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Internal Calcium Release and Activation of Sea Urchin Eggs by cGMP are Independent of the Phosphoinositide Signaling Pathway

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We show that microinjecting cyclic GMP (cGMP) into unfertilized sea urchin eggs activates them by stimulating a rise in the intracellular free calcium ion concentration ($[Ca^{2+}]_i$). The increase in $[Ca^{2+}]_i$ is similar in both magnitude and duration to the transient that activates the egg at fertilization. It is due to mobilization of calcium from intracellular stores but is not prevented by the inositol trisphosphate ($InsP_3$) antagonist heparin. Furthermore, cGMP does not stimulate the eggs Na^+/H^+ antiport when the $[Ca^{2+}]_i$ transient is blocked by the calcium chelator bis-(*O*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA), suggesting that cGMP does not activate eggs by interacting with their phosphoinositide signaling pathway. However, the $[Ca^{2+}]_i$ increase and activation are prevented in eggs in which the $InsP_3$ -sensitive calcium stores have been emptied by the prior microinjection of the $InsP_3$ analogue inositol 1,4,5-trisphosphorothioate. These data indicate that cGMP activates eggs by stimulating the release of calcium from an $InsP_3$ -sensitive calcium store via a novel, though unidentified, route independent of the $InsP_3$ receptor.

INTRODUCTION

An unfertilized sea urchin egg is activated to undergo cycles of cell division by the increase in intracellular free calcium concentration ($[Ca^{2+}]_i$)¹ that occurs at fertilization (Whitaker and Steinhardt, 1982; Whitaker and Patel, 1990). The $[Ca^{2+}]_i$ increase is thought to be due to the production of the phosphoinositide messenger inositol trisphosphate [$InsP_3$] (Turner *et al.*, 1984; Whitaker and Irvine, 1984; Swann and Whitaker, 1985; Ciapa and Whitaker, 1986). It has been suggested that the generation of $InsP_3$ by phosphoinositidase C at fertilization may be accomplished through a mechanism involving a G-protein (Turner *et al.*, 1986), analogous to that described for mammalian cells (Cockcroft and Stutchfield, 1988). This suggestion is based on the ob-

ervation that a nonhydrolyzable analogue of GTP, guanosine-5'-thiotriphosphate ($GTP\gamma S$), stimulates fertilization envelope elevation when microinjected into sea urchin eggs (Turner *et al.*, 1986). Furthermore, substrates for pertussis and cholera toxins, two agents that covalently modify G-proteins, have been found in sea urchin egg plasma membranes (Turner *et al.*, 1987). However, we recently have shown that the activation mechanisms of $GTP\gamma S$ and sperm are probably different (Crossley *et al.*, 1991) and that a G-protein mechanism (Jaffe *et al.*, 1988) is insufficient to explain egg activation at fertilization. Other equally plausible mechanisms have been proposed to explain egg activation at fertilization that do not rely on a G-protein-mediated signal transduction pathway (Whitaker *et al.*, 1989; Swann, 1991) but involve the injection of an activator into the egg when sperm and egg fuse.

Apart from the guanine nucleotide analogue $GTP\gamma S$, there are a number of compounds that can activate eggs and are thus candidates for the crucial fertilization messenger; among these are $InsP_3$ (Whitaker and Irvine, 1984; Busa *et al.*, 1985; Swann and Whitaker, 1986; Iwamatsu *et al.*, 1988; Miyazaki, 1988), calcium (Jaffe, 1980; Igusa and Miyazaki, 1983), cyclic ADP-ribose

¹ Abbreviations used: ASW, artificial seawater; BAPTA, bis-(*O*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; $[Ca^{2+}]_i$, intracellular free calcium concentration; CaFASW, calcium-free artificial seawater; cGMP, cyclic GMP; DAG, diacylglycerol; $GTP\gamma S$, guanosine 5'-thiotriphosphate; HPLC, high-performance liquid chromatography; $InsP_3$, inositol (1,4,5)-trisphosphate; $InsP_3S$, inositol 1,4,5-phosphorothioate; pH_i , intracellular pH.

(Clapper *et al.*, 1987), cyclic GMP (cGMP) (Swann *et al.*, 1987; Iwamatsu *et al.*, 1988), and an unidentified higher molecular weight factor (Dale *et al.*, 1985; Swann, 1991). It appears that a variety of activation mechanisms is available to the egg. Sperm contain large amounts of both InsP_3 (Iwasa *et al.*, 1989) and cGMP when they are metabolically activated (Kopf and Garbers, 1979).

In this study we report the effects of cGMP on the sea urchin egg calcium store. Activation by cGMP relies on mobilization of calcium from intracellular stores and gives rise to a $[\text{Ca}^{2+}]_i$ transient that is hard to distinguish from a fertilization $[\text{Ca}^{2+}]_i$ transient, but cGMP fails to mobilize calcium directly from eggs permeabilized with digitonin. cGMP also fails to stimulate the production of the conjugate second messengers InsP_3 and diacylglycerol (DAG), as judged by the absence of protein kinase C activation and insensitivity to heparin, an antagonist of the InsP_3 receptor. We also show that cGMP is only able to elicit an activation response in eggs when the InsP_3 -sensitive calcium store is full. These data suggest that cGMP activates eggs by releasing intracellular Ca^{2+} through a novel and as yet unidentified signaling pathway.

MATERIALS AND METHODS

Microinjection Techniques

Eggs were held with polylysine on glass coverslips in an inverted microscope and were microinjected with volumes of up to 5 μl of 0.5 M KCl, 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.7, using pulses from a pressure injection system (Swann and Whitaker, 1986). To microinject activated eggs, we fertilized them in artificial seawater (ASW; see below) containing 1 mM para-aminobenzoic acid (this prevented elevation and hardening of the fertilization envelope) and injected them in ASW containing 1.5% dextran (molecular weight 200 000; this collapsed the fertilization envelope). Injection volumes were estimated using an eyepiece graticule. The egg volume was taken to be 500 μl .

Measurement of Intracellular Calcium and pH

$[\text{Ca}^{2+}]_i$ 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 μM . $[\text{Ca}^{2+}]_i$ in single eggs was determined using a ratiometric method (Swann and Whitaker, 1986).

Intracellular pH was measured using the fluorescent indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF). The techniques of fluorescence measurement are the same as those for fura2 except that the eggs are excited alternately 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) is measured. Calibration was performed *in vitro* as previously described (Crossley *et al.*, 1991).

