Guanosine 5'-thiotriphosphate may stimulate phosphoinositide messenger production in sea urchin eggs by a different route than the fertilizing sperm

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We show that microinjecting guanosine-5'-thiotriphosphate (GTP γ S) into unfertilized sea urchin eggs generates an intracellular free calcium concentration ([Ca]_i) transient apparently identical in magnitude and duration to the calcium transient that activates the egg at fertilization. The GTP γ S-induced transient is blocked by prior microinjection of the inositol trisphosphate (InsP₃) antagonist heparin. GTP γ S injection also causes stimulation of the egg's Na⁺/H⁺ antiporter *via* protein kinase C, even in the absence of a [Ca]_i increase. These data suggest that GTP γ S acts by stimulating the calcium-independent production of the phosphoinositide messengers InsP₃ and diacylglycerol (DAG). However, the fertilization [Ca]_i transient is not affected by heparin, nor can the sperm cause calcium-independent stimulation of protein kinase C. It seems that the bulk of InsP₃ and DAG production at fertilization is triggered by the [Ca]_i transient, not by the sperm itself. GDP β S, a G-protein antagonist, does not affect the fertilization [Ca]_i transient. Our findings do not support the idea that signal transduction at fertilization operates *via* a G-protein linked directly to a plasma membrane sperm receptor.

Introduction

The cell cycle arrest of the unfertilized sea urchin egg is broken by the increase in intracellular free calcium concentration ([Ca]_i) that occurs at fertilization (Whitaker and Patel, 1990). The [Ca]_i increase seems to be due to the production of the phosphoinositide messengers inositol tris-

phosphate (InsP₃)¹ and diacylglycerol (DAG) (Turner *et al.*, 1984; Whitaker and Irvine, 1984; Swann and Whitaker, 1985; Ciapa and Whitaker, 1986; Berridge, 1987). There is evidence in mammalian cells that the phosphoinositide messengers can be generated by a signal transduction mechanism involving GTP-binding proteins (G-proteins) (Gilman, 1987; Cockcroft and Stutchfield, 1988). It has been suggested that a similar transduction mechanism may operate at fertilization (Turner *et al.*, 1986). The suggestion is based on the observation that a thioanalogue of GTP, guanosine-5'-thiotriphosphate (GTP γ S), will stimulate fertilization envelope elevation when microinjected into sea urchin eggs (Turner *et al.*, 1986). The idea gains support from the identification in the sea urchin egg plasma membrane of two proteins that are substrates for pertussis and cholera toxins, two agents that covalently modify G-proteins (Turner *et al.*, 1987). Looked at in this way, fertilization comprises the interaction of the fertilizing sperm with a receptor that generates the phosphoinositide messengers by stimulating phosphoinositidase C *via* a G-protein. Comparable experiments in frog and hamster eggs are consistent with this point of view (Kline and Jaffe, 1987; Kline *et al.*, 1988; Miyazaki, 1988).

Our aim was to consolidate this way of looking at fertilization by demonstrating that G-protein stimulation by GTP γ S led to the production of the phosphoinositide messengers and caused an increase in [Ca]_i. We found this to be so. However, our experiments indicate that, although the fertilizing sperm also triggers the

¹ Abbreviations: BAPTA, bis-(*o*-aminophenoxy)-*N,N,N,N'*-tetraacetic acid; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein; [Ca]_i, intracellular free calcium concentration; DAG, diacylglycerol; EGTA, ethyleneglycol-bis-(β -aminoethylether)-*N,N,N,N'*-tetraacetic acid; G-proteins, GTP-binding proteins; GTP γ S, guanosine 5'-thiotriphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; InsP₃, inositol (1,4,5)-trisphosphate; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PtdInsP₂, phosphatidylinositol bisphosphate; TAPS, *N*-tris(hydroxymethyl)aminopropane sulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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Table 1. Magnitude and latency of $[Ca]_i$ transient after microinjection of GTP γ S

[GTP γ S], μ M	Peak $[Ca]_i$, μ M	Latency, s
6–15 (8 of 12)	1.5 $\pm$ 0.17	304 $\pm$ 56
20–30 (13 of 18)	1.6 $\pm$ 0.08	194 $\pm$ 17
100 (8 of 8)	2.3 $\pm$ 0.20	139 $\pm$ 45
150 (8 of 8)	1.4 $\pm$ 0.07	52 $\pm$ 8.3
Vehicle (6)	0.27 $\pm$ 0.04	0
Microinjection transient (6)	0.34 $\pm$ 0.08	0

Means $\pm$ SE are shown.

production of phosphoinositide messengers, it may do so by a different route than GTP γ S. It appears that the signal transduction pathway at fertilization bypasses the egg's G-proteins, at least initially, despite their close link with the phosphoinositide messenger pathway.

