Regulation of intracellular calcium release during fertilization in sea urchin eggs

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Regulation of intracellular calcium release during fertilization in sea urchin eggs

by

Shyh-Jye Lee

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements of the degree of

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER I. THE CYCLIC GMP-MEDIATED CALCIUM RELEASE PATHWAY IN SEA URCHIN EGGS IS NOT REQUIRED FOR THE RISE IN CALCIUM DURING FERTILIZATION</td>
<td>6</td>
</tr>
<tr>
<td>CHAPTER II. THE CALCIUM TRANSIENT IN SEA URCHIN EGGS DURING FERTILIZATION REQUIRES THE PRODUCTION OF INOSITOL 1,4,5-TRISPHOSPHATE</td>
<td>46</td>
</tr>
<tr>
<td>GENERAL CONCLUSIONS</td>
<td>93</td>
</tr>
<tr>
<td>GENERAL REFERENCES</td>
<td>98</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>105</td>
</tr>
</tbody>
</table>
ABSTRACT

The requirement for both inositol-1,4,5 trisphosphate (InsP₃) and ryanodine-sensitive intracellular Ca²⁺ release mechanisms during fertilization was studied in sea urchin eggs. The postulated pathway of cGMP-dependent protein kinase (PKG) activation of ADP-ribosyl cyclase for production of cADPR to activate the ryanodine receptor Ca²⁺ channel was tested with a variety of activators and inhibitors. Our observations are consistent with Ca²⁺ release by cGMP in the egg being dependent on an isoform of PKG that is distinct from the mammalian enzyme. PKG activity in the sea urchin egg was activated by cIMP and was insensitive to cGMP analogs, which are normally an inhibitor and potent activators of mammalian isozymes, respectively. Surprisingly inhibitors of either PKG or ADP-ribosyl cyclase activities did not prevent the transient rise in intracellular Ca²⁺ activity ([Ca²⁺]ᵢ) in 0.7-1.0 mg/mL heparin-loaded eggs during fertilization. These results suggest the synthesis of cADPR during fertilization is not necessary for regulating the Ca²⁺ event. The production of inositol 1,4,5-trisphosphate (InsP₃) to mediate the transient rise in [Ca²⁺]ᵢ in sea urchin eggs during fertilization was studied by inhibiting the hydrolysis of phosphatidylinositol-4,5-bisphosphate to InsP₃ and 1,2-diacylglycerol by phospholipase C (PLC). U73122, a PLC inhibitor, eliminated the sperm-induced Ca²⁺ release in a dose-dependent manner. It also prevented the accompanying rise in intracellular pH (pHᵢ), which is mediated by the activation of the Na⁺-H⁺ antiporter. The antiporter is regulated through activation of protein kinase C by 1,2-diacylglycerol. U73122 inhibition was
not due to a failure of fertilization, since incorporated sperm pronuclei were evident in U73122-treated eggs. The inhibition of InsP$_3$ production during the first 2 min of fertilization by U73122 was confirmed by InsP$_3$ mass measurements. In addition, U73122 also inhibited the GTP$_\gamma$S-induced Ca$^{2+}$ release and pH$_i$ rise in unfertilized eggs. These results suggested that the transient rise in Ca$^{2+}$ in sea urchin during fertilization requires the production of InsP$_3$ via a PLC$\beta$-dependent pathway. In summary, the InsP$_3$-mediated pathway is the primarily required mechanism to regulate the rise in [Ca$^{2+}$]$_i$ during fertilization in sea urchin eggs.
GENERAL INTRODUCTION

A transient rise in intracellular calcium activity ([Ca$^{2+}$]$_i$) controls many cellular responses, including fertilization (Berridge, 1993). During fertilization, a spermatozoon triggers a momentary elevation of [Ca$^{2+}$]$_i$ that sweeps across the egg from the sperm-egg fusion site to the antipode (Jaffe, 1980, 1983). This sperm-induced Ca$^{2+}$ transient is necessary and sufficient to relieve the metabolic depression of unfertilized sea urchin eggs (Whitaker and Steinhardt, 1985). Despite the significance of this fertilization response, the mechanism for the rise in [Ca$^{2+}$]$_i$ remains poorly understood. Two types of intracellular Ca$^{2+}$ release channels, which are mediated by InsP$_3$ and ryanodine, respectively, have been found in a variety of cell types, including sea urchin eggs (Shen, 1995). The sea urchin egg contains proteins recognized by antibodies against type I InsP$_3$ receptor (Parys et al., 1994) or skeletal muscle type 1 ryanodine receptor (McPherson et al., 1992). Substantial evidence suggests that InsP$_3$-induced Ca$^{2+}$ release participates in the sperm-induced Ca$^{2+}$ transient in sea urchin eggs. The turnover of InsP$_3$ precursor, polyphosphoinositides, increases during fertilization (Turner et al., 1984; Ciapa et al., 1992). The relative amount of InsP$_3$ and its accompanied product, 1, 2-diacylglycerol (DAG) have also been shown to increase 66% and 44%, respectively, within 20 seconds following insemination in sea urchin eggs (Ciapa and Whitaker, 1986). Micrinjection of InsP$_3$ triggers a transient rise in [Ca$^{2+}$]$_i$ in unfertilized sea urchin eggs (Whitaker and Irvine, 1984; Swann and Whitaker, 1986). Phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) hydrolysis may be
catalyzed by either type of phospholipase C (PLC) isozyme, PLCβ or PLCγ, through G protein- or tyrosine kinase-coupled receptors, respectively (Lee and Rhee, 1995). A spermatozoon may bind to a G protein-linked receptor in the egg plasma membrane and the G protein-activated PLCβ can hydrolyze PtdInsP₂ to InsP₃ and DAG (Jaffe, 1989).

Several lines of evidence support the G protein-linked pathway: 1) sea urchin egg cortex contains PLC activity (Whitaker and Aitchison, 1985); 2) microinjection of GTPγS, a G-protein activator, generates a fertilization-like [Ca²⁺]ᵢ transient (Crossley et al., 1991); 3) sea urchin eggs contain both cholera toxin (CTX) and pertussis toxin sensitive G proteins and CTX can activate the eggs (Turner et al., 1987). On the contrary, objections have been raised against this idea: 1) bindin, the sea urchin sperm acrosomal protein, mediates the sperm-egg recognition, but does not activate the egg (Ohlendiek and Lennarz, 1995); 2) GDPβS, a G protein antagonist, fails to inhibit the sperm-induced Ca²⁺ transient (Whitaker et al., 1989; Crossley et al., 1991). Tyrosine kinase activity has been detected early in fertilization of sea urchin eggs (Satoh and Garbers, 1985; Ciapa and Epel, 1991). Membrane proteins are phosphorylated by egg cortical tyrosine kinase activity at 5 s and the maximum phosphorylation is reached within 20 s after insemination (Abassi and Foltz, 1994). However, the significance of tyrosine kinase phosphorylation to the sperm-induced Ca²⁺ transient is uncertain, because inhibitors of protein tyrosine kinase do not affect the early events during sea urchin fertilization (Moore and Kinsey, 1995).
InsP₃-independent Ca²⁺ release has also been demonstrated in sea urchin eggs by using agonists of the ryanodine receptor, including caffeine, ryanodine and cADPR (Fujiwara et al., 1990; Dargie et al., 1990; Galione et al, 1991). Unlike the InsP₃-induced Ca²⁺ release, the natural agonist for the ryanodine receptor is not known. It has been proposed that cADPR is the physiological agonist for the ryanodine receptor Ca²⁺ channels (Lee, 1994; Galione and White, 1994). cADPR is a β-NAD⁺ metabolite, which is synthesized by a widely distributed enzyme, ADP-ribosyl cyclase (Lee and Aarhus, 1993). cADPR has also been found in a variety of cell types, including sea urchin eggs (Walseth et al., 1991). However, before accepting cADPR as the natural messenger that mediates the fertilization response of sea urchin eggs, a signal transduction pathway and the change in cADPR levels in eggs during fertilization need to be demonstrated. It has been suggested that a cGMP-mediated pathway may regulate the cADPR level during sea urchin fertilization. cGMP is one of the low molecular weight molecules that can trigger a rise in [Ca²⁺]ᵢ in sea urchin eggs (Swann et al., 1987; Whalley et al., 1992). The cGMP-induced [Ca²⁺]ᵢ rise has the characteristic latent period between the addition of agonist and the initiation of rise in [Ca²⁺]ᵢ as that during fertilization. The link between cGMP and the possible natural agonist of ryanodine receptor, cADPR, makes cGMP an attractive sperm messenger at fertilization.

InsP₃-dependent Ca²⁺ release via InsP₃ receptor and InsP₃-independent Ca²⁺ release presumably via ryanodine receptor may both occur during sea urchin fertilization (Rakow and Shen, 1990; Crossley et al., 1991). These two types of mechanisms release Ca²⁺
from the same intracellular Ca\(^{2+}\) store (Whalley et al., 1992; Buck et al., 1994). Blocking either type of Ca\(^{2+}\) release mechanism with respective antagonists has little effect on the rise in [Ca\(^{2+}\)]\(_i\) during fertilization in sea urchin eggs (Rakow and Shen, 1990; Crossley et al., 1991; Galione et al., 1993a; Lee et al., 1993). However, the sperm-induced Ca\(^{2+}\) transient was abolished if both types of Ca\(^{2+}\) release mechanisms were inhibited (Galione et al., 1993a; Lee et al., 1993). Therefore, it was proposed that the dual Ca\(^{2+}\) release mechanisms may be redundant to assure the rise in [Ca\(^{2+}\)]\(_i\) at fertilization (Galione et al., 1993a; Lee et al., 1993). However, Mohri et al. (1995), observed a concentration-dependent inhibition by heparin or pentosan polysulfates, InsP\(_3\) receptor antagonists, on the kinetics and amplitude of sperm-induced Ca\(^{2+}\) release. This finding is in contrast to the report (Lee, et al., 1993) that the sperm-induced Ca\(^{2+}\) transient occurred in eggs preloaded with 4.7 mg/mL heparin. These contradictory results may be due to the use of heparin with different molecular weight. An uncleaved high molecule weight heparin was used by Lee et al. (1993) and a low molecular weight cleaved product was used by Mohri et al. (1995). The high molecular weight heparin has been shown to be less than 20% effective to block the InsP\(_3\) receptor (Ghosh et al., 1988). Nevertheless, the blockade of sperm-induced Ca\(^{2+}\) transient by high concentration of heparin suggests that the fertilization response is primarily InsP\(_3\)-dependent. However, the effect of high level of heparin on the InsP\(_3\)-independent Ca\(^{2+}\) release channels is of concern (Shen, 1995). Therefore, an alternative approach should be considered to confirm the results obtained using heparin.
The objective of this thesis was first to validate the proposed pathway for cGMP-induced Ca\(^{2+}\) release in sea urchin eggs. More importantly, the necessity of this cGMP-dependent pathway during fertilization was investigated. Secondly, the role of the InsP\(_3\)-dependent pathway during fertilization was reexamined by blocking the InsP\(_3\) synthesizing enzyme, phospholipase C.