^{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, 500 mM glycine, pH 6.7) for 5 min to make their plasma membranes permeable and were left for a further 5 min in the same medium without digitonin, before

Table 1. Effect of phosphodiesterase on cAMP efficacy.

| Injection | Peak height (μM) |
|--------------------------|--------------------------------|
| cGMP | 2.6 ± 0.3 (4) ^a |
| cGMP + phosphodiesterase | 0.5 ± 0.1 (4) |

Both samples, one of 20 mM cGMP, the other 20 mM cGMP with 100 units/ml of phosphodiesterase, were made up in 50% glycerol/water, 45 mM MgSO_4 , 45 mM Tris/HCl, pH 7.5, and incubated for 4 h at 30°C in a water bath before microinjection at 0.5% of the egg volume. The hydrolysis of cGMP by the enzyme was monitored by thin-layer chromatography. Samples were taken at hourly intervals for analysis. The rate of hydrolysis was approximately linear (0.3 nmol/min), and at 4 h, we estimated using visualization with ultraviolet light and appropriate concentration standards that >95% of the sample had been converted to a product that migrated with the same mobility as a 5'-GMP standard. Comparison with the data of Table 3 confirms that the final cGMP concentration after 4 h hydrolysis was <1 mM, as judged by its ability to cause a Ca_i increase. Controls performed with the cGMP + phosphodiesterase sample before incubation indicated that the enzyme itself was not inhibitory. Mean \pm SEM.

^a Values in parentheses are n.

addition of 2.5 mM MgATP and ^{45}Ca (Amersham, Arlington Heights, IL; 0.1 $\mu\text{Ci}/\text{dish}$) in IM. After incubating for 10 min, the permeabilized eggs were washed three times in IM containing ATP, and ^{45}Ca efflux was assessed under various conditions by measuring both the ^{45}Ca and protein content of the dish. The eggs were dissolved in 1% Triton X-100. Samples were measured by liquid scintillation counting, and a Bradford protein assay and ^{45}Ca release were determined by comparing the counts per minute per milligram of protein remaining in the experimental dishes with counts per minute per milligram of protein in untreated controls.

Obtaining and Handling Gametes

Lytechinus pictus were supplied by Pacific Biomarine (Venice, CA) and Marinus (Longbeach, CA). Experiments were carried out on eggs from which the jelly coat had been removed by passage through nylon mesh (Swann and Whitaker, 1986). An ASW was used, comprising 435 mM NaCl, 40 mM MgCl_2 , 15 mM MgSO_4 , 11 mM CaCl_2 , 10 mM KCl, 2.5 mM NaHCO_3 , 1 mM EDTA, pH 8.0. Calcium-free sea water contained 445 mM NaCl and 2 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA).

Materials

All chemicals were of analytical grade. Fura2 and BCECF were from Molecular Probes (Eugene, OR) and ATP was from Sigma Chemical (Poole, Dorset). InsP_3 was generated from erythrocyte plasma membrane and purified by high-performance liquid chromatography (HPLC).

cGMP was from Boeringer Mannheim UK (Lewes, Sussex) or Sigma. All solutions of cGMP were freshly made on the day of use. We found no obvious differences in the response of the eggs to cGMP from these two sources. cGMP from Boeringer Mannheim has no major contaminants as determined by paper chromatography (n-butanol: acetic acid:water [50:15:25]; their data). We analyzed this cGMP ourselves by thin-layer chromatography on PEI-cellulose (Aldrich Chemical, Gillingham, Dorset, UK) with 0.2 M LiCl and by HPLC on a Waters Associates (Northwich, Cheshire, UK) reverse-phase C18 column, eluting with 0.5 M sodium phosphate, pH 5. We found a

Table 2. Nucleotides and egg activation.

| Compound | Concentration (μM) | No FE (%) | Partial FE (%) | Full FE (%) | n |
|-----------|---------------------------------|-----------|----------------|-------------|----|
| Vehicle | — | 100 | 0 | 0 | 20 |
| GTP | 100 | 100 | 0 | 0 | 14 |
| 5'GMP | 100 | 100 | 0 | 0 | 10 |
| 3'5'cGMP | 10 | 0 | 20 | 80 | 10 |
| 3'5'cGMP | 20 | 0 | 0 | 100 | 15 |
| 8-Br-cGMP | 100 | 100 | 0 | 0 | 10 |
| 2'3'cGMP | 100 | 100 | 0 | 0 | 16 |
| 3'5'cAMP | 100 | 89 | 11 | 0 | 17 |

Eggs were microinjected with 5 pl (1% egg volume) of 0.5 M KCl, 20 mM PIPES containing nucleotide to give the final cytoplasmic concentration shown above. FE: Fertilization envelope.

single spot with thin-layer chromatography and one major peak with HPLC, both suggesting that the samples were >98% pure (by HPLC [% total absorbance at 256 nm]; Sigma: cGMP 98.9, GDP 0.7, GMP 0.3, GTP 0.1; Boehringer: cGMP 99.1, GDP 0.3, GMP 0.3, GTP 0.3). We also found that two enantiomeric phosphothiorate-analogues (Sp- and Rp-cGMP[S], the gift of Dr. Barry Potter, University of Leicester, Leicester, UK) were about as potent as the commercially obtained cGMP. To exclude the possibility that it was a very minor contaminant of these cGMP samples from different sources that was responsible for the effects we observed, we treated a cGMP sample with a phosphodiesterase (Sigma; from porcine brain [P1790]) to destroy cyclic nucleotides specifically and followed its hydrolysis using paper chromatography with 0.2 M LiCl. The results of these experiments are shown in Table 1. They indicate that phosphodiesterase treatment abolishes the activity present in the sample of cGMP.

RESULTS

cGMP Activates Sea Urchin Eggs

We tested the ability of various nucleotides to activate unfertilized sea urchin eggs using calcium-dependent exocytosis (fertilization envelope activation) as an assay. The results of these experiments are shown in Table 2. For each nucleotide, eggs were injected to give the desired final cytoplasmic concentration. A minimum of 10 eggs was injected with each of the compounds being tested. The injection volume was <1% of the egg volume.