Results

GTP γ S activates sea urchin eggs by generating a $[Ca]_i$ transient

Microinjecting GTP γ S into sea urchin eggs causes exocytosis of the egg's cortical granules, suggesting that GTP γ S triggers an increase in $[Ca]_i$ (Turner *et al.*, 1986; Figure 1, inset). We used the calcium indicator dye fura2 to measure $[Ca]_i$ in a single egg microinjected with GTP γ S to a final concentration of 200 μ M (Figure 1a). There is a small and rapid increase in $[Ca]_i$ (Figure 1 and Table 1) immediately after injection. We attribute this to the microinjection technique itself, because control injections of vehicle caused similar immediate increases in $[Ca]_i$ (Table 1). A $[Ca]_i$ transient comparable in magnitude and duration to the fertilization $[Ca]_i$ transient occurred 20–400 s (Table 1) after GTP γ S microinjection. The latency of the GTP γ S-induced $[Ca]_i$ transient at the highest concentrations we tested was two to three times longer

than the latency of the $[Ca]_i$ transient at fertilization (Whitaker *et al.*, 1989; Table 3) and decreased as the concentration of GTP γ S increased. In an experiment on eggs from the same female, the latency of fertilization envelope elevation was 104 $\pm$ 7.6 (SE) s (n = 21) at a concentration of 50 μ M and 38 $\pm$ 8.2 s at concentrations between 200 and 400 μ M. These values are significantly different (p < 0.01, Student's t test). Low concentrations (6–30 μ M) of GTP γ S did not always elicit a $[Ca]_i$ transient. In 7 of 30 eggs injected with this range of concentrations, no delayed transient was detected, and, in 2 other eggs, a small (ca. 400-nM) transient occurred after a long delay. The size and duration of the $[Ca]_i$ transient appeared to be independent of GTP γ S concentration in the range 50–400 μ M. The peak of the GTP γ S-induced $[Ca]_i$ transient is not affected by replacing the normal sea water bathing the eggs with calcium-free seawater, although the duration of the transient decreases (Figure 1b). The peak of the fertilization $[Ca]_i$ transient is also unaffected and its duration is similarly shortened by removal of external calcium (Figure 1b, inset). Finally, the GTP γ S-induced $[Ca]_i$ transient and cortical granule exocytosis was completely abolished in two experiments by simultaneous injection of the calcium chelator bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) to a final concentration of 1 mM. These data indicate that GTP γ S activates eggs by generating a $[Ca]_i$ transient that is indistinguishable from the fertilization $[Ca]_i$ transient in all but its latency. They exclude the possibility that GTP γ S stimulates exocytosis directly via the exocytotic G-protein G_e (Cockcroft *et al.*, 1987).

GTP γ S, unlike *InsP*₃, does not directly release calcium from an intracellular store in sea urchin eggs

Guanine nucleotides can directly induce calcium release from intracellular stores (Joseph *et al.*, 1988). We tested this possibility in permeabil-

Figure 1. Activation of unfertilized eggs by microinjection of GTP γ S. Top: Changes in $[Ca]_i$ in a single unfertilized egg in response to microinjection of 150 μ M GTP γ S. There is a small initial increase in $[Ca]_i$ at the time of microinjection. This can be attributed to a slight influx or release of calcium caused by the injection technique itself (see Table 1). One hundred seconds after injection, $[Ca]_i$ rises to 1.5 μ M. The inset shows fertilization envelope elevation after injection of GTP γ S. Bottom: The solid trace represents the mean $[Ca]_i$ increase in four eggs microinjected with 150–200 μ M GTP γ S in normal artificial seawater; the dotted trace, the mean response in four eggs microinjected with the same concentrations of GTP γ S in calcium-free artificial seawater containing 2 mM EGTA. The SE of each point did not exceed 10% of the mean value. The traces are significantly different at the 1% level from 180–500 s (Student's t test). Because the GTP γ S-induced $[Ca]_i$ transient has a variable latency, the time scale origin is arbitrary: the traces were aligned by setting the peaks of the transients in register. The inset shows the mean fertilization $[Ca]_i$ transient in calcium-containing (●, 3 eggs) and calcium-free (○, 3 eggs) sea water for comparison. The SE of each point is shown when it exceeds the symbol size. Zero time was taken arbitrarily as the first data point to show a $[Ca]_i$ above the resting level. *L. pictus*. 16°C.

ized eggs by measuring calcium efflux from an egg compartment that sequestered ^{45}Ca in the presence of mitochondrial inhibitors when supplied with ATP (Clapper and Lee, 1985; Payan *et al.*, 1986). ^{45}Ca efflux was stimulated by the calcium-releasing messenger InsP_3 , but was unaffected by addition of $100\ \mu\text{M}$ $\text{GTP}\gamma\text{S}$ (Figure 2). We conclude that $\text{GTP}\gamma\text{S}$ acts indirectly to release calcium, a view consistent with the finding that $\text{GTP}\gamma\text{S}$, unlike InsP_3 (Swann and Whitaker, 1986), stimulates calcium release in the intact egg only after a delay of ≥ 20 s (see above).