Dissertation Organization

This dissertation is composed of two separate articles, which have been published in or accepted by Developmental Biology, listed as following:


2. Lee, S.J. and Shen, S.S. (1997). The Ca\(^{2+}\) transient during fertilization is mediated by the production of inositol 1,4,5-trisphosphate via a PLC\(\beta\)-dependent pathway in sea urchin eggs. (accepted for publication)

Following the articles is the general conclusions and a list of references cited in the general introduction and general conclusions.
CHAPTER 1. THE CYCLIC GMP-MEDIATED CALCIUM RELEASE PATHWAY IN SEA URCHIN EGGS IS NOT REQUIRED FOR THE RISE IN CALCIUM DURING FERTILIZATION

A paper published in Developmental Biology

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ABSTRACT

The mechanisms required for cGMP-induced Ca\textsuperscript{2+} release in the sea urchin egg were investigated using both egg homogenates and intact eggs. The postulated pathway of cGMP-dependent protein kinase (PKG) activation of ADP-ribosyl cyclase for production of cADPR to activate the ryanodine receptor Ca\textsuperscript{2+} channel was tested with a variety of activators (cGMP analogs and cIMP) and inhibitors (Rp-8-pCPT-cGMPS, 3-aminopyridine NAD, nicotinamide and spermine). Our observations are consistent with Ca\textsuperscript{2+} release by cGMP in the egg is dependent on an isoform of PKG that is distinct from the mammalian enzyme. PKG activity in the sea urchin egg was activated by cIMP, but was insensitive to cGMP analogs, which are potent activators of mammalian isoenzymes. Surprisingly it appears the activation of the cGMP-dependent Ca\textsuperscript{2+} release pathway was unnecessary

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during fertilization. Inhibitors of either PKG or ADP-ribosyl cyclase activities did not prevent the transient rise in intracellular Ca\(^{2+}\) activity in heparin-loaded eggs during fertilization. These results suggest the synthesis of cADPR during fertilization is not necessary for regulating the Ca\(^{2+}\) event.

**INTRODUCTION**

The regulation of cytoplasmic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)) in a wide variety of cells is well established as an important event during signal transduction of numerous external stimuli. Often the principal source of Ca\(^{2+}\) is from organelles, which is generally dependent on either of two types of intracellular Ca\(^{2+}\) release channels that bind inositol 1,4,5-trisphosphate (InsP\(_3\)) or ryanodine (Berridge, 1993). A propagated wave of intracellular Ca\(^{2+}\) release characterizes the fertilization response of eggs from invertebrates to mammals (Jaffe, 1991). The transient rise in [Ca\(^{2+}\)]\(_i\) is both necessary and sufficient for metabolic activation of the quiescent unfertilized sea urchin egg (Whitaker and Steinhardt, 1985). Despite the significance of the propagated wave of intracellular Ca\(^{2+}\) release, we still do not understand how the rise in [Ca\(^{2+}\)]\(_i\) is generated during fertilization. The sea urchin egg contains specific proteins recognized by an antibody against the skeletal muscle type 1 ryanodine receptor (McPherson et al., 1992) and an antibody against the type I InsP\(_3\) receptor (Parys et al., 1994). InsP\(_3\)-induced Ca\(^{2+}\) release during sea urchin fertilization is suggested by the observations of an increased turnover of
polyphosphoinositides (Turner et al., 1984; Kamel et al., 1985; Ciapa and Whitaker, 1986) and a transient rise in $[Ca^{2+}]_i$ by microinjection of InsP$_3$ (Whitaker and Irvine, 1984). InsP$_3$-independent $Ca^{2+}$ release during fertilization is suggested by the occurrence of a transient rise in $[Ca^{2+}]_i$ in eggs, which were preloaded with 0.1-0.3 mg/ml heparin (Rakow and Shen, 1990; Crossley et al., 1991). Heparin is a competitive and potent inhibitor of InsP$_3$-dependent $Ca^{2+}$ release (Ghosh et al., 1988) and InsP$_3$ binding (Worley et al., 1987). The sea urchin egg also contains a caffeine and ryanodine-sensitive $Ca^{2+}$ release mechanism (Galione et al., 1991; Buck et al., 1992).

The two mechanisms were proposed to be redundant to ensure the occurrence of an increase in $[Ca^{2+}]_i$ during the fertilization reaction in sea urchin eggs. Inhibitors of both InsP$_3$-sensitive and ryanodine-sensitive intracellular $Ca^{2+}$ release mechanisms were reported to be necessary to block the rise in $[Ca^{2+}]_i$ during fertilization (Galione et al., 1993a; Lee et al., 1993). There may be some uncertainty for the dual redundant role of InsP$_3$-dependent and -independent $Ca^{2+}$ release during fertilization. In one report (Galione et al., 1993a) ryanodine-dependent $Ca^{2+}$ release was inhibited with ruthenium red, which only partially blocks the action of ryanodine in intact eggs (Buck et al., 1992). In the other report (Lee et al., 1993) eggs were preloaded with 4.7 mg/ml heparin, which alone would be sufficient to inhibit all parameters of $Ca^{2+}$ release in sea urchin eggs during the fertilization reaction (Mohri et al., 1995).

Unlike the well known InsP$_3$ signaling pathway (Berridge and Irvine, 1992), the regulation of ryanodine-sensitive $Ca^{2+}$ release remains unclear (Lee, 1994). The naturally
occurring cyclic ADP-ribose (cADPR) has been suggested as the endogenous agonist of the ryanodine receptor Ca\(^{2+}\) channel (Galione, 1992; Galione and White, 1994), although the significance of cADPR is uncertain (Sitsapesan et al., 1995). In order to establish cADPR as a physiological signal molecule, a transduction pathway from insemination to cADPR production is necessary. Guanosine 3\:'5\:'-cyclic monophosphate (cGMP) has attracted attention because microinjection of cGMP into the unfertilized sea urchin egg triggers a transient [Ca\(^{2+}\)]\(_i\) rise, which closely resembles the Ca\(^{2+}\) transient during fertilization in both kinetics and amplitude (Swann et al. 1987; Whalley et al., 1992). The action of cGMP is InsP\(_3\)-independent and absent in digitonin-permeabilized eggs, which suggested a key molecule has leaked out from the permeabilized eggs (Whalley et al., 1992). The missing molecule in the digitonin-permeabilized sea urchin eggs is likely to be β-NAD\(^{+}\), because the addition of β-NAD\(^{+}\) restores cGMP-induced Ca\(^{2+}\) release in egg homogenates (Galione et al., 1993b). Protein kinase inhibitors with broad specificities blocked the β-NAD\(^{+}\) dependent Ca\(^{2+}\) release by cGMP in egg homogenates and it has been proposed (Galione et al., 1993b) that cGMP through cGMP-dependent protein kinase (PKG) activates ADP-ribosyl cyclase, which metabolizes β-NAD\(^{+}\) to cADPR that opens the ryanodine receptor Ca\(^{2+}\) channels in sea urchin eggs.

This signal transduction pathway intrigued us because of its novelty and possibility for testing the presence of dual redundant Ca\(^{2+}\) release in sea urchin eggs during fertilization. Regulation of Ca\(^{2+}\) by cGMP has been suggested in other cell systems, including *Xenopus* oocytes (Dascal and Landau, 1982) and Medaka eggs (Iwamatsu et al.,
The regulation of ion channel conductances in sensory cells (Kaupp, 1991) and relaxation of tension in smooth muscles (Lincoln, 1989) by cGMP are well established. It has been recognized that nitric oxide stimulates the soluble form of guanylate cyclase and increases the level of cGMP (Bredt and Snyder, 1989), which may be responsible for eliciting a variety of physiological responses. An early increase in cGMP in the sea urchin egg during fertilization was recently reported (Ciapa and Epel, 1996), which could be mimicked by application of nitric oxide (Willmott et al., 1996). Inhibition of cGMP production and action did not appear to block the Ca$^{2+}$ transient during fertilization, presumably due to compensation by the InsP$_3$-induced Ca$^{2+}$ release (Ciapa and Epel, 1996). This study presents a more in-depth investigation of the enzymatic links in the cGMP-induced Ca$^{2+}$ release pathway in sea urchin eggs. We have further characterized with several agonists and antagonists, the β-NAD$^+$ dependent, cGMP-induced Ca$^{2+}$ release in egg homogenates and extended the observations to intact eggs. We have directly examined the possible requirement for elements of the cGMP-dependent pathway in the transient rise in Ca$^{2+}$ in eggs during the fertilization response by simultaneously blocking the InsP$_3$-mediated Ca$^{2+}$ release with heparin. We report that although the cGMP-induced Ca$^{2+}$ release appears to rely on the PKG and ADP-ribosyl cyclase activities in both egg homogenates and intact eggs, our results are not consistent with redundant dual Ca$^{2+}$ release mechanisms during fertilization. Inhibitors of PKG and ADP-ribosyl cyclase did not block the transient rise in [Ca$^{2+}$]$_i$ during the fertilization response of eggs preloaded
with 0.7-1.0 mg/ml heparin. Portions of the data presented here have been published previously in abstract form (Lee and Shen, 1994).

MATERIALS AND METHODS

Preparation of Gametes and Solutions

Eggs and sperm of the sea urchin *Lytechinus pictus* were obtained by injection of 0.5 M KCl into the coelomic cavity. The sea urchins were purchased from Marinus, Inc. (Long Beach, CA), and were maintained in Instant Ocean culture with biweekly feeding of *Macroystis*. Eggs were shed into artificial sea water (ASW) of the following composition: 470 mM NaCl, 10 mM KCl, 11 mM CaCl2, 29 mM MgSO4, 27 mM MgCl2, 5 mM NaHCO3, pH 8. The jelly coats were removed from the eggs by several passages through fine-mesh silk and they were washed twice in ASW. Dejellied eggs were used at once for making homogenates or kept in suspension by constant stirring at 60 rpm at 16-18°C until use for single cell recordings. The sperm were kept dry at 4°C. For insemination of intact egg recordings, 0.05 ml of freshly diluted sperm at ≈10⁷ sperm per ml were placed 5-10 mm from the egg. Fluorescent chromophores fluo-3, fura-2 and BCECF-dextran (Mw, 40,000) were purchased from Molecular Probes (Eugene, OR). Fluo-3 was dissolved in homogenization buffer (250 mM N-methylglucamine, 250 mM K gluconate, 20 mM HEPES, 1 mM MgCl2, pH titrated to 7.2 with acetic acid) with addition of 100 µM EGTA. Fura-2 and BCECF-dextran were prepared in intracellular buffer (220 mM K
acetate. 500 mM glycine, 40 mM NaCl, 100 μM EGTA and 20 mM Tris, pH 6.9). Stock solutions of other chemicals were prepared in homogenization buffer with 100 μM EGTA or intracellular buffer for egg homogenate or microinjection studies, respectively. InsP₃ and the Ca²⁺ ionophore, Br-A23187, which was dissolved in DMSO at 5 mM, were obtained from Calbiochem (La Jolla, CA). Low molecular weight (MW ~6000) heparin (H2149 and H5640) was purchased from Sigma Chemical Co. (St. Louis, MO). (Rp)-8-(para-chlorophenylthio)guanosine-3',5'-cyclic monophosphate ((Rp)-8-pCPT-cGMPS) was purchased from BIOLOG (La Jolla, CA) and prepared in 50% homogenization buffer or intracellular buffer and 50% DMSO (v/v). Cyclic ADPR was purchased from Amersham (Arlington Heights, IL). The cGMP analogs were kindly provided by Drs. S.H. Francis and J.D. Corbin (Vanderbilt University). All other chemicals were purchased from Sigma.