Of the nucleotides tested, only 3'5'cGMP was able to activate eggs. It activated 80% of those eggs injected at a concentration of 10 μM and was fully active at 20 μM . The other cyclic nucleotide second messenger, 3'5'cyclic AMP, did not activate eggs. Both the 2'3' isomer and also the nonhydrolyzable 8'-Br derivative of cGMP failed to cause any activation in these experiments. The lack of effect of 8'-Br-cGMP could be taken to suggest a role for phosphodiesterase activity in the activation mechanism because the 8'-bromo derivative is not hydrolyzed readily (Szaduykis-Szadurski and Berti, 1972). However, the product of cGMP-directed phosphodi-

esterase activity, 5'GMP, is also without effect when injected into eggs.

cGMP Activates Eggs by Mobilization of Intracellular Calcium

It has been shown previously that cGMP causes a calcium transient similar to the activating transient at fertilization (Swann *et al.*, 1987). We have determined the source of the calcium responsible for the cGMP-induced

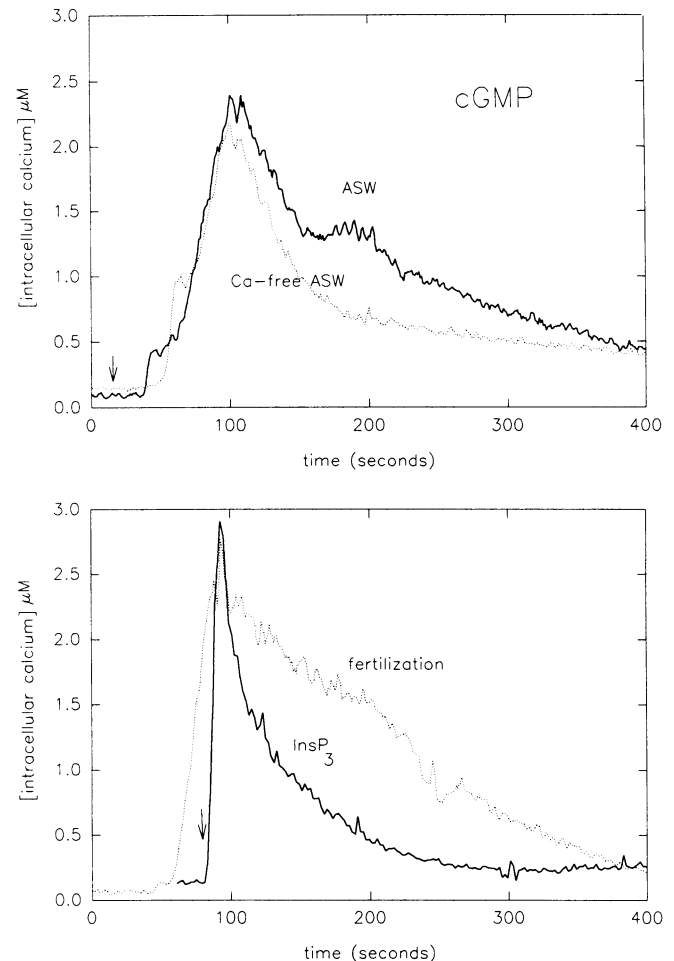


Figure 1. (A) The cGMP-induced $[\text{Ca}^{2+}]_i$ transient measured in single eggs in calcium-containing ASW and calcium-free ASW. The arrow marks the time of injection of cGMP to a final concentration of 20 μM in ASW; the egg in calcium-free ASW was micro-injected at $t = 0$. The rise time and peak height of the transient are unaffected by removal of extracellular calcium. The eggs contained 20 μM of the fluorescent calcium indicator dye, fura2. One of seven experiments in ASW and five in Ca-free ASW. (B) Comparison of the $[\text{Ca}^{2+}]_i$ transient at fertilization and after microinjection of 0.5 pl (0.1% egg volume) of 10 μM InsP₃. The InsP₃-induced transient rises more rapidly and subsides more quickly than the transient at fertilization. The records are representative of more than 20 individual experiments. *L. pictus*. 16°C.

transient by measuring intracellular calcium concentrations with the fluorescent dye fura2. We microinjected cGMP into fura2-loaded eggs to give final cytoplasmic concentrations of 20 μM in both calcium-containing ASW and calcium-free ASW (CaFASW). Typical records of such experiments are shown in Figure 1. It can be seen that in both cases the $[\text{Ca}^{2+}]_i$ rises to $\sim 2 \mu\text{M}$ and then declines over the next few minutes. The mean and SEM values for the peak of the transients are $1.95 \pm 0.11 \mu\text{M}$ in ASW ($n = 7$) and $1.92 \pm 0.18 \mu\text{M}$ in CaFASW ($n = 5$). It is only in the later stages of the calcium transient that there appears to be a difference between ASW and CaFASW. There is a comparable late requirement for extracellular calcium at fertilization (Crossley *et al.*, 1991). These data clearly show that the source of calcium mobilization during cGMP-stimulated activation is intracellular and that removing extracellular calcium affects only the falling phase of the transient.

Figure 1 also shows a fertilization $[\text{Ca}^{2+}]_i$ transient and a $[\text{Ca}^{2+}]_i$ transient induced by microinjection of InsP_3 , for comparison. InsP_3 causes an immediate rapid increase in $[\text{Ca}^{2+}]_i$ to a peak height similar to the peak height at fertilization or after injection of cGMP. However, the falling phase of the InsP_3 -induced transient is appreciably more rapid than after fertilization or microinjection of cGMP, so that it lasts half as long.

At all concentrations of cGMP tested, the response has a latency (Table 3). We determined the latency by measuring the time between microinjection of cGMP and the first detectable response, that is, when $[\text{Ca}^{2+}]_i$ increased by $\sim 20 \text{ nM}$. The latency decreases with increasing concentrations of cGMP in the range 5–20 μM but was never $< 15 \text{ s}$, suggesting a mechanism in which there is a rate-limiting step governed by the concentration of some other species than cGMP. At cGMP concentrations of 10 μM and above, the calcium transient always rose from $\sim 100 \text{ nM}$ to $\sim 2 \mu\text{M}$, and the magnitude of the transient was independent of the concentration of cGMP injected. At 5 μM cGMP, four out of five eggs gave a smaller increase in $[\text{Ca}^{2+}]_i$ to $\sim 1 \mu\text{M}$, whereas the fifth transient was similar to those obtained with higher cGMP concentrations.