InsP_3 levels increase during the fertilization $[\text{Ca}]_i$ transient (Ciapa and Whitaker, 1986), so it seemed reasonable to suppose that $\text{GTP}\gamma\text{S}$ might act in a similar way and increase polyphosphoinositide hydrolysis. We measured the production of inositol phosphates in permeabilized eggs prelabeled with $[^3\text{H}]$ inositol to determine whether $\text{GTP}\gamma\text{S}$ stimulated phosphoinositide turnover. The results of three such experiments are shown in Figure 3. Electroporeabilization and resealing in themselves induced a four-fold increase in inositol phosphate production. A further 50% increase in inositol phosphates was measured in cells permeabilized and resealed in the presence of $\text{GTP}\gamma\text{S}$. Fertilization of intact eggs led to a 30% increase in inositol phosphates, an increase similar to that reported for InsP_3 (Ciapa and Whitaker, 1986). In one experiment using digitonin to ir-

Figure 2. $\text{GTP}\gamma\text{S}$ does not induce calcium efflux from unfertilized eggs treated with digitonin. Mean and SE of three experiments are shown. *L. pictus*. 16°C .

Figure 3. $\text{GTP}\gamma\text{S}$ stimulates inositol phosphate production in transiently permeabilized eggs. $\text{GTP}\gamma\text{S}$ was trapped ($100\ \mu\text{M}$ final concentration) in unfertilized eggs using a transient electroporeabilization technique, and inositol phosphates were extracted 2 min later. The differences (permeabilized: control vs. $\text{GTP}\gamma\text{S}$; nonpermeabilized: control vs. fertilized [40 s]) are significant (Student's paired, 1-tailed *t* test, $n = 3$) at the $p < 0.05$ level. Permeabilization itself stimulates inositol phosphate production ($p < 0.01$). *L. pictus*. 16°C .

reversibly permeabilize unfertilized eggs, addition of $\text{GTP}\gamma\text{S}$ caused a 300% increase in the production of inositol phosphates (not shown). In another similar experiment, no increase was detected above that caused by digitonin treatment. These results indicate that $\text{GTP}\gamma\text{S}$ increases inositol polyphosphate production in unfertilized sea urchin eggs. The absence of a reproducible effect in irreversibly permeabilized eggs may be due to the washout of the G-protein mechanism that occurs when essential cytoplasmic components diffuse out of the cell (Marty and Zimmerberg, 1989; Marty *et al.*, 1989). In addition, both transient and irreversible permeabilization in themselves led to a stimulation of inositol polyphosphate production. To circumvent the problems inherent in the permeabilization technique, we used a more indirect strategy in intact single cells to confirm that $\text{GTP}\gamma\text{S}$ stimulates the formation of the phosphoinositide messengers.

GTPγS-induced $[\text{Ca}]_i$ transient is blocked by the InsP_3 antagonist heparin

Heparin is a polysulfated polysaccharide that competes with InsP_3 at its binding sites (Hill *et al.*, 1987; Cullen *et al.*, 1988; Ghosh *et al.*, 1988;

Guillemet *et al.*, 1989). Less sulfated polysaccharides are ineffective. Eggs that were microinjected with heparin to a final concentration of 250 μ g/ml before microinjection of 200 μ M GTP γ S failed to respond with a $[Ca]_i$ transient (Figure 4), whereas controls injected with the same concentration of desulfated heparin responded normally (not shown). A concentration of 10 μ g/ml heparin failed to block the $[Ca]_i$ transient in response to 150 μ M GTP γ S, whereas in four experiments at a concentration of 50 μ g/ml it blocked effectively. Heparin also blocked the InsP $_3$ -induced $[Ca]_i$ transient. The inset to Figure 4 shows data that indicate that heparin is a competitive inhibitor of InsP $_3$ -induced calcium release in sea urchin eggs. Heparin (250 μ g/ml, 40–60 μ M) shifts the InsP $_3$ dose response curve 1.3 log units to the right. The data are consistent with the idea that GTP γ S causes the $[Ca]_i$ transient via the production of InsP $_3$. It could also be argued that heparin is

inhibiting the interaction of GTP γ S with a G-protein (Willuweit and Aktories, 1988); we exclude this possibility below.

GTP γ S stimulates production of DAG

Some or all of the InsP $_3$ and DAG production at fertilization is due to the increase in $[Ca]_i$ (Whitaker and Aitchison, 1985; Swann and Whitaker, 1986); there is a close temporal correlation between production of the phosphoinositide messengers and the fertilization $[Ca]_i$ transient (Ciapa and Whitaker, 1986). Calcium-stimulated hydrolysis of PtdInsP $_2$ contributes to or comprises the positive feedback mechanism that underlies the calcium wave at fertilization (Whitaker, 1989). In a trivial sense it is highly likely, therefore, that the $[Ca]_i$ transient induced by microinjection of GTP γ S leads to the formation of InsP $_3$ and DAG. A more pertinent test of the idea that GTP γ S directly stimulates