**Preparation of Egg Homogenates and Ca²⁺ Release Assay**

Homogenates of unfertilized sea urchin eggs were prepared with slight modifications of previously published procedures (Buck et al., 1994). Dejellied eggs were washed once in ASW, twice in Ca²⁺-free ASW containing 1 mM EGTA, twice in Ca²⁺-free ASW, once in homogenate buffer, and resuspended at 10% (v/v) in homogenization buffer with additions of 25 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 4 units/ml creatine kinase, 100 μM ATP and 8 mM phosphocreatine. The suspension was chilled on ice, homogenized with 5 strokes of a Dounce homogenizer
with a type A pestle, and homogenates were centrifuged for 14 sec at 13,000g at 4°C. The supernatant was collected and stored at -70°C until use. Frozen homogenates were thawed in a water bath at 20°C for 10 min; diluted to 5% (v/v) with homogenization buffer with additions as previously described, and incubated at 20°C for 2 hr. Before use the Ca^{2+} indicator fluo-3 was added at 3 μM. The cuvette contents were maintained at 20°C and fluorescence was measured at 510 nm excitation and 530 nm emission (5 nm bandpass) using an Aminco SLM 500C spectrofluorometer (Urbana, IL). Inhibitor or vehicle was added to the egg homogenate at the desired concentration at 10 min before recording. β-NAD^{+} and various Ca^{2+} mobilizing agents were added to the egg homogenate, while monitoring fluorescence changes. At the end of each recording, 5 μM Br-A23187 was added to calibrate the total releasable Ca^{2+} (Ca_{total}) in the egg microsome preparation. The total volume of additions was less than 2% of homogenate volume (0.6 ml) unless indicated.

**Microinjection Procedures and Fluorometric Monitoring of [Ca^{2+}]_{i}**

Microelectrodes for microinjection were pulled on a Narishige PE-2 puller (Narishige, Japan) from 1 mm (o.d.) x 0.8 mm (i.d.) Pyrex glass tubings (Drummond Scientific, Broomall, PA), heated to 200°C and silane-treated by exposing to hexamethyldisilizane vapors. The electrode tips were filled with intracellular buffer containing one or more of the following: 10 mM fura-2, 125 or 250 mg/ml heparin, 10 mg/ml BCECF-dextran, 10 mM cGMP or analogs, 25 mM 3-aminopyridine nicotinamide
adenine dinucleotide (3-AP NAD) and 50 mM (Rp)-8-pCPT-cGMPS. Microelectrode procedures and fluorimetric monitoring of [Ca\(^{2+}\)]\(_i\) were similar to that previously published (Buck et al., 1994). Injection volume was estimated using dye dilution (Lee, 1989) by measuring the fluorescent emission of fura-2 or BCECF-dextran at 535 nm to excitation at 380 or 440 nm, respectively, and comparing the fluorescence intensity to a dilution standard in intracellular buffer with 100 nM free Ca\(^{2+}\). The total injected volume was less than 1.5% of the egg volume. [Ca\(^{2+}\)]\(_i\) of injected eggs was continuously monitored by the 340/380 fluorescence ratio for fura-2-loaded eggs (Gryniewicz et al., 1985). The 340/380 fluorescence ratio was collected at 2 Hz and calibrated by determining \(R_{\text{min}}\) and \(R_{\text{max}}\). All recordings were made at 18–21°C and experimental data are expressed as mean ± SD.

RESULTS

The addition of cGMP triggers a release of Ca\(^{2+}\) in sea urchin egg homogenates supplemented with β-NAD\(^+\) (Galione et al., 1993b). Separate additions of either 10 μM β-NAD\(^+\) or 50 μM cGMP had little effect on Ca\(^{2+}\) release in 5% egg homogenate, but their sequential additions resulted in a delayed release of Ca\(^{2+}\) (Fig. 1A). At 10 μM β-NAD\(^+\) the Ca\(^{2+}\) responses of 50 μM cGMP and 100 μM cGMP were similar in both amplitude of the total releasable Ca\(^{2+}\) (\(C_{\text{total}}\), 37.5 ± 8.2% and 40.2 ± 12.9%, respectively, n = 3) and the time to reach the peak level (\(T_{\text{peak}}\), 6.6 ± 0.8 and 5.7 ± 2
Figure 1. $Ca^{2+}$ release by cGMP or cIMP in egg homogenates. $Ca^{2+}$ release in the microsome preparation was measured as increasing relative fluorescence of fluo-3 in the cuvette. In all recordings 10 µM β-NAD$^+$ was added at $t = 50$ s of the recording. Subsequently at $t = 100$ s of the recording, either (A) 25-100 µM of cGMP or (B) 50 or 100 µM cIMP was added. At the end of all recordings, 5 µM Br-A23187 was added to calibrate the total releasable $Ca^{2+}$ ($C_{total}$) in each microsome preparation. For simplicity, $C_{total}$ is shown only in (A), but in nearly all recordings $C_{total}$ was normalized to relative fluorescence of 9.5. These records are typical of three (A) and eight (B) experiments.
A lower concentration of 25 μM cGMP also induced a Ca^{2+} rise that was smaller (25.6 ± 8.8% of Ca_{total}) and slower (T_{peak} = 12 ± 5.6 min, n = 3), thus 50 μM was chosen for further studies on the Ca^{2+} releasing activity of cGMP. Ca^{2+} release by cGMP has been proposed to be mediated by production of cADPR through cGMP-dependent protein kinase (PKG) regulation of ADP-ribosyl cyclase activity and the opening of ryanodine receptor Ca^{2+} channels by cADPR (Galione et al., 1993b). The proposed cGMP-induced Ca^{2+} release pathway is schematized in Figure 2, which also illustrates the purported site of action of the activators and inhibitors of the proposed pathway that were tested in this report.

Mobilization of Ca^{2+} by cGMP and Analogs

A variety of cGMP analogs have been synthesized and used to characterize cGMP activation of PKG (Francis and Corbin, 1994). Several analogs have been shown to be selective activators of mammalian PKG. We tested the Ca^{2+} response in sea urchin homogenates and eggs to two groups of cGMP analogs: 8-(2,4-dihydroxyphenylthio)-cGMP and 8-(2-aminophenylthio)-cGMP, which are specific to mammalian type 1 PKG; and 1, N^2-(4-CH_3O-PET)-cGMP and 8-Br-1,N^2-PET-cGMP, which activate both mammalian type 1 PKG and type 1 PKG (Sekhar et al., 1992). In 5% egg homogenates only 8-Br-1,N^2-PET-cGMP induced a slight Ca^{2+} mobilizing activity (7.6% of Ca_{total}) in one of three recordings at a concentration of 12 μM, which is 900-fold greater than its K_s to the type 1 PKG. The effect of these four cGMP analogs on Ca^{2+} homeostasis was also
Figure 2. Proposed pathway for cGMP-induced Ca\textsuperscript{2+} release and sites of action for activators and inhibitors. Ca\textsuperscript{2+} release induced by cGMP is postulated to sequentially activate cGMP-dependent protein kinase (PKG) and ADP-ribosyl cyclase (ADPRC) to trigger the production of cyclic ADP-ribose (cADPR) from β-NAD\textsuperscript{+} (Galione et al., 1993a). The action of cADPR is mediated by cADPR-binding protein (cADPR-BP) and calmodulin (CaM) to release Ca\textsuperscript{2+} (Lee et al., 1994) from endoplasmic reticulum (ER) by opening ryanodine receptor Ca\textsuperscript{2+} channels (Ryr). Activators and inhibitors as indicated were used to examine this postulated cGMP-mediated pathway. Other abbreviations: H8, N-[2-(methylamino)ethyl]-5-isoquinolsulfonamide dihydrochloride; Rp-8-pCPT-cGMPS, (Rp)-8-(para-chlorophenythio)guanosine-3',5'-cyclic monophosphate; 3-AP NAD, 3-aminopyridine NAD; NiAm, nicotinamide.
tested by microinjecting them into intact eggs. No Ca\textsuperscript{2+} response in sea urchin eggs was observed following injection of specific type 1α PKG activators. A Ca\textsuperscript{2+} response was observed in some recordings subsequent to injection of types 1α and 1β PKG activators.

Microinjection of 20-30 μM 1,N\textsuperscript{2}-(4-CH\textsubscript{3}O-PET)-cGMP caused a slight rise of intracellular Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]\textsubscript{i}) to a peak level of 0.81 ± 0.24 μM (n = 3) over a duration of about 135 s in 3 of 10 eggs. Microinjection of 10-25 μM 8-Br-1,N\textsuperscript{2}-PET-cGMP had the most dramatic effect on Ca\textsuperscript{2+} regulation. A delayed increase in [Ca\textsuperscript{2+}]\textsubscript{i} was recorded in 10 of 22 injected eggs. In eggs activated by the cGMP analog, [Ca\textsuperscript{2+}]\textsubscript{i} rose from 110 ± 22 nM to 1.79 ± 0.6 μM (n = 10) after a 1.5-4 min delay (Fig. 3).

The longer time delay between injecting cGMP analog and the rise in [Ca\textsuperscript{2+}]\textsubscript{i} suggested that the Ca\textsuperscript{2+} release may occur due to inhibition of cGMP-dependent phosphodiesterase (cGMP-PDE) activity rather than direct stimulation of PKG in the eggs. A high affinity inhibitor of both binding and hydrolytic activities of mammalian cGMP-PDE is inosine 3':5'-cyclic monophosphate (cIMP; Thomas et al., 1992). Surprisingly microinjection of 10-20 μM cIMP into intact eggs triggered a fertilization-like rise in [Ca\textsuperscript{2+}]\textsubscript{i}, which was indistinguishable from the action of cGMP (data not shown), and microinjection of 50-100 μM 5'-IMP was without effect. Similarly the addition of cIMP induced a dose dependent rise in Ca\textsuperscript{2+} in 10 μM β-NAD\textsuperscript{+}-supplemented egg homogenates (Fig. 1B). Addition of 100 μM cIMP triggered a 37.4 ± 5.9% release of Ca\textsubscript{total} and a T\textsubscript{peak} of 7.5 ± 1.2 min (n = 8), which are similar to the release in Ca\textsuperscript{2+} triggered by the addition of 50-100 μM cGMP. A variety of cGMP-PDE inhibitors (Weishaar et al.,
Figure 3. A transient rise in $[\text{Ca}^{2+}]_i$ induced by a cGMP analog. Microinjection of some cGMP analogs, especially 8-Br-1,N2-PET-cGMP, into unfertilized eggs induced a delayed $\text{Ca}^{2+}$ response. In this recording the microinjection of 10 µM 8-Br-1,N2-PET-cGMP triggered a delayed rise in $[\text{Ca}^{2+}]_i$ from a pre-injection level of 120 nM to a peak of 2.55 µM at 145 s. A broad plateau during the recovery phase was typical of most recordings and elevation of the fertilization envelope was confirmed at $t = 4$ min.
1985), including 0.5 mM zaprinast, 1 mM dipyridamole, and 200 μM 3-isobutyl-1-methylxanthine had no apparent effect on Ca\(^{2+}\) regulation in egg homogenates or intact eggs. Thus cIMP-induced Ca\(^{2+}\) release does not appear to be mediated by inhibiting cGMP-PDE and the cause of a delayed response to the cGMP analog, 8-Br-1,N\(^2\)-PET-cGMP, remains unclear.