Table 3.

| Concentration (μM) | Latency (s) | Peak $[\text{Ca}^{2+}]_i$ (μM) |
|---------------------------------|----------------|---|
| 5 | 27.5 ± 1.2 | 1.06 ± 0.23 (5)* |
| 10 | 22.0 ± 1.7 | 2.40 ± 0.23 (5) |
| 20 | 18.0 ± 0.8 | 1.95 ± 0.10 (8) |
| 50 | 17.5 ± 0.9 | 2.25 ± 0.09 (4) |

Eggs were microinjected with 0.5–5 μl (0.1–1% egg volume) of 5 mM cGMP in 0.5 M KCl, 20 mM PIPES to the final concentration shown.

* Values in parentheses are n for each data set.

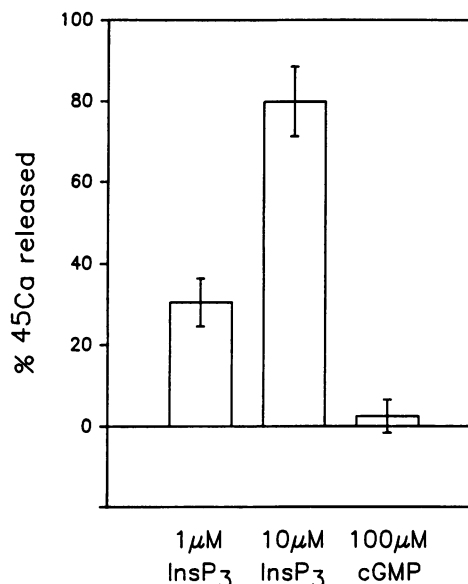


Figure 2. InsP_3 induces ^{45}Ca release from digitonin-permeabilized eggs, whereas cGMP is without effect. ^{45}Ca is sequestered by digitonin-permeabilized eggs into a nonmitochondrial compartment when ATP is added. Subsequent addition of InsP_3 releases most of the sequestered ^{45}Ca (Crossley *et al.*, 1991). Mean and SEM of four experiments are shown. *L. pictus*. 16°C .

cGMP Does Not Directly Release Calcium From an Intracellular Store in Sea Urchin Eggs

We tested the possibility that cGMP could, like InsP_3 , release calcium directly from intracellular stores. We did this by measuring the efflux in permeabilized eggs of ^{45}Ca sequestered in the presence of ATP (Clapper and Lee, 1985; Payan *et al.*, 1986; Crossley *et al.*, 1991). ^{45}Ca efflux was stimulated by the calcium-releasing messenger InsP_3 (10 μM InsP_3 stimulated the release of $\sim 80\%$ of the sequestered calcium) but was unaffected by addition of 100 μM cGMP (Figure 2), indicating that cGMP does not act directly to release calcium from intracellular stores. The corollary is that cytoplasmic factors cooperate with cGMP to induce calcium release and are lost or inactivated when the egg is permeabilized. This idea fits with our finding that cGMP stimulates a calcium transient in the intact egg only after a delay of $\sim 20 \text{ s}$, unlike InsP_3 , which acts with no measurable latency (Swann and Whitaker, 1986). cGMP thus resembles $\text{GTP}\gamma\text{S}$ in generating a delayed $[\text{Ca}^{2+}]_i$ transient and having no effect on calcium release in permeabilized eggs (Crossley *et al.*, 1991).

cGMP Fails to Activate Phosphoinositidase C

The finding that cGMP fails to release Ca^{2+} from permeabilized eggs suggests that cGMP stimulates calcium release by activating an enzyme activity or via its me-

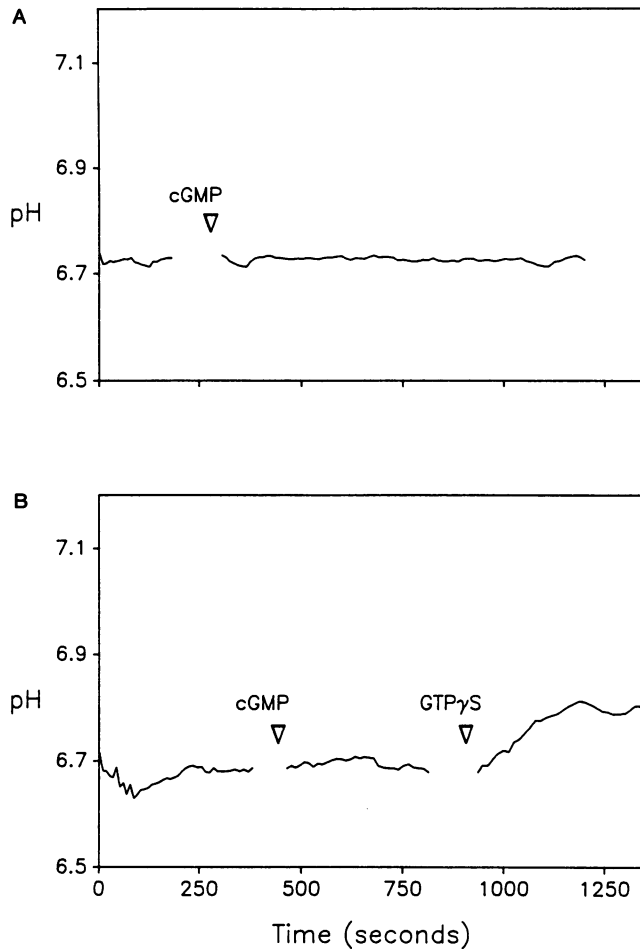


Figure 3. cGMP does not induce a pH increase in BAPTA-injected eggs. (A) cGMP is without effect on the pH of an egg injected with 1 mM BAPTA and 50 μ M BCECF, a pH indicator. One of five experiments. (B) An additional experiment in which, again, cGMP was ineffective but that also includes a positive control, the subsequent injection of GTP γ S. GTP γ S stimulates a pH increase in BAPTA-injected eggs by activating phosphoinositidase C, causing the production of DAG and activation of the protein kinase C-dependent Na/H antiport (Crossley *et al.*, 1991). Control injections of cGMP (not shown) caused an increase in pH_i similar to those seen at fertilization or after injection of InsP_3 , as might be expected, because the $[\text{Ca}^{2+}]_i$ increase will stimulate the exchanger directly (Shen and Buck, 1990) and also lead to production of DAG (Whitaker and Irvine, 1984; Whitaker and Aitchison, 1985; Swann and Whitaker, 1986). *L. pictus*. 16°C.

tabolism to an active product. The most obvious candidate as cytoplasmic cofactor and target for cGMP are a kinase activity that might stimulate phosphatidylinositol triphosphate (PtdInsP₂) hydrolysis and the production of the Ca²⁺-releasing second messenger InsP₃.