Figure 4. The GTP γ S-induced $[Ca]_i$ transient is blocked by heparin. Heparin was coinjected with fura2 to a final concentration of 300 μ g/ml. GTP γ S was then injected to a final concentration of 150 μ M. The prior injection of heparin has completely abolished the $[Ca]_i$ transient, although the small microinjection-induced $[Ca]_i$ rise persists. The egg subsequently showed a normal $[Ca]_i$ transient on insemination. The inset shows the effects of prior injection of heparin (300 μ g/ml) on the peak height of the InsP $_3$ -induced $[Ca]_i$ transient. Mean, SE, and the number of determinations are shown. $\circ$, control eggs; Δ , heparin-injected eggs. This heparin concentration is the highest we can achieve and shifts the InsP $_3$ dose-response curve by 1.3 log units. The final concentration of InsP $_3$ is 0.1% of the pipette concentration. *L. pictus*. 16°C.

phosphoinositidase C via a G-protein is to assay DAG production under conditions in which the regenerative $[Ca]_i$ transient is prevented.

Microinjection of the calcium chelator ethylglycol-bis-(β -aminoethyle ether)- N,N,N',N' -tetraacetic acid (EGTA) to a final concentration of 0.5–1 mM prevents egg activation at fertilization (Zucker and Steinhardt, 1978). A similar concentration of BAPTA blocks the $GTP\gamma S$ -induced $[Ca]_i$ transient (see above). We monitored DAG production in BAPTA-injected eggs by looking at the activation of the Na^+/H^+ -antiporter: the antiporter is activated at fertilization (Payan *et al.*, 1983; Johnson *et al.*, 1974) and by protein kinase C activators and synthetic DAGs (Swann and Whitaker, 1985; Lau *et al.*, 1986; Shen and Burgart, 1986). The data of Figure 5 show that $GTP\gamma S$ injection causes an increase in pH_i in eggs previously injected with 1 mM BAPTA (0.11 ± 0.01 pH units, mean $\pm$ SE, $n = 4$, $t_{90\%}$: 420 ± 30 s). The pH_i increase is both larger and faster when the egg is exposed to the DAG kinase inhibitor R59022, a compound that inhibits the metabolic conversion of DAG to phosphatidic acid (De Chaffoy de Courcelles, 1985). In experiments with R59022, the pH_i increase was 0.29 ± 0.04 pH units (mean $\pm$ SE, $n = 7$, $t_{90\%}$: 170 ± 34 s). The $GTP\gamma S$ -induced pH_i increase did not occur in the presence of dimethylamiloride (Figure 5), an inhibitor of the Na^+/H^+ -antiporter (Vigne *et al.*, 1984), and was also inhibited by microinjection of the PKC-(19-36) inhibitory peptide (not shown; Shen and Buck, 1990). These data indicate that $GTP\gamma S$ causes an increase in pH_i in BAPTA-injected eggs by activating the Na^+/H^+ -antiporter via protein kinase C. They strongly suggest that $GTP\gamma S$ can stimulate production of DAG in the absence of an increase in $[Ca]_i$, an inference strengthened by the observation that the effect on pH_i is enhanced by an inhibitor of DAG metabolism.

GTP γ S-induced DAG production is not blocked by heparin

We showed above that heparin prevented the $GTP\gamma S$ -induced $[Ca]_i$ transient (Figure 5). Besides its blocking $InsP_3$ -induced calcium release, heparin has been shown to affect G-proteins in platelets (Willuweit and Aktories, 1988). To explore this possibility, we measured the pH_i increase in response to $GTP\gamma S$ in heparin-injected eggs. The pH_i increase under these conditions (0.32 ± 0.03 ; mean $\pm$ SEM, $n = 3$) was very similar in both magnitude and time course to the pH_i increase that we measured in BAPTA-injected eggs, indicating that heparin was not

Figure 5. Microinjection of $GTP\gamma S$ induces a pH_i increase in BAPTA-injected eggs, where the $[Ca]_i$ transient is prevented. Top: The $GTP\gamma S$ -induced pH_i increase in an egg that had been previously injected with the pH_i indicator BCECF (final concentration 50 μM) and the calcium chelator BAPTA (final concentration 2 mM). Middle: A similar experiment in which the diacylglycerol kinase inhibitor R59022 was present in the bath at a concentration of 10 μM . The $GTP\gamma S$ -induced pH_i increase is both larger and faster. Bottom: A further experiment in which, in addition, the Na^+/H^+ -antiporter inhibitor dimethylamiloride (DMA; 100 μM) is present at the beginning of the experiment and removed at the arrow. The latent stimulation of the Na^+/H^+ -antiporter by $GTP\gamma S$ is revealed by removing DMA. *L. pictus*. 16°C.

interfering with G-protein-stimulated production of DAG.