**Inhibition of cGMP-Induced Ca\(^{2+}\) Release in Egg Homogenates**

The dependence of cGMP-induced Ca\(^{2+}\) release on PKG activity was further tested with (Rp)-8-pCPT-cGMPS, which has been characterized as a selective inhibitor of PKG activity in other cell types (Butt et al., 1994; Meriney et al., 1994). In the following experiments, 5% egg homogenates were preincubated with the inhibitor at the desired concentration for 10 min and subsequently the release of Ca\(^{2+}\) was triggered by the additions of \(\beta\)-NAD\(^+\) and cGMP. Recordings designated as 0 μM were addition of vehicle only, which used the same amount of solvent as in the highest concentration of inhibitor treatment. (Rp)-8-pCPT-cGMPS blocked the cGMP-induced Ca\(^{2+}\) release in a dose-dependent manner with an IC\(_{50}\) of 103.8 μM (Fig. 4). In the presence of 250 μM (Rp)-8-pCPT-cGMPS, the cGMP-induced Ca\(^{2+}\) release was reduced 94.9 ± 7.3% (n = 3). (Rp)-8-pCPT-cGMPS at 250 μM may directly inhibited cADPR-induced Ca\(^{2+}\) release because it slightly inhibited the action of 100 nM cADPR without having any effect on Ca\(^{2+}\) release by 1 μM InsP\(_3\) (Table 1). A more potent inhibitor of cADPR action was spermine, which has been reported to inhibit the ryanodine receptor Ca\(^{2+}\) channels
Figure 4. \((Rp)-8\text{-pCPT-cGMPS}\) blocked cGMP-induced Ca\(^{2+}\) release in egg homogenates. Different concentrations of 0-250 \(\mu\text{M}\) of \((Rp)-8\text{-pCPT-cGMPS}\) were added to the egg homogenate at 10 min before recording. 10 \(\mu\text{M}\) \(\beta\text{-NAD}^{+}\) and 50 \(\mu\text{M}\) cGMP were added at \(t = 50\) s and 100s, respectively. Increasing concentrations of the PKG inhibitor blocked cGMP-induced Ca\(^{2+}\) release. These records are typical of three experiments.
Spermine at 4.8 mM completely blocked Ca\(^{2+}\) release by 100 nM cADPR and also significantly reduced the action of 1 \(\mu\)M InsP\(_3\) (Table 1). Ca\(^{2+}\) release by 100 nM cADPR was insensitive to 1mg/ml heparin (Dargie, et al., 1990), which completely blocked InsP\(_3\)-induced Ca\(^{2+}\) release (Table 1). A possible target of PKG is ADP-ribosyl cyclase, which catalyzes synthesis of cADPR from \(\beta\)-NAD\(^+\) (Lee and Aarhus, 1991). An increase in the cADPR metabolite, ADP-ribose, by the addition of cGMP has been reported in sea urchin egg homogenates (Galione et al., 1993b). This observation implied that the sea urchin ADP-ribosyl cyclase may have similar properties as the bifunctional \(\beta\)-NAD\(^+\) glycohydrolase (NADase), which not only synthesize cADPR from \(\beta\)-NAD\(^+\) but also hydrolyze cADPR to ADP-ribose (Kim et al., 1993). Therefore, bifunctional NADase inhibitors, which include NAD analogs (Yost and Anderson, 1982), may function as blockers of ADP-ribosyl cyclase. We found 3-aminopyridine NAD (3-AP NAD) inhibited cGMP-induced Ca\(^{2+}\) release in a dose-dependent manner (Fig. 5). 3-AP NAD had an IC\(_{50}\) of 115.2 \(\mu\)M and at 200 \(\mu\)M inhibited 95.4 \(\pm\) 8\% \((n = 3)\) of the cGMP-induced Ca\(^{2+}\) release with no effect on Ca\(^{2+}\) release induced by 100 nM cADPR addition (Fig. 5, Table 1). 200 \(\mu\)M 3-aminopyridine, which is a nicotinamide analog and a less potent NADase inhibitor (Yost and Anderson, 1982), did not block the cGMP-induced Ca\(^{2+}\) release.
### TABLE 1
Effects of PKG and NADase Inhibitors on cADPR- and InsP$_3$- Induced Ca$^{2+}$ Release in Sea Urchin Homogenates

<table>
<thead>
<tr>
<th>Condition</th>
<th>N</th>
<th>% of $C_{\text{total}}$</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>100 nM cADPR-induced Ca$^{2+}$ release</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>61.2 ± 5.9</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>250 µM Rp-8-pCPT-cGMPS</td>
<td>3</td>
<td>47.2 ± 3.1**</td>
<td>83.5 ± 2.2**</td>
</tr>
<tr>
<td>200 µM 3-AP NAD</td>
<td>3</td>
<td>55.1 ± 4.2</td>
<td>97.4 ± 5.3</td>
</tr>
<tr>
<td>1 mg/ml heparin</td>
<td>3</td>
<td>61.6 ± 1.8</td>
<td>103.8 ± 3.0</td>
</tr>
<tr>
<td>4.8 mM spermine</td>
<td>3</td>
<td>3.0 ± 0.8**</td>
<td>5.5 ± 0.6**</td>
</tr>
<tr>
<td><strong>1 µM InsP$_3$-induced Ca$^{2+}$ release</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>49.1 ± 3.1</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>250 µM Rp-8-pCPT-cGMPS</td>
<td>3</td>
<td>50.6 ± 3.2</td>
<td>102.7 ± 14.3</td>
</tr>
<tr>
<td>200 µM 3-AP NAD</td>
<td>3</td>
<td>55.1 ± 4.2</td>
<td>104.5 ± 7.1</td>
</tr>
<tr>
<td>1 mg/ml heparin</td>
<td>3</td>
<td>2.9 ± 1.5**</td>
<td>4.8 ± 2.5**</td>
</tr>
<tr>
<td>4.8 mM spermine</td>
<td>3</td>
<td>39.9 ± 2.5*</td>
<td>84.7 ± 5.3*</td>
</tr>
</tbody>
</table>

*Note.* The $C_{\text{total}}$ refers to the change in relative fluorescence induced by 5 µM of Br-A23187, which is defined as 100% $C_{\text{total}}$, at the end of each recording as described in "Material and Methods". Data is presented as mean ± standard deviation. Experimental values are compared to control values for each set. The significance of the difference between means is analyzed using Student's t test (Two-Samples Assuming Unequal Variances), with *"* for $p < 0.05$ and "**" for $p < 0.01$. 
Figure 5. 3-AP NAD blocked the cGMP-induced Ca\textsuperscript{2+} release in egg homogenates. Different concentrations from 0-200 μM of 3-AP NAD were added to the egg homogenates at 10 min before recording. 10 μM β-NAD\textsuperscript{+} and 50 μM cGMP were added at t = 50 s and 100 s, respectively, and increasing concentrations of 3-AP NAD inhibited cGMP-induced Ca\textsuperscript{2+} release. In the presence of 200 μM 3-AP NAD, cGMP failed to release Ca\textsuperscript{2+}; however, the subsequent addition at t= 920 s of 100 nM cADPR triggered Ca\textsuperscript{2+} release that was similar to control. These records are typical of three experiments.
**Ca$$^2+$$ Release by cGMP in Intact Eggs and Inhibition of the Ca$$^2+$$ Transient during Fertilization**

The *in vitro* assays using egg homogenates suggest that cGMP-triggered Ca$$^2+$$ rise occurs through the production of cADPR by sequential activations of PKG and ADP-ribosyl cyclase. We tested if cGMP utilized the same pathway *in vivo* and more importantly, evaluated the contribution of the cGMP-induced Ca$$^2+$$ release to the transient rise in Ca$$^2+$$ during fertilization. The pathway of cGMP-induced Ca$$^2+$$ release in intact eggs was examined by antagonizing PKG and ADP-ribosyl cyclase activities. Blocking either PKG with (Rp)-8-pCPT-cGMPS or ADP-ribosyl cyclase with 3-AP NAD consistently blocked Ca$$^2+$$ release by injection of 10-20 μM cGMP (Table 2). Ca$$^2+$$ release by cGMP was also blocked by the bath addition of 10 mM nicotinamide (NiAm, Table 2), which has been reported to inhibit ADP-ribosyl cyclase in renal epithelial cells (Beers *et al.*, 1995) and sea urchin eggs (Willmott *et al.*, 1996). In order to reduce possible compensation by InsP$_3$-dependent Ca$$^2+$$ release, eggs were also preloaded with 0.5-1.3 mg/ml heparin, which had no effect on the block of cGMP-induced Ca$$^2+$$ release by PKG inhibitors (Fig. 6, Table 2). As shown in figure 6A, an egg was preloaded with ~0.7 mg/ml heparin and ~420 μM (Rp)-8-pCPT-cGMPS, and the injection of ~20 μM cGMP triggered only a slight rise in [Ca$$^2+$$]$_i$ from 110 nM to a peak of 340 nM, which was probably due to the injection procedure. An egg preloaded with ~1 mg/ml heparin and ~100 μM 3-AP NAD had only a slight rise in [Ca$$^2+$$]$_i$ from 110 nM to a peak of 380 nM in response to microinjection of ~25 μM cGMP (Fig. 6B). In figure 6C, a control egg
TABLE 2
Effects of Inhibitors on Sperm- and cGMP-Induced Ca\(^{2+}\) Release in Sea Urchin Eggs

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Heparin mg/ml</th>
<th>Inhibitor mM</th>
<th>Resting [Ca(^{2+})](_i) nM</th>
<th>Latent Period (\mu) sec</th>
<th>(R_{wp}+) (\mu) sec</th>
<th>Peak of [Ca(^{2+})](_i) (\mu)M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sperm-induced Ca(^{2+}) release</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>115 ± 9</td>
<td>12.9 ± 2.7</td>
<td>11.6 ± 3.9</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Heparin</td>
<td>5</td>
<td>0.4-1.3</td>
<td>-</td>
<td>118 ± 13</td>
<td>38.4 ± 7.2</td>
<td>8.8 ± 3.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Rp-8-pCPT-cGMPS</td>
<td>7</td>
<td>-</td>
<td>0.5-0.6</td>
<td>125 ± 27</td>
<td>14.3 ± 3.6</td>
<td>13.0 ± 3.5</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Rp-8-pCPT-cGMPS + heparin</td>
<td>5</td>
<td>0.8-1.2</td>
<td>0.4-0.6</td>
<td>100 ± 8</td>
<td>38.8 ± 6.1</td>
<td>11.2 ± 3.8</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>3-AP NAD</td>
<td>7</td>
<td>-</td>
<td>0.1-0.2</td>
<td>97 ± 7</td>
<td>12.3 ± 3.1</td>
<td>13.6 ± 5.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>3-AP NAD + heparin</td>
<td>5</td>
<td>0.6-0.9</td>
<td>0.1-0.2</td>
<td>95 ± 11</td>
<td>37.2 ± 7.1</td>
<td>12.4 ± 3.7</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>3</td>
<td>-</td>
<td>10</td>
<td>99 ± 4</td>
<td>14.3 ± 3.6</td>
<td>8.3 ± 2.9</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Nicotinamide + heparin</td>
<td>3</td>
<td>0.6-0.7</td>
<td>10</td>
<td>102 ± 3</td>
<td>31.3 ± 4.2</td>
<td>13.3 ± 3.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td><strong>10-20 (\mu)M cGMP-induced Ca(^{2+}) release</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>126 ± 18</td>
<td>-</td>
<td>28.7 ± 15.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Heparin</td>
<td>7</td>
<td>0.4-0.7</td>
<td>-</td>
<td>119 ± 2</td>
<td>-</td>
<td>24.0 ± 8.6</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Rp-8-pCPT-cGMPS</td>
<td>10</td>
<td>-</td>
<td>0.4-0.6</td>
<td>128 ± 18</td>
<td>-</td>
<td>-</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Rp-8-pCPT-cGMPS + heparin</td>
<td>6</td>
<td>0.8-1.0</td>
<td>0.4-0.6</td>
<td>113 ± 17</td>
<td>-</td>
<td>-</td>
<td>0.32 ± 0.22</td>
</tr>
<tr>
<td>3-AP NAD</td>
<td>14</td>
<td>-</td>
<td>0.1-0.2</td>
<td>112 ± 15</td>
<td>-</td>
<td>-</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>3-AP NAD + heparin</td>
<td>5</td>
<td>0.5-1.3</td>
<td>0.1-0.2</td>
<td>119 ± 6</td>
<td>-</td>
<td>-</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>5</td>
<td>-</td>
<td>10</td>
<td>95 ± 9</td>
<td>-</td>
<td>-</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Nicotinamide + heparin</td>
<td>5</td>
<td>0.5-0.7</td>
<td>10</td>
<td>109 ± 17</td>
<td>-</td>
<td>-</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

*Note.* Data is presented as mean ± standard deviation.