We investigated the possibility of an effect on the phosphoinositide messenger pathway by using a single cell assay for the production of DAG, the conjugate second messenger of InsP₃ (Berridge, 1987). This assay

detects changes in intracellular pH caused by a stimulation of the Na/H antiport (Payan *et al.*, 1983) using the pH-sensitive fluorescent dye BCECF (Crossley *et al.*, 1991). The Na/H antiport is under the direct control of protein kinase C, as well as $[\text{Ca}^{2+}]_i$ (Swann and Whitaker, 1985; Lau *et al.*, 1986; Shen and Burgart, 1986; Shen and Buck, 1990), and this assay enables us to assess the production of DAG, an activator of protein kinase C. Eggs were injected with the calcium chelator BAPTA to prevent any Ca²⁺-stimulated DAG production (Whitaker and Aitchison, 1985; Swann and Whitaker, 1986), which would be a consequence of cGMP microinjection into normal eggs. Thus, any pH change that occurs is independent of changes in $[\text{Ca}^{2+}]_i$ and is attributable to the calcium-independent production of DAG.

Figure 3A shows that cGMP fails to produce any change in pH when injected to give a final concentration of 100 μ M. This is a concentration of cGMP sufficient to activate 100% of normal eggs (see Table 2). The record shown is typical of five such determinations. The initial pH before microinjection is \sim 6.7 and remains at this level for \geq 10 min after microinjection (recall that cGMP induces an activation response with a latency of \sim 20 s in control eggs). This result suggests that cGMP fails to stimulate the production of DAG under conditions in which $[\text{Ca}^{2+}]_i$ is clamped at resting levels. Figure 3B shows a similar experiment in which the egg was first injected with cGMP to give a final concentration of 100 μ M followed by a second injection of 100 μ M GTP γ S, a compound known to stimulate DAG production under the conditions of this assay (Crossley *et al.*, 1991). The pH remains stable at about pH 6.7 after cGMP microinjection, but after the microinjection of GTP γ S there is a rapid increase of \sim 0.25 pH units. This result shows that GTP γ S can stimulate alkalization in eggs in which cGMP has no effect and confirms that cGMP does not stimulate phosphoinositidase C (or other DAG-producing enzymes) under conditions in which $[\text{Ca}^{2+}]_i$ is clamped at resting levels.

Table 4. Effect of heparin on latency of cGMP and fertilization response.

| Injection | Latency (s) | Time to peak (s) | Peak height (μ M) |
|-------------------------|-----------------------------|------------------|----------------------------------|
| cGMP | 18.7 \pm 1.02 | 37.0 \pm 5.5 | 1.92 \pm 0.11 (7) ^a |
| cGMP + heparin | 19.8 \pm 1.0 | 35.6 \pm 3.4 | 2.32 \pm 0.12 (5) |
| Fertilization | 20.1 \pm 2.5 ^b | 27.3 \pm 4.7 | 1.84 \pm 0.04 (5) ^b |
| Fertilization + heparin | 84.5 \pm 9.3 ^b | 49.6 \pm 8.4 | 1.61 \pm 0.16 (6) ^b |

^a Values in parentheses are n for each set of data.

^b Originally published in Crossley *et al.*, 1991.