All these data are therefore consistent with the idea that $GTP\gamma S$ activates sea urchin eggs

Figure 6. Microinjection of GDP β S induces a $[Ca]_i$ transient but does not abolish the sperm-induced $[Ca]_i$ increase. The GDP β S-induced (10 mM final concentration) transient differs from that induced by GTP γ S in that it is both rapid in onset and short in duration. Subsequent insemination (Δ) gives a $[Ca]_i$ transient that rises to the levels associated with fertilization. The fertilization $[Ca]_i$ transient in another GDP β S-injected egg is also shown ($\blacktriangle$). All of the three other eggs of the total of five injected with GDP β S also showed transients with $[Ca]_i$ peaks $> 1 \mu M$ on insemination. *L. pictus*. 16°C.

by stimulating formation of $InsP_3$ and DAG and support the idea that the egg contains a G-protein that is linked to phosphoinositidase C.

GDP β S does not prevent the fertilization of $[Ca]_i$ transient

Turner *et al.* (1986) found that microinjecting the G-protein antagonist GDP β S prevented egg activation at fertilization without preventing activation triggered by microinjection of $InsP_3$. These observations indicate that GDP β S is blocking the signal pathway proximal to $InsP_3$ and strongly suggest that egg activation at fertilization is occurring via a G-protein. We measured $[Ca]_i$ in eggs injected with GDP β S to confirm these findings.

We found (Figure 6), contrary to expectation, that microinjecting GDP β S itself triggered a $[Ca]_i$ transient. The transient varied in size and duration. We inseminated those GDP β S-injected eggs in which $[Ca]_i$ returned toward resting levels and recorded a second $[Ca]_i$ transient after insemination. During both transients, $[Ca]_i$ rose to levels that would be expected to induce cortical granule exocytosis, but neither transient resulted in exocytosis or elevation of the fertilization envelope. These results indicate that the apparent inhibition of egg activation at fertilization in GDP β S-injected eggs is due to inhibition of cortical granule exocytosis, not to inhibition of the signal transduction pathway proximal to the $[Ca]_i$ increase.

We were unable to reproduce the earlier observation that $InsP_3$ microinjection could over-

come the activation block in GDP β S-injected eggs (Turner *et al.*, 1986).

Phosphoinositide messenger production by sperm

We applied the approach we used in studying the mechanism of action of GTP γ S to look at phosphoinositide messenger production stimulated by the fertilizing sperm. If sperm activate eggs by stimulating a G-protein linked to phosphoinositidase C, then there should be an initial phase of $InsP_3$ and DAG production during the 10- to 15-s latent period that precedes the onset of the fertilization $[Ca]_i$ transient (Whitaker *et al.*, 1989). We can extend the latent period indefinitely by preventing the onset of the transient with the calcium chelator BAPTA. Microinjection of BAPTA does not prevent the interaction of sperm with eggs. In fact, BAPTA-

Table 2. Polyspermy as a result of microinjection of BAPTA or heparin

	Number of fluorescent sperm nuclei (mean $\pm$ SD)	Number of eggs injected
Control noninjected eggs	1.0 $\pm$ 0.0	20
Control vehicle-injected eggs	1.1 $\pm$ 0.3	8
Heparin-injected eggs (250–350 $\mu g/ml$)	7.1 $\pm$ 3.5	24
BAPTA-injected eggs (2 mM)	9.1 $\pm$ 2.4	10

injected eggs are highly polyspermic (Table 2): ≥ 10 sperm are incorporated under conditions that would lead to monospermic fertilization in control eggs, presumably because the block to polyspermy represented by calcium-induced fertilization envelope elevation (Whitaker and Steinhardt, 1982) is absent.

Fertilization does not lead to a measurable calcium-independent pH_i increase

Insemination of BAPTA-injected eggs leads to the appearance of multiple fertilization cones, but no increase in pH_i (Figure 7). Inseminated eggs with fertilization cones *did*, on the other hand, respond to microinjection of $GTP\gamma S$ with a pH_i increase. These experiments indicate either that sperm do not cause calcium-independent DAG production at fertilization or that the 10 sperm that interact with a BAPTA-injected egg activate too small a fraction of the G-protein pool to stimulate detectable DAG-induced alkalization.

Heparin does not prevent the fertilization $[Ca]_i$ transient

If, as might be expected, sperm activate only a small number of G-protein molecules in the immediate vicinity of the sperm attachment point, it follows, just as with the DAG-mediated pH_i increase, that $GTP\gamma S$ microinjection should be a more potent stimulus to $InsP_3$ production and calcium release than the sperm. We tested this by comparing the relative sensitivity of the sperm- and $GTP\gamma S$ -induced calcium transient to inhibition by heparin. To our surprise, heparin failed to prevent the fertilization $[Ca]_i$ transient at concentrations up to 350 $\mu g/ml$ (50–70 μM ; Figure 8). At a heparin concentration fivefold greater than that required to inhibit the $GTP\gamma S$ -induced $[Ca]_i$ transient, the fertilization calcium transient was indistinguishable from the transient in control eggs (Table 3 and Figure 8, inset). We noted that heparin-injected eggs were polyspermic at our usual sperm density. We reduced sperm density until we achieved monospermic fertilization; this did not alter the result (Table 3).

