane pore formation (Suh et al., 1996), or activation of phospholipase D2 (Chahdi et al., 2003). A commonly accepted control for the specificity of mastoparan is the ability to inhibit its effects by Gαi-inhibitory reagents: competitor peptides, inhibitory antibodies or pertussis toxin (Kreft et al., 1999). The pertussis toxin treatment described by Lopez-Godinez et al. (2003) failed to suppress mastoparan effects; therefore, the functional relevance of this report to physiological G-protein signaling is unclear.

In our hands, inhibiting G-protein signaling (Gαs, Gαq or Gβγ) in the egg never extinguished the Ca2+ transient completely, but it was significantly attenuated. We cannot presently distinguish between two possible explanations for this observation: (1) our reagents not being 100% effective in inhibiting G-protein signaling by competing with endogenous protein interaction partners, and/or (2) G-proteins function in the modulation – either by feedback or cross-talk – of the SFK–PLCγ–Ins(1,4,5)P3 pathway required for Ca2+ release. In either scenario, the G-proteins are clearly required for timely, full-scale Ca2+ release at fertilization. The complexity of Ca2+-generating signaling at fertilization has emerged in recent years, implicating contribution of many signaling pathways to the eventual release of cytoplasmic Ca2+ (reviewed by Galione and Churchill, 2002). In addition to the dominant role of Ins(1,4,5)P3, cyclic ADP-ribose (cADPR) is involved in generation of prolonged Ca2+ release from the ER (Leckie et al., 2003), and nicotinic acid adenine dinucleotide phosphate (NAADP) is hypothesized to act as a local trigger for Ca2+-induced Ca2+ release and to contribute to the generation of the Ca2+ activation potential (Churchill et al., 2003; Galione and Churchill, 2002). Production of cADPR at fertilization occurs in response to nitric oxide and cGMP, and heterotrimeric G-protein activity is linked to the cADPR pathway in certain mammalian tissues (Higashida et al., 2001).

Signaling mechanisms in vertebrate eggs (mouse, fish and Xenopus) at fertilization appear distinct from that in invertebrates; neither PLCγ SH2 domains, nor G-protein inhibitors such as pertussis toxin, anti-Gαq antibody, or sequestration of Gβγ subunits are inhibitory to the Ca2+ release (reviewed by Runft et al., 2002; Sato et al., 2004). Nevertheless, signaling through cytoplasmic tyrosine kinases still appears important, as tyrosine kinase inhibitors suppress Ca2+ release in Xenopus eggs (Sato et al., 2000), and SH2 domains of Fyn suppress Ca2+ transients in zebrafish eggs (Kinsey et al., 2003). The contribution of sperm factor (a sperm cytoplasmic protein inducing egg activation when introduced into the cytoplasm) is well documented for mammalian egg activation. Recent evidence supports a novel sperm PLCζ as a candidate for this activity, which produces a Ca2+ rise, enhanced by Ca2+-dependent autoamplification of sperm PLC activity (Carroll, 2001; Cox et al., 2002; Saunders et al., 2002; Swann et al., 2001). Other factors that might activate PLC in vertebrate eggs are Ca2+ introduced by sperm, increase in the amount of the lipid PLC substrate phosphatidylinositol (4,5)-bisphosphate, or improved accessibility of this substrate to PLC (reviewed by Mehlmann et al., 1998). Clearly, identification of signaling pathways, participants and interactions at work in different fertilization mechanisms will enable deeper understanding of evolved traits in this fundamental process.

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References


G-proteins at fertilization