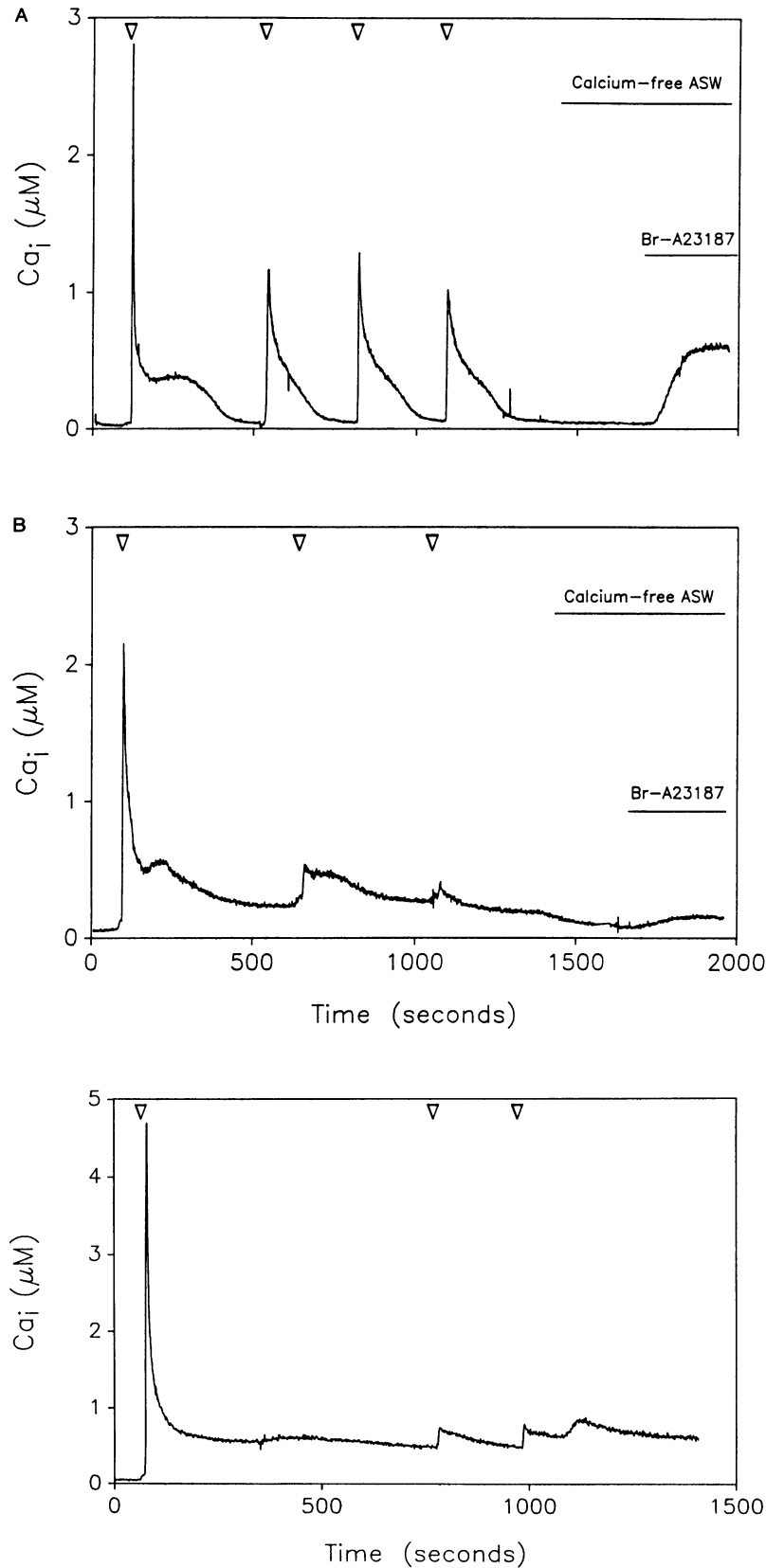


Figure 4. The consequences of multiple injections of inositol trisphosphates into single eggs. $[Ca^{2+}]_i$ is measured with fura2. (A) Multiple injections of $InsP_3$ to a final concentration of 0.8 μM cause repeated $[Ca^{2+}]_i$ transients. Subsequent addition of the calcium-releasing agent Br-A23187 (50 μM in calcium-free ASW) causes calcium release from the internal store. The area under the Br-A23187 transient equaled or exceeded the area under the second and subsequent $InsP_3$ transients in all five experiments. (B) Multiple injections of $InsP_3$ to a final concentration of 4 μM . Resting $[Ca^{2+}]_i$ is relatively greater in $InsP_3$ -injected eggs after the first $[Ca^{2+}]_i$ transient, and subsequent injections of $InsP_3$ cause only small $[Ca^{2+}]_i$ increases. In this case Br-A23187 caused little increase in $[Ca^{2+}]_i$, and the area under the Br-A23187 transient was negligible, compared even to the second and third $InsP_3$ -induced transient, suggesting that the calcium store is progressively depleted by successive injections of $InsP_3$. One of five experiments. *L. pictus*. 16°C.

Figure 5. The consequences of successive microinjection of $InsP_3$ (final concentration 4 μM), $InsP_3$ (0.8 μM), and cGMP (200 μM). $InsP_3$ depletes the calcium store and subsequent injection of both $InsP_3$ (second arrow) and cGMP (third arrow) fails to elicit a significant increase in $[Ca^{2+}]_i$. One of three experiments is shown. *L. pictus*. 16°C.

Heparin Does Not Affect Egg Responses to cGMP

Heparin, an antagonist of InsP_3 -induced calcium release (Hill *et al.*, 1987; Cullen *et al.*, 1988), has been shown to abolish completely activation of sea urchin eggs in response to $\text{GTP}\gamma\text{S}$ (Crossley *et al.*, 1991). Concentrations of heparin that block $\text{GTP}\gamma\text{S}$ -induced activation did not affect the calcium transient generated by microinjecting cGMP at concentrations of up to $100 \mu\text{M}$ (Table 4). Heparin also fails to block the fertilization $[\text{Ca}^{2+}]_i$ transient, though it increases the latency of the fertilization response (the latent period [Allen and Griffin, 1958]) fourfold and doubles the rise time of the fertilization $[\text{Ca}^{2+}]_i$ transient (Crossley *et al.*, 1991 and Table 4), suggesting that both processes involve the production of InsP_3 . We tested the effects of heparin microinjection (final concentration $250 \mu\text{g}/\text{ml}$) on the responses of eggs to $20 \mu\text{M}$ cGMP by characterizing the latency of activation, the time from the end of the latent period to the peak of the $[\text{Ca}^{2+}]_i$ increase (rise time), and the magnitude of the calcium transient.

Table 4 shows that the latency of activation, that is, the time from injection to the time of the onset of the $[\text{Ca}^{2+}]_i$ transient, in response to microinjection of cGMP is unaffected by $250 \mu\text{g}/\text{ml}$ heparin, as is the rise time of the transient. This result is markedly different to those we obtain with this concentration of heparin at fertilization (Crossley *et al.*, 1991 and Table 4).

The heparin data confirm that cGMP acts via a route independent of the phosphoinositide messenger system and the InsP_3 receptor when it generates a Ca^{2+} transient. They suggested to us the hypothesis that cGMP mobilizes Ca^{2+} from an InsP_3 -insensitive Ca^{2+} store (Clapper *et al.*, 1987; Berridge and Galione, 1988).

Identity of the cGMP-Sensitive Calcium Store

We pursued the hypothesis by microinjecting cGMP into eggs in which we had previously injected inositol 1,4,5-phosphorothioate (InsPS_3), an analogue of InsP_3 resistant to inactivation by either phosphorylation or dephosphorylation (Taylor *et al.*, 1989). We first examined the effects of multiple injections of InsP_3 and InsPS_3 on intracellular calcium levels. Typical examples of these experiments are shown in Figure 4. The microinjection of InsP_3 (Figure 5) at a pipette concentration of $100 \mu\text{M}$ (0.8% injection) caused a large rise in $[\text{Ca}^{2+}]_i$ with a peak of $2.42 \pm 0.34 \mu\text{M}$ (mean \pm SEM, $n = 5$). A second injection of the same quantity of InsP_3 caused an increase in $[\text{Ca}^{2+}]_i$ that had a peak of $1.33 \pm 0.195 \mu\text{M}$ (mean \pm SEM, $n = 5$). A third and fourth injection were indistinguishable from the second injection in terms of both magnitude and duration.

The injection of InsPS_3 with a similar activity to that of InsP_3 (it is ~ 5 -fold less potent) caused a transient of similar magnitude and duration (Figure 4B). However, subsequent injections of InsPS_3 resulted in many small

risers in $[\text{Ca}^{2+}]_i$. The second injection caused a transient with a peak of only $0.68 \pm 0.25 \mu\text{M}$ (mean \pm SEM, $n = 5$) and the third injection, $0.43 \pm 0.07 \mu\text{M}$. Calcium responses are attenuated in response to InsPS_3 . This could be due to either the continued occupancy of the receptor preventing store refilling or the desensitization of the InsP_3 receptor that would allow the store to refill but prevent further inositol phosphate-induced calcium release. To determine which of these possibilities was the most likely, we measured the change in $[\text{Ca}^{2+}]_i$ in response to the calcium ionophore A23187 (we used Br-A23187, a nonfluorescent derivative), which releases calcium from intracellular stores in sea urchin eggs without inducing significant calcium influx across the plasma membrane. However, to obviate any contribution that ionophore-induced calcium entry might make, this part of the experiment was performed in CaFASW. The data of Figure 4 show that Br-A23187 causes substantial calcium release in the egg previously microinjected with InsP_3 , but in the egg injected with InsPS_3 the response is reduced greatly. The lack of response to Br-A23187 in InsPS_3 -injected eggs suggests that the only major A23187-sensitive calcium store present in intact eggs is the InsP_3 -sensitive store and that the absence of a significant response to all but the first injection of InsPS_3 is due to the store being empty, rather than to desensitization of the InsP_3 receptor or a quantal release phenomenon (Meyer and Stryer, 1990).² That $[\text{Ca}^{2+}]_i$ returns to submicromolar levels when net uptake into the InsP_3 -sensitive store is prevented by the continued presence of InsPS_3 implies that the calcium released by InsPS_3 is either taken up into mitochondria (Eisen *et al.*, 1984), which may be immune to the effects of A23187 under these conditions (Pressman, 1976) or extruded across the plasma membrane.