Heparin prolongs the latent period

One way of determining the latent period at fertilization is to measure the time from the onset of the sperm-induced current to the onset of the $[Ca]_i$ transient (Whitaker *et al.*, 1989). The sperm-induced current is almost coincident with the calcium action potential it triggers (Cham-

bers and de Armendi, 1979; David *et al.*, 1988; Whitaker *et al.*, 1989). The calcium action potential causes a small increase in $[Ca]_i$ (Whitaker *et al.*, 1989; Table 3) that is detectable at fertilization but absent when the action potential is inactivated by fertilizing eggs at a membrane potential of -20 mV (not shown). In heparin-injected eggs, the time between the initial, small $[Ca]_i$ increase due to the action potential and the start of the fertilization $[Ca]_i$ transient (the latent period) was twice that in control eggs (Table 3).

Discussion

G-protein-mediated production of phosphoinositide messengers

Our data are consistent with the views that unfertilized sea urchin eggs contain one or more G-proteins that couple to phosphoinositidase C and that $GTP\gamma S$ activates eggs by stimulating the production of both $InsP_3$ and DAG. The $[Ca]_i$ transient triggered by $GTP\gamma S$ is similar in both magnitude and duration to the fertilization $[Ca]_i$ transient and shows the same late requirement for extracellular calcium. It is all-or-none: the magnitude and duration of the transient are independent of $GTP\gamma S$ concentration. The latency of the transient is concentration dependent. This behavior can be explained if it is assumed that the regenerative $GTP\gamma S$ -induced $[Ca]_i$ transient is triggered by a critical intracellular concentration of $InsP_3$ (Parker and Miledi, 1989). Heparin, a competitive inhibitor of $InsP_3$ -induced calcium release, will inhibit the $GTP\gamma S$ -induced transient by increasing the critical concentration of $InsP_3$ necessary to induce a regenerative response. $GTP\gamma S$ -stimulated production of the phosphoinositide messengers is independent of $[Ca]_i$, because a stimulation of the Na/H antiporter consistent with DAG production occurs in the presence of a calcium chelator that blocks any increase in $[Ca]_i$.

Stimulation of the Na/H antiporter via protein kinase C

In the presence of the DAG kinase inhibitor R59022, the pH_i increase attributable to DAG-induced stimulation of protein kinase C is about half that recorded at fertilization (Shen and Steinhardt, 1978). The kinetics and magnitude of the pH_i increase under these conditions resemble the those of the pH_i increase recorded when the Na/H antiporter is maximally stimulated with phorbol esters and exogenous DAGs (Swann and Whitaker, 1985; Shen and Burgart,

1986; Lau *et al.*, 1986). At fertilization, about half the pH_i increase can be directly ascribed to stimulation of protein kinase C, because it is this proportion that is blocked by protein kinase C-inhibitory peptide (Shen and Buck, 1990). The comparison suggests that the amount of DAG produced by the calcium-independent action of GTP γ S (in the presence of R59022) maximally stimulates protein kinase C and is similar to the amount of DAG generated at fertilization, a supposition supported by the similarities in time course of the increase of $[\text{Ca}]_i$ in these two cases. Without the inhibitor of DAG metabolism, stimulation of the kinase is submaximal and the pH_i increase slower and smaller.

Signal transduction at fertilization

There is a striking resemblance between the properties of the GTP γ S-induced $[\text{Ca}]_i$ transient and that at fertilization. The simplest way to account for this similarity is to suppose that the sperm interacts with a G-protein to generate the fertilization $[\text{Ca}]_i$ transient (Jaffe *et al.*, 1988). However, three pieces of evidence point away from this idea. The first is that the G-protein antagonist GDP β S does not prevent the sperm from generating a $[\text{Ca}]_i$ transient; the second is the finding that the InsP_3 antagonist heparin prevents the GTP γ S-induced $[\text{Ca}]_i$ transient but leaves the fertilization $[\text{Ca}]_i$ transient unaffected; the third is our observation that there is no detectable sperm-induced DAG production in the absence of a $[\text{Ca}]_i$ increase, even when ≥ 10 sperm interact with and incorporate into the egg.

This last observation indicates that the bulk of DAG production at fertilization is calcium stimulated, and it accords with data that show both that InsP_3 and DAG production at fertilization in sea urchin eggs coincides with the rise in $[\text{Ca}]_i$ (Ciapa and Whitaker, 1986) and that the egg plasma membrane contains a calcium-dependent polyphosphoinositidase C activity (Whitaker and Aitchison, 1985). It supports the idea that the calcium-stimulated production of the phosphoinositide messengers is one component of the autocatalytic mechanism that generates and propagates the fertilization calcium wave (Swann and Whitaker, 1986). Nonetheless, on this evidence alone, it is still possible to argue that there may be a small initial quantity of InsP_3 and DAG generated at fertilization, undetectable biochemically and too small to be revealed by the methods we have reported here.