\(^{1}\)Time from half of the total rise in [Ca\(^{2+}\)]\(_i\) to the peak of [Ca\(^{2+}\)]\(_i\).


Figure 6. (A) (Rp)-8-pCPT cGMPS blocked cGMP-induced Ca^{2+} release in heparin-loaded eggs, but not the transient rise in [Ca^{2+}], during fertilization. This egg was preloaded with ~0.7 mg/ml heparin and ~420 μM (Rp)-8-pCPT cGMPS. At t = 0, the egg was injected with ~20 μM cGMP, which only caused a brief rise in Ca^{2+} from 110 nM to 340 nM. At t = 3 min, dilute sperm suspension (S) was added to the dish and a rise in [Ca^{2+}], from 128 nM to a peak of 3.2 μM was observed after a delay of ~3 min. (B) 3-AP NAD blocked cGMP-induced Ca^{2+} release in heparin-loaded eggs, but not the transient rise in [Ca^{2+}], during fertilization. This egg was preloaded with ~1 mg/ml heparin and ~100 μM 3-AP NAD. At t = 0, the egg was injected with ~25 μM cGMP, which caused a slight Ca^{2+} release from 110 nM to 380 nM. At t = 6.8 min, dilute sperm suspension (S) was added to the dish and a rise in [Ca^{2+}], from 100 nM to a peak of 1.8 μM was observed after a delay of ~4.5 min. The amount of microinjected cGMP was quantitated between 3-4 min and the recording from 2-6 min was compressed. (C) NiAm blocked cGMP-induced Ca^{2+} release in heparin-loaded eggs, but not the transient rise in [Ca^{2+}], during fertilization. A control egg (egg 1) that was preloaded with ~0.7 mg/ml low MW heparin had a Ca^{2+} transient from 90 nM to 1.55 μM with injection of ~15 μM cGMP. At t = 12 min, 10 mM NiAm was added to the bath and a second egg was preloaded with fura2 and ~0.7 mg/ml heparin. The injection of ~20 μM cGMP did not cause a rise in [Ca^{2+}], which fell from 140 nM to 102 nM. The subsequent addition of dilute sperm suspension (S) induced a transient rise in [Ca^{2+}] to 1.85 μM after a delay of 90 s. The recording from 9 to 18 min was compressed.
(egg 1) that was preloaded with ~0.7 mg/ml heparin has a $[Ca^{2+}]_i$ transient from 90 nM to 1.55 μM with injection of ~15 μM cGMP. 10 mM NiAm was then added to the bath and a second egg was preloaded with fura2 and ~0.7 mg/ml heparin. The subsequent injection of ~20 μM cGMP did not trigger a rise in $[Ca^{2+}]_i$, which fell from 140 nM to 102 nM. The rise time of the $Ca^{2+}$ transient as measured by the time from half of the total rise in $[Ca^{2+}]_i$ to peak fluorescence by microinjection of cGMP is significantly (P < 0.01) slower than that during fertilization (Table 2). Preloading eggs with heparin had no significant effect on the rise time of the $Ca^{2+}$ transient by microinjection of cGMP or fertilization.

Although cGMP-induced $Ca^{2+}$ release in intact eggs was blocked by inhibiting either PKG or NADase activity, a transient rise in $[Ca^{2+}]_i$ was still observed in these eggs during fertilization (Fig. 6). In the egg preloaded with (Rp)-8-pCPT-cGMPS and heparin, the subsequent addition of dilute sperm suspension triggered a rise in $[Ca^{2+}]_i$ from 128 nM to a peak of 3.2 μM after a delay of ~3 min (Fig. 6A). Similarly the eggs that had been preloaded with 3-AP NAD or NiAm in the presence of heparin had a transient peak $[Ca^{2+}]_i$ of 1.8 μM after a delay of ~4.5 min (Fig. 6B) and 1.85 μM after a delay of ~1.5 min (Fig. 6C), respectively. The increase in latent period or delay in the onset of the $Ca^{2+}$ transient during fertilization is characteristic of eggs preloaded with heparin (Crossley et al., 1991; Mohri et al., 1995). Since these eggs were also preloaded with heparin to prevent compensatory $Ca^{2+}$ release by InsP₃, the presence of a $Ca^{2+}$ transient during fertilization suggests the cGMP-mediated pathway is not required for $Ca^{2+}$ release.
In these experiments the prior injection of cGMP may have compromised the action of the cGMP pathway inhibitors and a second set of experiments were conducted. The effects of inhibitors on sperm-induced Ca\(^{2+}\) release were determined separately from the microinjection of cGMP and are summarized in Table 2. The pretreatment of unfertilized eggs with cGMP pathway inhibitors alone or in combination with heparin did not block the Ca\(^{2+}\) transient during fertilization. The cGMP pathway inhibitors had no significant effect on the latent period, unless they were tested in combination with heparin, nor did the inhibitors significantly affect the rate of rise in \([\text{Ca}^{2+}]_i\) for sperm-induced Ca\(^{2+}\) release. In some cases the inhibitors did slightly reduce the peak \([\text{Ca}^{2+}]_i\) of the response. Preloading eggs with 3-AP NAD alone reduced (P < 0.05) the peak response during fertilization. The combination of heparin and the cGMP pathway inhibitors also reduced (P < 0.01) the peak \([\text{Ca}^{2+}]_i\) (Table 2). A typical experiment series is illustrated in figure 7, where "S" marks the small increase in \([\text{Ca}^{2+}]_i\) at the beginning of the latent period (Whitaker et al., 1989). An egg preloaded with ~200 \(\mu\text{M}\) 3-AP NAD had a similar Ca\(^{2+}\) transient during fertilization to that of a control egg with peak \([\text{Ca}^{2+}]_i\) of 1.49 \(\mu\text{M}\) and 1.63 \(\mu\text{M}\), respectively. The latent period of eggs preloaded with heparin increased to > 40 s from a latent period of 13 s for the control fertilization reaction. Otherwise the kinetics of sperm-induced Ca\(^{2+}\) release was unchanged by preloading ~0.8 mg/ml heparin. The combination of ~190 \(\mu\text{M}\) 3-AP NAD with ~0.9 mg/ml heparin also did slightly reduce the peak \([\text{Ca}^{2+}]_i\) of the fertilization response to 1.24 \(\mu\text{M}\).
A Ca\(^{2+}\) response during fertilization occurred in eggs, which were preloaded with heparin and/or 3-AP NAD. In these sample recordings, unfertilized eggs were preloaded with heparin (H) and/or 3-AP NAD, then fertilized. Although the presence of inhibitors had effects on the kinetics of Ca\(^{2+}\) release, which are summarized in Table 2 and the text, a transient rise in [Ca\(^{2+}\)]\(_i\) was always observed subsequent to the addition of sperm. The individual recordings were aligned with \(t = 0\) when the small increase in [Ca\(^{2+}\)]\(_i\) at the beginning of the latent period was observed and marked with "S".
DISCUSSION

In this study we have provided further evidence for the involvements of cGMP-dependent protein kinase and ADP-ribosyl cyclase in the cGMP-induced Ca\(^{2+}\) release in sea urchin homogenates and extended the hypothesis (Galione et al., 1993b) to intact eggs. In the intact eggs, inhibitors of PKG and NADase activities blocked cGMP-induced Ca\(^{2+}\) release. Since the mechanism of sperm-induced egg activation remains unclear (Shen, 1995), we examined the possible participation of the cGMP-activated Ca\(^{2+}\) release pathway in the transient rise in [Ca\(^{2+}\)]\(_i\) during fertilization. We used low molecular weight heparin, which has no effect on cGMP-dependent (Whalley et al., 1992) or cADPR-induced (Dargie et al., 1990) Ca\(^{2+}\) release, to reduce InsP\(_3\) activity (Rakow and Shen, 1990; Crossley et al., 1991). In all eggs treated with either PKG or NADase inhibitors, which had (Fig. 6) or should have had (Table 2) no significant Ca\(^{2+}\) response to injection of cGMP, a delayed transient rise in [Ca\(^{2+}\)]\(_i\) during subsequent fertilization was still observed. Our observations are inconsistent with the proposal of dual redundant InsP\(_3\)- and cADPR-dependent Ca\(^{2+}\) release mechanisms during fertilization (Galione et al., 1993a; Lee et al., 1993). Instead these results are consistent with the idea that the primary required Ca\(^{2+}\) release during fertilization is InsP\(_3\)-dependent (Mohri et al., 1995).

The idea of InsP\(_3\)-insensitive Ca\(^{2+}\) release during fertilization was proposed due to observations of a rise in [Ca\(^{2+}\)]\(_i\) during fertilization in heparin-loaded eggs (Rakow and Shen, 1990; Crossley et al., 1991). This conclusion was based on the observations that
injections of 0.1 to 0.3 mg/ml heparin (final intracellular concentration) blocked Ca\(^{2+}\) release by injected InsP\(_3\) and GTP\(_{\gamma}\)S. The latter has been shown to activate endogenous InsP\(_3\) production (Crossley \textit{et al.}, 1991). Subsequent findings concerning the ryanodine-sensitive Ca\(^{2+}\) release mechanism that is cADPR-activated (Galione and White, 1994; Shen, 1995) further supported the idea of InsP\(_3\)-independent Ca\(^{2+}\) release. A recent report (Mohri \textit{et al.}, 1995) has suggested that InsP\(_3\)-dependent Ca\(^{2+}\) release is the primary prerequisite pathway during fertilization because higher levels of heparin to 1 mg/ml causes a concentration-dependent inhibition of the sperm-induced rise in [Ca\(^{2+}\)], such that high levels of heparin can entirely suppress Ca\(^{2+}\) release during monospermic fertilization. In this study we used 0.7-1.0 mg/ml heparin to reduce InsP\(_3\)-dependent Ca\(^{2+}\) release during fertilization. This level of heparin did not affect Ca\(^{2+}\) release by microinjection of either cGMP or cADPR. Several possible explanations may account for our observations that the additional presence of PKG or NADase inhibitors did not prevent the rise in [Ca\(^{2+}\)]\(_i\) in these heparin-loaded eggs during fertilization. The most direct explanation is that InsP\(_3\)-mediated Ca\(^{2+}\) release is indeed the primary prerequisite mechanism underlying generation of the rise in [Ca\(^{2+}\)]\(_i\) during fertilization and at 0.7-1.0 mg/ml heparin, the InsP\(_3\)-dependent Ca\(^{2+}\) transient was delayed but not fully suppressed (Mohri \textit{et al.}, 1995). Another possibility is that another heparin-insensitive Ca\(^{2+}\) release pathway is utilized during fertilization. In sea urchin eggs a new mechanism for Ca\(^{2+}\) release by nicotinic acid adenine dinucleotide phosphate (NAADP) has been reported (Lee and Aarhus, 1995; Chini \textit{et al.}, 1995a; Perez-Terzic \textit{et al.}, 1995). Ca\(^{2+}\) release by NAADP is insensitive to
heparin and antagonists of cADPR-dependent Ca\(^{2+}\) release (Chini et al., 1995b; Graeff et al., 1995). Thirdly, it may also be possible that compartmentation of the Ca\(^{2+}\) mobilizing agents or inhibitors might occur, such that local concentrations of natural Ca\(^{2+}\) release agonists might be sufficient during fertilization. This possibility is particularly intriguing because it otherwise appears that the generation of cADPR is not required during fertilization, since neither 3-AP NAD or NiAm inhibited the Ca\(^{2+}\) response during fertilization (Table 2). Although the effect of 3-AP NAD on cADPR production in the sea urchin egg has not been directly measured, we have found that 10 mM NiAm also blocked cGMP-induced Ca\(^{2+}\) release in intact eggs without inhibiting the sperm-induced rise in [Ca\(^{2+}\)]\(_i\) in heparin-loaded eggs. NiAm was recently reported to be a potent inhibitor of cADPR production in opossum kidney renal epithelial cells (Beers et al., 1995) and sea urchin homogenates (Willmott et al., 1996). Furthermore, we have shown that 3-AP NAD blocked cGMP-induced Ca\(^{2+}\) release without inhibiting the direct action of cADPR (Table 1). Surprisingly, the presence of 10 mM NiAm did not appear to perturb early embryogenesis of the sea urchin to gastrulation.