Examining the effects of InsPS_3 itself led us to the conclusion that a major functional calcium store in sea urchin eggs is identified by its sensitivity to InsP_3 . By using this approach, we can determine whether cGMP mobilizes calcium from the InsP_3 -sensitive store or another discrete A23187- or InsP_3 -insensitive calcium store. We injected eggs with $50 \mu\text{M}$ InsPS_3 , followed this with an injection of $10 \mu\text{M}$ InsP_3 , and a further injection of $200 \mu\text{M}$ cGMP. The results of one such experiment are shown in Figure 4. The injection of InsPS_3 led to a very rapid and large increase in $[\text{Ca}^{2+}]_i$ from $\sim 50 \text{ nM}$ to nearly $3 \mu\text{M}$. $[\text{Ca}^{2+}]_i$ then rapidly decreased

² The design of this experiment rests on the assumption that Br-A23187 is capable of releasing calcium directly from intracellular stores, rather than requiring the cooperation of calcium-stimulated InsP_3 production. If the latter were true, our conclusion that the InsP_3 -sensitive store is emptied would be invalid; it could equally well be the case that it is desensitized to InsP_3 . We tested this assumption by adding the calcium ionophore Br-A23187 to eggs injected with heparin, an InsP_3 antagonist. Br-A23187 caused the release of calcium (an increase in Ca_i) under these conditions.

to a value of ~ 600 nM. Subsequent injection of $10 \mu\text{M}$ InsP_3 caused a small increase of ~ 200 nM. After this the microinjection of $200 \mu\text{M}$ cGMP caused a very similar rise of only 200 nM. This experiment shows that after the release of calcium from an InsP_3 -sensitive store, cGMP is unable to elicit its calcium transient, implying that cGMP causes calcium release from the egg's major functional InsP_3 -sensitive calcium store.

DISCUSSION

cGMP Activates Eggs by Mobilizing Intracellular Calcium

We show that cGMP activates sea urchin eggs by stimulating a $[\text{Ca}^{2+}]_i$ transient. The increase in $[\text{Ca}^{2+}]_i$ is independent of external calcium, that is, cGMP mobilizes calcium from intracellular stores. It is only the later falling phase of the transient that depends on extracellular calcium, and, in this respect, the rise in calcium stimulated by cGMP is similar to that at fertilization (Crossley *et al.*, 1991). The rise in $[\text{Ca}^{2+}]_i$ occurs only after a delay of ~ 20 s. During the delay, it seems likely that cGMP activates an enzyme or that some metabolism of cGMP is occurring to generate a more potent activating compound. This hypothesis is borne out by the observation that cGMP does not release ^{45}Ca when added to permeabilized eggs. In contrast, InsP_3 causes calcium release with no detectable delay (Whitaker and Irvine, 1984; Crossley *et al.*, 1988) and releases ^{45}Ca from permeabilized eggs quite effectively. 8'-Br-cGMP, a non-hydrolysable derivative, of cGMP do not cause a $[\text{Ca}^{2+}]_i$ transient when microinjected; but 5'GMP, the product of the action of cGMP phosphodiesterase, is also ineffective, thus ruling out this enzyme as the source of an activating compound. Another known target of cGMP is the cGMP-dependent kinase. Yet the kinase is as sensitive to 8'-Br-cGMP as it is to cGMP itself (Lincoln and Corbin, 1983), so this too is an unlikely mechanism.

cGMP Does Not Stimulate Polyphosphoinositide Metabolism

There are similarities in the modes of egg activation by cGMP and $\text{GTP}\gamma\text{S}$, namely, mobilization of intracellular calcium, a latency before activation and a lack of ability to release calcium from permeabilized eggs (Crossley *et al.*, 1991). We have shown that $\text{GTP}\gamma\text{S}$ activates phosphoinositidase C by virtue of the fact that it can stimulate a process, Na/H antiport activation, known to be under the control of protein kinase C via DAG (Crossley *et al.*, 1991). Using the same assay, we show here that cGMP fails to activate the Na/H antiport; under conditions in which cytosolic $[\text{Ca}^{2+}]_i$ is clamped to resting levels, the microinjection of activating concentrations of cGMP fails to cause any cytoplasmic alkalinization. cGMP fails to activate DAG production when changes

in $[\text{Ca}^{2+}]_i$ are prevented, and in this crucial respect it differs from activation induced by $\text{GTP}\gamma\text{S}$. It does not appear to stimulate phosphoinositidase C.

Heparin has no Effects on Egg Activation by cGMP

Another way of determining whether cGMP activates eggs through the generation of InsP_3 (perhaps by a different route than via the G-protein-linked phosphoinositidase C) is to examine the effects of heparin on activation. Heparin competitively inhibits the binding of InsP_3 to its receptors in the endoplasmic reticulum (Hill *et al.*, 1987; Cullen *et al.*, 1988) and has effects on the activation of eggs by both $\text{GTP}\gamma\text{S}$ and sperm. Heparin completely inhibits activation due to $\text{GTP}\gamma\text{S}$ and increases the latency of activation at fertilization (Crossley *et al.*, 1991). Here we have shown that heparin affects neither the magnitude of the calcium transient caused by cGMP nor the latency of activation. These data indicate that cGMP does not release calcium via the InsP_3 receptor, either directly or indirectly.

cGMP Requires an Intact InsP_3 -Sensitive Calcium Store for Activation

To assess whether cGMP caused calcium release from a distinct calcium pool, we examined the response to the microinjection of cGMP in eggs that had an empty InsP_3 -sensitive calcium store. We did this by first injecting eggs with InsPS_3 , a nonhydrolyzable analogue of InsP_3 (Taylor *et al.*, 1989). After the release of calcium from the InsP_3 -sensitive calcium store, the microinjection of cGMP had very little effect on $[\text{Ca}^{2+}]_i$ with an increase of only 200 nM. This suggests that cGMP relies on a filled InsP_3 -sensitive calcium store to be able to activate an egg. In other words, it does not stimulate calcium release from a distinct InsP_3 -insensitive calcium store (Clapper *et al.*, 1987).