Comparing the relative potency of the sperm and GTP γ S using heparin

The magnitude and duration of the fertilization $[\text{Ca}]_i$ transient were unaffected by heparin at the highest concentration we were able to introduce into the cell (250 $\mu\text{g}/\text{ml}$). The same concentration of heparin completely blocked the $[\text{Ca}]_i$ transient induced by up to 300 μM GTP γ S (the highest concentration that we tested). These data and the longer latency of the GTP γ S-induced transient suggest that the guanine nucleotide analogue represents a weaker stimulus to the egg than the fertilizing sperm, even at relatively high concentration. Yet a simple geometric comparison suggests that a single sperm would have immediate access to only 0.2% of the egg's G-proteins, on the rather generous assumption that a sperm might recruit G-proteins from up to 10 μm from its point of interaction. In addition, GTP γ S-induced DAG production is comparable in its effects on the Na^+/H^+ -antiporter to the DAG produced during the fertilization $[\text{Ca}]_i$ wave, though the possibility that GTP γ S may recruit some DAG from other lipids than PtdInsP_2 (Irving and Exton, 1987; Diaz-Meco *et al.*, 1989) must be borne in mind. The simplest explanation of these quantitative discrepancies is that InsP_3 production is not the initial response of the egg to the sperm. On this hypothesis, generation of InsP_3 is secondary to the $[\text{Ca}]_i$ increase at fertilization, a conclusion that fits with our observation that the sperm causes no detectable DAG production when the $[\text{Ca}]_i$ increase is prevented. This conclusion points toward alternative models of egg activation (Whitaker *et al.*, 1989; Whitaker and Crossley, 1990).

The most straightforward interpretation of the lack of effect of heparin on the amplitude of the fertilization $[\text{Ca}]_i$ transient is that InsP_3 itself is not, as has previously been assumed (Whitaker and Irvine, 1984; Whitaker, 1989), an essential component of the fertilization response. This interpretation cannot be discounted. The strongest argument in favor of InsP_3 as a messenger at fertilization is that its levels clearly coincide with the activating $[\text{Ca}]_i$ wave (Ciapa and Whitaker, 1986): it may well be that InsP_3 levels within the egg during the $[\text{Ca}]_i$ wave are sufficient to overcome heparin's competitive antagonism. As clear as the absence of the effect on wave amplitude at fertilization is the doubling of wave latency by heparin; this suggests some interaction between heparin and the mechanism of wave initiation.

Figure 7. The pH_i increase in a BAPTA-injected egg after insemination and microinjection of $GTP\gamma S$. The experiment is similar to those of Figure 4 and the bathing medium contained $10\ \mu M$ R59022. Insemination does not lead to a detectable alteration in pH_i . However, subsequent injection of $GTP\gamma S$ causes a pH_i increase. *L. pictus*, $16^\circ C$.

G-proteins and egg activation

Our data indicate that $GTP\gamma S$ activates sea urchin eggs by stimulating the production of the conjugate phosphoinositide messengers $InsP_3$ and DAG. However, they are not readily accommodated by a model that involves the initial stimulation of a G-protein by a sperm. Teleologically, this is an unsatisfactory conclusion. It is possible that G-proteins participate in the calcium-induced production of the phosphoinositide messengers during the fertilization $[Ca]_i$ wave, although our experiments with the inhibitory analogue $GDP\beta S$ tend to argue against this idea. It is also possible that the egg plasma membrane acts as a repository for G-proteins that assume a functional importance only later in development.

Materials and methods

Measurement of intracellular calcium and pH

$[Ca^{2+}]_i$ was measured using the calcium-sensitive fluorescent dye fura2. The dye (pentapotassium salt; Molecular Probes, Junction City, OR) was introduced into the eggs by microinjection to a final concentration of $50\ \mu M$. $[Ca^{2+}]_i$ in single eggs was determined by a ratiometric method (Swann and Whitaker, 1986).

pH_i was similarly measured with the fluorescent indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF). The free acid form of the dye (Molecular Probes) dissolved in injection vehicle ($0.5\ M\ KCl$, $100\ \mu M\ EGTA$) was introduced into eggs by microinjection. The techniques of fluorescence measurement are the same as those for fura2, except that the eggs are alternately excited with 450 and 490 nm (6.5- and 7.3-nm bandwidth, respectively) light and the intensity of light emitted at 530 nm (8.5-nm bandwidth) was measured. Calibration was performed in vitro: a medium designed to mimic ooplasm (IM: 220 mM potassium glu-

tamate, 500 mM glycine, 10 mM NaCl, 2.5 mM $MgCl_2$, and 4 mM KCl) was used with a number of buffers (PIPES, HEPES, TAPS and Tris) to give a pH range of 4.0–9.0. BCECF ($10\ \mu M$) was added to these solutions and the fluorescence ratios for drops of these solutions was determined on the microscope used for pH_i measurements. An effective pK for the dye was calculated and R_{min} and R_{max} (the ratios for free and saturated forms of the dye) were determined.

Electrophysiological measurements

Microelectrodes were pulled from glass capillary tubing with an inner glass filament (1.0-mm i.d., Clarke Electromedical, Pangbourne, U.K.) using a Kopf Microelectrode Puller (Tjunga, CA). The microelectrodes were filled with 3 M KCl and had a resistance of 40–80 M Ω . Egg membrane potentials were measured by a standard current clamp technique. The methods of Chambers and de Armendi (1979) were used to penetrate the egg and to establish a -70-mV resting membrane potential.