This study also provides some additional information about PKG-regulated Ca\(^{2+}\) release in sea urchin eggs. Activation of PKG was suggested to mediate Ca\(^{2+}\) release by cGMP; however, the effective cGMP concentrations of 10-20 \(\mu\)M in intact eggs (Whalley et al., 1992) and 50 \(\mu\)M in \(\beta\)-NAD\(^+\) supplemented egg homogenates (Galione et al., 1993) were much higher than the \(K_a\) values of 110 nM for type I and 250 nM for type I to activate the mammalian PKG isoenzymes (Sekhar et al., 1992). We have observed that
several cGMP analogs, which are 10-25-fold more potent mammalian PKG activators, generally failed, but the less potent cIMP (Francis et al., 1988) triggered Ca\textsuperscript{2+} release in sea urchin egg homogenates and intact eggs. Ca\textsuperscript{2+} release by cIMP was inhibited by antagonists of PKG and ADP-ribosyl cyclase (data not shown). It does not appear that cIMP functions by blocking cGMP-PDE (Thomas et al., 1992), since a variety of phosphodiesterase inhibitors did not cause Ca\textsuperscript{2+} release nor affect cGMP-induced Ca\textsuperscript{2+} release. Preliminary affinity chromatography and immunohistochemistry suggest the PKG present in sea urchin eggs is unlike mammalian isoforms. Different forms of PKG have been identified in several nonmammalian species (Francis and Corbin, 1994). Significantly higher concentrations of (Rp)-8-pCPT-cGMPS was also necessary to block cGMP-induced Ca\textsuperscript{2+} release in homogenates and intact eggs. A less specific inhibitor, Rp-cGMPS, was reported to directly activate sea urchin eggs (Ciapa and Epel, 1996).

A postulated target of PKG activity in the sea urchin egg is the cADPR synthesizing enzyme, ADP-ribosyl cyclase (Galione et al., 1993b). Since the cGMP-induced Ca\textsuperscript{2+} release was blocked by the snake venom NADase inhibitor, 3-AP NAD (Yost and Anderson, 1982), the sea urchin egg ADP-ribosyl cyclase may be a bifunctional enzyme like the canine spleen NADase (Kim et al., 1993) and human lymphocyte CD38 (Howard et al., 1993), which contains both ADP-ribosyl cyclase and cADPR hydrolase activities. More importantly, the block of cGMP-induced Ca\textsuperscript{2+} release by 3-AP NAD suggested that PKG may activate ADP-ribosyl cyclase to convert β-NAD\textsuperscript{+} to cADPR. The direct proof of PKG regulation of ADP-ribosyl cyclase activity requires in vivo experiments to show
that ADP-ribosyl cyclase can be phosphorylated by PKG. An *Aplysia* egg-specific NADase has been cloned and two potential phosphorylation sites, where basic residues are amino terminal to a serine (Glick *et al.*, 1991) have been identified. One of the serine residues along with a carboxyl terminal phenylalanine residue has been shown to be conserve among rat, mouse, human and *Aplysia* NADase (Koguma *et al.*, 1994). A carboxyl terminal phenylalanine in a phosphorylation site sequence may provide preferential phosphorylation by PKG (Francis and Corbin, 1994).

The Ca$^{2+}$ response during fertilization in *Xenopus* (Nuccitelli *et al.*, 1993) and hamster (Miyazaki *et al.*, 1992, 1993) eggs has been established to be only dependent on an InsP$_3$-regulated mechanism. Despite the presence of multiple pathways of Ca$^{2+}$ release in sea urchin eggs, this report and the earlier report of Mohri *et al.* (1995) suggest that the sperm-induced Ca$^{2+}$ release in sea urchin eggs may also be primarily dependent on the InsP$_3$-regulated Ca$^{2+}$ release channel. The significance of other Ca$^{2+}$ release mechanisms in sea urchin eggs remains to be elucidated.

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CHAPTER II. THE CALCIUM TRANSIENT IN SEA URCHIN EGGS DURING FERTILIZATION REQUIRES THE PRODUCTION OF INOSITOL 1,4,5-TRISPHOSPHATE

A paper accepted by Developmental Biology

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ABSTRACT

The production of inositol 1,4,5-trisphosphate (InsP\(_3\)) has been reported to mediate the transient rise in intracellular Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)) in sea urchin eggs during fertilization. However, direct evidence of an absolute requirement for generation of InsP\(_3\) during fertilization is still lacking. We investigated this question by blocking the InsP\(_3\) synthesizing enzyme, phospholipase C (PLC) during fertilization with U73122, an aminosteroid. U73122 inhibited the sperm-induced Ca\(^{2+}\) release in a dose-dependent manner; although, above 15 \(\mu\)M U73122 eggs showed an elevated resting [Ca\(^{2+}\)]\(_i\) and a lower fertilization rate. The inhibition of Ca\(^{2+}\) transient by U73122 was not due to a failure of fertilization, since incorporated sperm nuclei were evident in eggs used to measure the Ca\(^{2+}\) response. U73122 also prevented the accompanying rise in intracellular pH (pH\(_i\)), which is mediated by the activation of the Na\(^{+}\)-H\(^{+}\) antiporter. The antiporter is regulated through activation of protein kinase C by 1,2-diacylglycerol, which is the other hydrolytic product of phosphatidylinositol-4,5-bisphosphate.

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by PLC. Further evidence of the specificity of U73122 action was inhibition of the increase in InsP₃ mass during the first 2 min of fertilization. In addition, U73122 also inhibited the GTPγS-induced Ca²⁺ release and pHᵢ rise in unfertilized eggs. These results suggested that the transient rise in Ca²⁺ in sea urchin during fertilization requires the production of InsP₃.

**INTRODUCTION**

A transient rise in intracellular Ca²⁺ activity ([Ca²⁺]ᵢ) occurs during fertilization of both vertebrate and invertebrate eggs. In sea urchins the [Ca²⁺]ᵢ transient is sufficient and necessary for activation of the metabolically quiescent egg (Whitaker and Steinhardt, 1985). Despite its significance, the initiation and subsequent regulation of this transient rise in [Ca²⁺]ᵢ are not fully understood. Inositol 1,4,5-trisphosphate (InsP₃) is a well known second messenger, which can trigger release of Ca²⁺ from internal stores in a variety of cell types (Berridge, 1993). It has been demonstrated that InsP₃-mediated pathway is the primary mechanism for sperm-induced Ca²⁺ release in *Xenopus* (Nuccitelli et al., 1993) and hamster eggs (Miyazaki et al., 1992). In sea urchin eggs two types of intracellular Ca²⁺ release channels, including the InsP₃ receptor (Parys et al., 1994) and the ryanodine receptor (McPherson et al., 1992) have been found. It has been hypothesized that the two types of Ca²⁺ release channels are redundant to ensure the occurrence of the transient rise in [Ca²⁺]ᵢ during fertilization (Galione et al., 1993; Lee et al., 1993). However, Mohri et al., (1995) observed a concentration-dependent inhibition by heparin or pentosan polysulfates, InsP₃ receptor antagonists, on the kinetics and amplitude of sperm-induced Ca²⁺ release. In addition, we have reported that the
ryanodine-mediated pathway is not essential during fertilization by blocking the pathway with a variety of inhibitors against key components of the pathway (Lee et al., 1996). Both studies suggested that the sperm-induced Ca\(^{2+}\) release is primarily regulated by InsP\(_3\) in sea urchin eggs as in other animal egg systems. Since antagonists of Ca\(^{2+}\) release mechanisms have pleiotropic effects on other cellular functions (Shen, 1995), we took an alternative approach to examine the requirement of InsP\(_3\)-mediated pathway during fertilization by blocking the InsP\(_3\) synthesizing enzyme, phospholipase C (PLC).

U73122, an aminosteroid, has been demonstrated to be a potent PLC inhibitor in a variety of cell systems. Thrombin-induced InsP\(_3\) production and subsequent rise in [Ca\(^{2+}\)]\(_i\) were inhibited by U73122 in human platelets (Bleasdale et al., 1990). U73122 also blocked the N-formyl-methionyl-leucyl-phenylalanine-induced aggregation of human polymorphonuclear neutrophiles and the associated production of InsP\(_3\) and 1,2-diacylglycerol (DAG, Bleasdale et al., 1990). The cholecystokinin or carbacol-induced phosphatidylinositol (PI) turnover was dose-dependently inhibited by U73122 in rat pancreatic acinar cells (Yule et al., 1992). Thompson et al., (1991) have shown that U73122 prevented the sequestration of muscarinic acetylcholine receptors by oxotremorine-M along with the associated production of InsP\(_3\) and rise in [Ca\(^{2+}\)]\(_i\) in SK-N-SH neuroblastoma cells. In addition, they demonstrated that U73122 markedly reduced the guanosine-5'-O-(3-thiotriphosphate) (GTP\(_\gamma\)S)-induced InsP\(_3\) generation, which indicated that U73122 inhibited PLC\(_\beta\)-dependent PI turnover.

U73122 did not affect either the \(\beta_2\)-integrin-dependent tyrosine phosphorylation of PLC\(_\gamma2\) or formation of InsP\(_3\) in human neutrophiles, which implied that PLC\(_\gamma\) activity is independent of U73122 inhibition (Hellberg et al., 1996). These results suggested that U73122 may block
the hydrolysis of PI and subsequent responses by inhibiting the Gqα subunit-regulated PLCβ (Smrcka et al., 1991; Yule et al., 1992). However, it has been reported that U73122 may also affect the activity of PLCγ as well as PLCβ. U73122 diminished both the epidermal growth factor (EGF)-induced motility and PLC responses via PLCγ in NR6 cells expressed with EGF receptors (Chen et al., 1994; Chen et al., 1996). Both U73122 and its biological inactive analog, U73343, have also been reported to affect the tyrosine kinase activity in human platelets (Heemskerk et al., 1997).