We investigated the identities of the calcium stores in intact cells using InsPS_3 . We showed that after one injection of this analogue, subsequent responses are much reduced. We also show that this is due to a prevention of refilling of the calcium store. One of the interesting aspects of this experiment is that A23187 fails to stimulate substantial calcium release from eggs in which the InsP_3 -sensitive calcium store has been desensitized, suggesting that the InsP_3 -sensitive and A23187-sensitive calcium stores are one and the same; in other words, in the intact egg there is only one major calcium store. Our data in intact eggs may conflict with those of Clapper *et al.* (1987), who identified two different microsomal calcium stores *in vitro* on the basis of sensitivity to calcium-releasing agents after density-gradient centrifugation. However, the relative capacities of the two stores were not compared directly. The fact that cGMP mobilizes calcium from an InsP_3 -sensitive calcium store without utilizing the InsP_3 receptor sug-

gests that this calcium store is multifunctional, in that it possesses a calcium-releasing receptor or mechanism distinct from the InsP_3 -activated pathway, possibly, though not necessarily, a distinct calcium release channel (Berridge and Galione, 1988; Missiaen *et al.*, 1991).

cGMP as the Activating Messenger at Fertilization

If we take the receptor-G-protein hypothesis of activation at fertilization as our touchstone (Jaffe *et al.*, 1988), we can make a good case for cGMP as the activating messenger at fertilization. The mode of action of cGMP more closely resembles the mode of action of the fertilizing sperm than does activation of a GTP-binding protein in that the latter signal pathway involves activation of phosphoinositidase C and is blocked by heparin, whereas the former do not and are not.

Considering instead the hypothesis that an activating messenger diffuses into the egg from the sperm after sperm-egg fusion (Whitaker *et al.*, 1989; Whitaker and Crossley, 1990), there are two candidate messengers in sea urchin sperm, InsP_3 (Whitaker and Irvine, 1984; Iwasa *et al.*, 1989) and cGMP (Swann *et al.*, 1987). The $[\text{Ca}^{2+}]_i$ response to cGMP quite closely resembles the fertilization response in its latency, amplitude, duration, and insensitivity to heparin, whereas the response to InsP_3 is completely blocked by heparin (Crossley *et al.*, 1991), which has no detectable latency and subsides more rapidly than the fertilization $[\text{Ca}^{2+}]_i$ transient. On the other hand, quantitatively, InsP_3 seems to have the edge over cGMP. A local concentration of 1 μM InsP_3 is sufficient to induce a regenerative $[\text{Ca}^{2+}]_i$ transient in sea urchin eggs (Whitaker and Irvine, 1984), and sperm appear to contain a sufficient quantity of InsP_3 to achieve this concentration (Iwasa *et al.*, 1989), whereas we report here that a final concentration of 5–10 μM cGMP is needed to do the same thing, requiring a minimum pipette concentration of 0.5–1 mM. However, InsP_3 acts directly on the egg's calcium store and is effective locally (Swann and Whitaker, 1986), whereas cGMP acts indirectly and with a long latency of ~ 25 s at the lowest effective concentrations. Diffusion of molecules the size of cGMP from the injection site to the antipode in sea urchin eggs is 90% complete in 20–30 s (Swann and Whitaker, 1986; Swann, 1987), so that the cGMP concentration at the site of injection will have fallen 50-fold within a few seconds. It is therefore appropriate to consider only the final concentration of cGMP because we cannot maintain a localized increase in cGMP using microinjection. There is support for this idea in our data in that the effect of cGMP appears to depend on the final concentration, not the pipette concentration; a 1% injection of 1 mM cGMP (Table 2) has an effect similar to that of a 0.2% injection of 5 mM cGMP (Table 3). Of course, if cGMP is a messenger at fertilization, it is likely that it acts locally. Sea urchin sperm contain cGMP

at a concentration of 1 $\mu\text{mol/kg}$ wet weight when activated (Kopf and Garbers, 1979), a high concentration in comparison with other cell types, but not in itself sufficient to act as the sustained source of cGMP necessary for egg activation. Nonetheless, the high levels of cGMP in sperm reflect the high levels of a persistent guanylate cyclase activity (Kopf and Garbers, 1979; Singh *et al.*, 1988) that should be capable of generating much larger quantities of cGMP over a long period once egg GTP becomes available to it as a substrate.

The best argument in favor of cGMP as the activating messenger is the close similarity we have found between its $[\text{Ca}^{2+}]_i$ response and the fertilization calcium transient. The one difference that we have identified between activation at fertilization and by cGMP is subtle; the response latency and rate of rise are increased significantly by heparin at fertilization, but heparin has no effect on the parameters of the cGMP response. This difference, though small, is enough to imply that cGMP cannot be the sole activating agent. Because heparin, the InsP_3 antagonist, increases the latency of the fertilization response, the simplest hypothesis would suggest that a combination of cGMP and InsP_3 is injected when sperm and egg fuse and that it is the cocktail that activates the egg.

cGMP as a Regulator of Intracellular Calcium

We have shown that cGMP can increase $[\text{Ca}^{2+}]_i$ in sea urchin eggs. The result is not without precedent and our data may be of more general application. At similar concentrations, cGMP also activates *Medaka* eggs (Iwamatsu *et al.*, 1988); it can mimic calcium-mediated responses when injected into *Xenopus* oocytes (Dascal and Landau, 1982) and *Limulus* photoreceptors (Johnson *et al.*, 1986). In *Dictyostelium* it has been suggested to regulate calcium influx (Menz *et al.*, 1991). The data that we present here suggest that cGMP can cause calcium release from an InsP_3 -sensitive calcium store but that its mechanism of action does not involve the generation of inositol phosphates and is independent of the InsP_3 receptor. cGMP acts in sea urchin eggs via a novel, if unidentified, signaling pathway, implying the existence of yet another mechanism by which cells can regulate intracellular calcium levels.

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