Assay for sperm egg fusion

We used the fluorescent DNA vital dye Hoechst 33342 (Sigma, Poole, UK) to assess whether sperm had fused with eggs (Hinckley *et al.*, 1986). Eggs were incubated for 30 min in $20\ \mu M$ Hoechst 33342 and then extensively washed (at least 4 times) to remove external dye, before being attached to coverslips and microinjected as above. The eggs were fertilized and, after 10 min, fixed with 3% formaldehyde. Only those sperm nuclei that fuse with the egg will stain with the dye. The fluorescent sperm nuclei were visualized on a Leitz (Leitz Instruments, Luton, UK) inverted microscope equipped with a Ploemopak fluorescence attachment. The eggs were excited with 380 nm (10-nm bandwidth) light, and a 450-nm longpass filter was placed in the emitted light path.

^{45}Ca efflux

Unfertilized, dejellied eggs were attached to 3-cm plastic petri dishes using 0.01 mg/ml polylysine. They were treated with 0.0025% digitonin in IM (220 mM potassium glutamate,

Figure 8. The $[Ca]_i$ transient after insemination in a heparin-injected egg. Heparin (final concentration: 300 μ g/ml) was coinjected with fura2 before insemination. This egg was subsequently shown to be monospermic using the Hoechst dye incorporation assay. The inset shows the mean $[Ca]_i$ increase in six eggs microinjected with 250–350 μ g/ml heparin (—) and five eggs microinjected with a similar quantity of injection buffer alone (· · ·). *L. pictus*. 16°C.

500 mM glycine, pH 6.7) for 5 min to make their plasma membranes permeable and left for a further 5 min in the same medium without digitonin, before addition of 2.5 mM MgATP and 45 Ca (Amersham, Arlington Heights, IL; 0.1 μ Ci/dish) in IM. After incubating for 10 min, the permeabilized eggs were washed three times in IM containing ATP (Sigma Chemical, Poole, Dorset, U.K.), and 45 Ca efflux was assessed under various conditions by measuring both the 45 Ca and the protein content of the dish. The eggs were dissolved in 1% Triton X100. Samples were measured by liquid scintillation counting and a Bradford protein assay; 45 Ca release was determined by comparing the counts per minute per milligram protein remaining in the experimental dishes with counts per minute per milligram protein in untreated controls.

$[^3H]$ inositol phosphate production

The production of water-soluble inositol derivatives was determined in digitonin permeabilized eggs after incubation for 18 h with 100 μ Ci $[^3H]$ inositol. In these experiments, digitonin was added to eggs suspended in plastic tubes. After incubation under various conditions, the sample was treated with 20% perchloric acid. The inositol phosphates were analyzed by high-performance liquid chromatography (HPLC), using ^{32}P -labeled inositol phosphates prepared from red blood cells as standards.

We also made use of a transient electroporation technique devised by Swezey and Epel (1988). Before permeabilization, eggs were washed twice in calcium-free sea water and once in permeabilization buffer (300 mM glycine, 175 mM potassium gluconate, 185 mM mannitol, 50 mM PIPES, 20 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 2.5% w/v Dextran T10, pH 6.8) before resuspension in permeabilization buffer at 25% v/v and transfer to the electroporation chamber. The chamber was of perspex with two stainless steel electrodes separated by 2 cm. The suspension was pulsed three times by discharging a 1- μ F capacitor previously charged to 100 V across the chamber. We verified that transient permeabilization had occurred using the fluorescent dye BCECF. The trapped dye concentration after

Table 3. Fertilization $[Ca]_i$ transient in heparin-injected eggs

	Latency, s	Amplitude, μ M
Vehicle-injected egg (5)	20.1 $\pm$ 2.5	1.8 $\pm$ 0.04
Heparin-injected egg (6)*	84.5 $\pm$ 9.3	1.6 $\pm$ 0.16

Mean and SE are shown. *Mean sperm/egg in these experiments: 1.05 $\pm$ 0.68 (n = 20).

washing was determined to be between 10 and 20% of its concentration in the permeabilization medium.

Obtaining and handling gametes

Lytechinus pictus were supplied by Pacific Biomarine (Venice, CA) and Marinus (Long Beach, CA). Experiments were carried out on eggs from which the jelly coat had been removed by passage through Nitex mesh (Swann and Whitaker, 1986). An artificial sea water was used, comprising 435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, and 1 mM EDTA, pH 8.0. Calcium-free sea water contained 445 mM NaCl and 2 mM EGTA.

Materials

All chemicals were of analytical grade. R59022 was from Janssen (Beerse, Belgium). GTP γ S and GDP β S were supplied by Boeringer Chemicals (Lewes, Sussex, U.K.). The purity of both was verified by HPLC. InsP₃ was generated from erythrocyte plasma membrane and purified by HPLC.

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