Questions have been raised against the specificity of U73122 as a selective PLC inhibitor. A number of U73122 actions besides the inhibition of PLC have been reported, including the inhibition of ATP-dependent Ca^{2+} uptake and stimulation of Ca^{2+} oscillation in rabbit pancreatic acinar cells (Willems et al., 1994), the release of actively stored Ca^{2+} primarily via the inhibition of internal Ca^{2+} pump in rat liver microsomes (de Moel et al., 1995), the attenuation of InsP3-induced Ca^{2+} release, which was unlike the thapsigargin-like effect, in human neutrophils (Hellberg et al., 1996), and the partial blockage of sperm-egg fusion in mouse oocytes (Dupont et al., 1996). Due to these nonspecific features of U73122, one needs to be cautious when using U73122 to antagonize the activity of PLC. The inhibition of PLC by U73122 have been shown to be significantly abolished in U73343 by substituting a less electrophilic succinimide group in place of the maleimide group in U73122 (Bleasdale et al., 1990). U73343 becomes a biological inactive analog of U73122 which can be used as a control agent. We investigated the effects of U73122 on the sperm-induced Ca^{2+} release and its upstream signaling pathway, the production of InsP3 and DAG. The possible nonspecific effects of U73122 on Ca^{2+} homeostasis and fertilization in sea urchin eggs were
also examined. We report here that the production of InsP3 and the Ca^{2+} release during fertilization were blocked by U73122 at a concentration which had minimal nonspecific effects. This suggested an absolute requirement for InsP3 production to generate the transient rise in [Ca^{2+}]_{i} in sea urchin eggs during fertilization. Portions of the data presented here have been published previously in abstract form (Lee and Shen, 1996).

**MATERIALS AND METHODS**

**Preparation of Gametes and Solutions**

The sea urchin *Lytechinus pictus* were purchased from Marinus, Inc. (Long Beach, CA), and were maintained in Instant Ocean culture with biweekly feeding of *Macrocystis*. Eggs and sperm of the sea urchin were obtained by injection of 0.5 M KCl into the coelomic cavity. Eggs were shed into artificial sea water (ASW) of the following composition: 470 mM NaCl, 10 mM KCl, 11 mM CaCl2, 29 mM MgSO4, 27 mM MgCl2, 5 mM NaHCO3, pH 8. The jelly coats were removed from the eggs by several passages through fine-mesh silk and the eggs were allowed to settle through several ASW washes. The first supernatant was collected and used as the ASW containing egg jelly for inducing sperm acrosome reaction. Dejellied eggs were kept at 16-18°C in suspension by constant stirring at 60 rpm until use. The sperm were kept dry at 4°C. For insemination, sperm acrosome reaction was triggered by a 1:100 dilution in the ASW containing egg jelly and then diluted sperm were added to the eggs to make the final dilution of 1:15,000 for all experiments unless specified. 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-
yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and 1-[6-[[17β-3-methoxyestra-1,3,5(10)-
trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione (U73343) were obtained from BIOMOL
(Plymouth Meeting, PA), dissolved in chloroform at 5 mM, distributed into small aliquots
and evaporated until a thin film was left on the tube wall. U73122 and U73343 were
stored at -20°C and reconstituted to 5 mM in dimethyl sulfoxide prior to use. Stock
solutions of all other chemicals were prepared in intracellular buffer (220 mM K acetate,
500 mM glycine, 40 mM NaCl, 100 μM EGTA and 20 mM Tris, pH 6.9). Fura-2,
BCECF-dextran (M₉, 40,000) and InsP₃ were purchased from Molecular Probes (Eugene,
OR). InsP₃ assay kit and cyclic ADPR (cADPR) were obtained from Amersham
(Arlington Heights, IL). All other chemicals were purchased from Sigma Chemical Co.
(St. Louis, MO).

Microinjection Procedures and Monitoring of [Ca²⁺], and pHᵢ

Microelectrodes for microinjection were pulled on a Narishige PE-2 puller
(Narishige, Japan) from 1 mm (o.d.) x 0.8 mm (i.d.) Pyrex glass tubings (Drummond
Scientific, Broomall, PA), heated to 200°C and silane-treated by exposing to
hexamethyldisilizane vapors. The electrode tips were filled with intracellular buffer
containing one or more of the following: 10 mM fura-2, 250 mg/mL heparin and 10
mg/mL BCECF-dextran (M₉, 40,000). Microelectrode procedures and fluorimetric
monitoring of [Ca²⁺], (Buck et al., 1994) or pHᵢ (Shen and Buck, 1990) were similar to
that previously published. Injection volume was estimated using dye dilution (Lee, 1989)
by measuring the fluorescent emission of fura-2 or BCECF-dextran at 535 nm to excitation
at 380 or 440 nm, respectively, and comparing the fluorescence intensity to a dilution standard in intracellular buffer with 100 nM free Ca^{2+} and pH 6.9. The total injected volume was less than 1.5% of the egg volume. \([\text{Ca}^{2+}]_i\) was continuously monitored by the 340/380 fluorescence ratio. The 340/380 fluorescence ratio was collected at 2 Hz and calibrated by determining \(R_{\text{min}}\) and \(R_{\text{max}}\) in fura-2-loaded eggs (Gryniewicz et al., 1985). Intracellular pH was monitored by the change in 490/440 fluorescence ratio at 2 Hz in BCECF-loaded eggs. All recordings were made at 18-21°C and experimental data are expressed as mean ± SD.

**Sperm-Egg Fusion Assay**

Eggs in a 0.5% egg suspension were incubated with 1 mg/mL Hoechst No. 33342 for 10 min. The unincorporated dye was removed by 3x washes of 50 mL ASW. Eggs were inseminated and fixed with 1% glutaraldehyde at 10 min post fertilization. For population studies, eggs were examined at once by fixing eggs between two cover slides. For the single cell recordings the observation could not be made until after the release of fura-2 from egg cytoplasm by fixation with 1% glutaraldehyde, which took about 3-5 min. Eggs were examined under a 40x fluor objective with a 400 nm dichroic mirror, at 340 nm excitation and 510 nm emission filters in the absence of any neutral density filter. Incorporated sperm were identified by the fluorescent sperm nuclei.
**InsP₃ Mass Assay**

Eggs in a 1% egg suspension were mixed with an equal amount of Ca²⁺ and Mg²⁺-free ASW consisting of 545 mM NaCl, 11.4 mM KCl and 6 mM NaHCO₃, pH 8 (Mohri *et al.*, 1994), to make a 0.5% egg suspension in a solution with only half of the Ca²⁺ and Mg²⁺ contents in normal ASW (1/2CaMgASW). Eggs were then incubated with or without 20 μM U73122 in a 0.5% egg suspension for 5 min and reconstituted to 7.5% before insemination. InsP₃ extraction and mass assay procedures were modified from Osawa *et al.*, 1997. Acrosome-reacted sperm were added to the egg suspension (1:1500 dilution) at 10-15 min post addition of U73122 and mixed thoroughly by swirling. 200 μL of sperm and eggs mixture was added to 100 μL ice-cold 11% phosphoric acid containing 120 mM LiCl. Samples were incubated on ice for at least 15 min and then centrifuged at 8000 xg for 15 min at 4°C. Egg pellets were dissolved by 0.15 N NaOH and the protein contents were determined using the Bio-Rad protein assay (BIO-RAD, CA). Supernatants were collected and neutralized by 2 N KOH with 200 mM Hepes, 40 mM EDTA and 10 mM EGTA. KClO₄ was removed by centrifugation at 8000 xg for 15 min at 4°C. Supernatants were incubated on ice prior to InsP₃ mass assay. InsP₃ mass was measured using a commercial InsP₃ assay kit (Amersham, IL). Samples were incubated with InsP₃ binding protein and [³H]InsP₃ on ice for 15 min and then centrifuged at 16,000 xg for 20 min. Supernatants were decanted and pellets were dissolved by 100 μL 0.15 N NaOH. The radioactivity associated with the pellets was determined by liquid scintillation. The amount of InsP₃ in each sample was determined by comparing the extent of the inhibition of [³H]InsP₃ binding with known amount of authentic InsP₃, which was prepared in 1/2CaMgASW.
RESULTS

Block of Sperm-Induced $Ca^{2+}$ Release by U73122

U73122 inhibited the $Ca^{2+}$ release during fertilization in a dose-dependent manner. As shown in these representative recordings (Fig. 1), the 5 $\mu$M U73122-treated egg had similar $Ca^{2+}$ responses as that in the untreated (designated 0 $\mu$M) eggs. In this case, the peak amplitude of sperm-induced $Ca^{2+}$ release was reduced (1.79 $\mu$M) at 10 $\mu$M U73122 when compared to the control fertilization response (2.8 $\mu$M), although in general the reduction was not statistically significant (Table 1). The peak amplitude of sperm-induced $Ca^{2+}$ release was significantly decreased at 15 $\mu$M U73122 and the rate of rise was noticeably slower. No significant rise in $[Ca^{2+}]_i$ during fertilization was observed at 20 $\mu$M U73122; however, an elevated resting $[Ca^{2+}]_i$ in this egg of 375 nM was recorded and it rose gradually to 480 nM until the end of recording. U73343, the biological inactive analog of U73122, had no effect on the rise in $[Ca^{2+}]_i$ during fertilization at 40 $\mu$M (dotted line, Fig. 1). The change in $[Ca^{2+}]_i$ during fertilization for U73122 or U73343-treated eggs is summarized in Table 1. The dosage of U73122 for a near complete inhibition of sperm-induced $Ca^{2+}$ release ranged from 15 to 40 $\mu$M, which varied among batches of eggs. Above 15 $\mu$M U73122 eggs showed an elevated resting $[Ca^{2+}]_i$ (Table 1). At a concentration higher than 25 $\mu$M, some eggs exhibited not only an elevated $[Ca^{2+}]_i$, but also a sudden rise in $[Ca^{2+}]_i$ and eventual lysis (data not shown). The data collected from those eggs were excluded from this study. No attempt was made to use U73122 at a concentration higher than 25 $\mu$M in further experiments. In contrast, U73343 had no effect on the sperm-induced $Ca^{2+}$ release up to 40 $\mu$M (only data from 40 $\mu$M
Fig. 1. U73122 blocked the sperm-induced Ca$^{2+}$ release in a dose-dependent manner. Eggs were pretreated with the designated concentration (in μM) of U73122 or 40 μM U73343 (dotted line) for 10-15 min and inseminated (1:15000 dilution) with acrosome-reacted sperm (S). U73122 inhibited the rise in [Ca$^{2+}$]$_i$ in a dose-dependent manner and a complete inhibition was observed at 20 μM. In contrast, U73343 had no significant effect on the sperm-induced Ca$^{2+}$ release. These recordings are representatives of at least 3 experiments for each treatment.
TABLE 1
Effects of U73122 and U73343 on the Sperm-Induced Ca\(^{2+}\) Release in Sea Urchin Eggs

<table>
<thead>
<tr>
<th>[Drug], μM</th>
<th>N</th>
<th>Resting ([\text{Ca}^{2+}]_i) nM</th>
<th>Peak of ([\text{Ca}^{2+}]_i) μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>132 ± 50</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>99 ± 32</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>120 ± 16</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>10 + 1 mg/ml heparin</td>
<td>4</td>
<td>107 ± 10</td>
<td>0.15 ± 0.3**</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>196 ± 122</td>
<td>1.0 ± 0.7*</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>250 ± 135</td>
<td>0.4 ± 0.3**</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>324 ± 80</td>
<td>0.5 ± 0.2**</td>
</tr>
<tr>
<td>10 + 1 mg/ml heparin</td>
<td>3</td>
<td>119 ± 10</td>
<td>1.8 ± 0.1**</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>163 ± 34</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

*Note.* All eggs were fertilized with at least one sperm pronucleus detected by the Hoechst dye sperm-egg fusion assay after recording the Ca\(^{2+}\) response. Data is presented as mean ± standard deviation. Experimental values are compared to control values for each set. The significance of the difference between means is analyzed using Student's t test with "**" for \(p < 0.05\) and "***" for \(p < 0.01\).
U73343 treatments are shown in Fig. 1 and Table 1). A small increase in [Ca\(^{2+}\)](i) (step) before the massive rise in [Ca\(^{2+}\)](i) is often used as a time marker of the beginning of sperm-egg fusion (Whitaker et al., 1989). However, the step was not consistently seen in recordings of U73122-treated eggs, which might be due to the block of L-type Ca\(^{2+}\) channel by U73122 (Macrez-Lepretre et al., 1996). Since no step was routinely observed in U73122-treated eggs, it is difficult to precisely measure the latent period between the sperm-egg fusion and the massive rise in [Ca\(^{2+}\)](i). Thus we cannot accurately determine by photomultiplier tube-spectrofluorometry if U73122 affected the latent period.

It was necessary to verify that the inhibition of the sperm-induced Ca\(^{2+}\) release in U73122-treated eggs was not due to a failure of fertilization. The Hoechst dye sperm-egg fusion assay was performed to determine that all of the eggs monitored in Table 1 were fertilized by at least one sperm. A control, 10 μM and 20 μM U73122-treated eggs are shown in Fig 2. In 20 μM U73122-treated eggs without a Ca\(^{2+}\) response during fertilization, the sperm nuclei remained condensed (Fig. 2C) at 10 min post insemination, a sufficient time for sperm nucleus decondensation to occur in 10 μM U73122-treated eggs (Fig. 2B) and control eggs (Fig. 2A).

We noticed at concentrations of U73122 above 15 μM a greater incidence of eggs without a sperm nucleus. Thus the effects of U73122 on fertilization were investigated on a population basis. The effect of U73122 on the fertilization envelope (FE) formation was examined as a criterion for the cortical reaction in response to Ca\(^{2+}\) release. Eggs in a 0.5% egg suspension were treated with different amount of U73122 for 10 min and inseminated in the presence of U73122 unless indicated. Eggs were scored for the elevated FE at 5 min after
Fig. 2. U73122 did not block sperm-egg fusion. Eggs were fixed at 10 min post insemination with 1 % glutaraldehyde. After the release of most of the fura-2 from the egg cytoplasm, the Hoechst dye stain became evident. (A) The female pronucleus (F) and a decondensed sperm pronucleus (M) were evident in a control egg. (B) A decondensed sperm pronucleus (M) was evident in a 10 μM U73122-treated egg. The female pronucleus (at 6:30 o’clock) and two other sperm pronuclei (at ~ 1 and 9 o’clock) were also present in this egg, but they were at a different focal plane. (C) A condensed sperm nucleus was seen in a 20 μM U73122-treated egg. The female pronuclei was at a different focal plane.
insemination. U73122 significantly reduced the % FE formation at concentrations ≥ 10 μM (Table 2) and FE formation was completely blocked by 20 μM.

The inhibition of FE formation might be due to the block of cortical reaction or sperm-egg fusion. To further investigate the blockage of FE formation, the % of fertilization of U73122-treated eggs was analyzed using the Hoechst dye sperm-egg fusion assay as judged by the incorporation of Hoechst dye into the sperm nuclei. U73122 significantly (p < 0.05) inhibited fertilization above 15 μM (Table 2). Polyspermy was frequently observed in the U73122-treated eggs presumably due to the lack of FE to prevent the entry of supernumerary sperm. The % of polyspermy was 34.7 ± 13.2 (n = 4, p < 0.05) and 37.5 ± 11.0 % (n = 4, p < 0.01) for eggs treated with 15 and 20 μM U73122, respectively (Table 2). The % of polyspermy was highest at 10 μM where the block of FE became significant, but a high level of fertilization was still observed. At less than 10 μM U73122, the % FE formation was higher, which reduced the % of polyspermy. At higher than 20 μM of U73122, sperm-egg fusion was significantly blocked, which resulted in a lower % of polyspermy. Similarly the number of incorporated sperm per fertilized egg was significantly higher at 10-20 μM (Table 2).

To examine the specificity of the inhibition on FE formation and sperm incorporation by U73122, the effects of U73343 were also studied. U73343 inhibited the FE formation at a higher concentration (≥ 15 μM), but to a lesser extent (Table 2). At the highest concentration (40 μM) tested, the FE formation was reduced to 17.3 ± 22.7 % (n = 4, p < 0.05). These results suggested that the block of FE formation by U73122 might be due to the general effect of aminosteroids. At a higher concentration (≥ 15 μM), U73343 also reduced the fertilization
### TABLE 2
Effects of U73122 and U73343 on the Sperm-Induced Ca^{2+} Release in Sea Urchin Eggs

<table>
<thead>
<tr>
<th>[Drug], μM</th>
<th>N</th>
<th>Resting [Ca^{2+}]_i</th>
<th>Latent Period sec</th>
<th>R_{1/2}^† sec</th>
<th>Peak of [Ca^{2+}]_i</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>U73122</td>
<td>5</td>
<td>132 ± 50</td>
<td>12.6 ± 3.0</td>
<td>12.4 ± 3.6</td>
<td>2.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>99 ± 32</td>
<td>10.7 ± 2.3</td>
<td>11.0 ± 6.9</td>
<td>2.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>120 ± 16</td>
<td>-</td>
<td>7.3 ± 0.6*</td>
<td>2.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>10 + 1 mg/ml heparin</td>
<td>4</td>
<td>107 ± 10</td>
<td>-</td>
<td>-</td>
<td>0.15 ± 0.3**</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>196 ± 122</td>
<td>-</td>
<td>-</td>
<td>1.0 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>250 ± 135</td>
<td>-</td>
<td>-</td>
<td>0.4 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>6</td>
<td>349 ± 204*</td>
<td>-</td>
<td>-</td>
<td>0.6 ± 0.5*</td>
<td></td>
</tr>
<tr>
<td>U73343</td>
<td>10 + 1 mg/ml heparin</td>
<td>2</td>
<td>119 ± 14</td>
<td>-</td>
<td>-</td>
<td>1.6 ± 1.8*</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
<td>163 ± 34</td>
<td>8.6 ± 3.4</td>
<td>13.2 ± 4.0</td>
<td>2.6 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Note. Data is presented as mean ± standard deviation. Experimental values are compared to control values for each set. The significance of the difference between means is analyzed using Student's t test with "*" for p < 0.05 and "**" for p < 0.01.

†Time from half of the total rise in [Ca^{2+}]_i to the peak of [Ca^{2+}]_i.
rate and increase the degree of polyspermy, but they were not statistically significant (p > 0.05) from the untreated control experiments.

The apparent nonspecific effects of high concentrations of U73122 on sperm incorporation and intracellular Ca\(^{2+}\) homeostasis are of concern. U73122 at 10 \(\mu\)M had no effect on the resting [Ca\(^{2+}\)]\(_i\) and only modest effects on sperm-induced Ca\(^{2+}\) release (Table 1) and fertilization rate (Table 2). Heparin, an InsP\(_3\) receptor antagonist (Ghosh et al., 1988), at 1 mg/mL, only partially blocked the rise in [Ca\(^{2+}\)]\(_i\) during fertilization (Mohri et al., 1995; Lee et al., 1996). If U73122 is inhibiting the production of InsP\(_3\), the combinatory use of U73122 and heparin should synergistically inhibit the sperm-induced Ca\(^{2+}\) release. We tested 1 mg/mL heparin in conjunction with 10 \(\mu\)M U73122 to block the sperm-induced Ca\(^{2+}\) release. Eggs were pretreated with 10 \(\mu\)M U73122 or U73343 and then loaded with 1 mg/mL heparin. Sperm triggered a rise in [Ca\(^{2+}\)]\(_i\) (Fig. 3) to 1.8 ± 0.1 \(\mu\)M with a latency of 35 ± 3.6 sec in U73343-treated eggs (n = 3, Table 1), which was similar to the responses in eggs loaded with only 1 mg/mL heparin (Lee et al., 1996). In contrast, the rise in [Ca\(^{2+}\)]\(_i\) was suppressed (Fig. 3) to an increase from 106.9 ± 9.5 nM to 151 ± 32 nM in the presence of U73122 and heparin (n = 4, Table 1). All U73122 and heparin treated eggs were polyspermic with 4.3 ± 2.2 (n = 4) sperm per egg.

**U73122 Abolished the Production of InsP\(_3\) During Fertilization**

As a consequence of inhibiting PLC activity in U73122-treated eggs, the production of InsP\(_3\) should be decreased during fertilization. We measured the changes in InsP\(_3\) mass during fertilization in the presence or absence of U73122. The InsP\(_3\) mass is presented as pmol/mg
Fig. 3. Sperm-induced Ca$^{2+}$ release was blocked at a lower level of U73122 in combination with 1 mg/mL heparin. Eggs were treated with 10 μM U73122 or U73343 and then loaded with 1 mg/mL heparin. The U73122-treated egg showed a slight rise in [Ca$^{2+}$]$_i$ from 94.4 nM to 178 nM after insemination at $T = 3$ min. In contrast, sperm triggered a rise in [Ca$^{2+}$]$_i$ from 119 nM to 1.6 μM in the U73343-treated egg after a 31 sec latent period. Both eggs were polyspermic, with 4 and 2 sperm nuclei in the U73343 and U73122-treated eggs, respectively. These recordings are representatives of at least 3 experiments for each treatment.
protein and the concentration is estimated by assuming that 1 mg protein equal to 4.3 μL in egg volume (Gillot et al., 1989). In untreated control eggs, sperm triggered a two-phased increase in InsP₃ mass during the first 2 min of fertilization (Fig. 4). At T = 0, the InsP₃ mass was 0.81 ± 0.07 pmol/mg protein (188.4 ± 16.3 nM, n = 4). The first significant (p < 0.05) rise in InsP₃ mass was detected at T = 30 sec, when InsP₃ mass rose to 1.10 ± 0.13 pmol/mg protein (255.8 ± 30 nM). InsP₃ mass slightly increased further to 1.32 ± 0.12 pmol/mg protein (307.0 ± 27.9 nM) by T = 50 sec. After that, a bulk increase in InsP₃ mass was observed, which reached 5.01 ± 0.62 pmol/mg protein (1.17 ± 0.14 μM) by T = 2 min.

Preliminary data show that the InsP₃ level returns to near unfertilized levels after 3 min (data not shown). In contrast, unfertilized eggs pretreated with 20 μM U73122 had an InsP₃ mass of 0.70 ± 0.09 pmol/mg protein (162.8 ± 20.9 nM), which rose at T = 30 sec to 1.07 ± 0.40 pmol/mg protein (248.8 ± 93.0 nM) that was not significant. No significant change in InsP₃ mass was observed until at T = 2 min when InsP₃ mass rose to 1.33 ± 0.27 pmol/mg protein (309.3 ± 62.8 nM, p < 0.05). A pair of individual experiments are shown in Fig. 4B. In addition to changes in InsP₃ mass in control and 20 μM U73122-treated eggs, the % of FE formation in the control eggs was also recorded. At 45 sec post insemination, 23 of 101 eggs had elevated FE, which reached nearly 90 % of FE formation at T = 55 sec. The FE formation lagged the initial rise in InsP₃ mass, but preceded the bulk of InsP₃ mass increase.

**U73122 Inhibited the Cytoplasmic Alkalization During Fertilization**

Hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) is known to produce InsP₃ and DAG. Earlier studies have shown that DAG activation of protein kinase C activates the
Fig. 4. 20 μM U73122 inhibited the production of InsP₃ during fertilization. Eggs in a 7.5 % suspension were inseminated by acrosome-reacted sperm (1:1500 dilution). (A) Fertilization triggered a two-phase increase in InsP₃ mass in pmol/mg protein during the first 2 min post insemination (N = 4). At T = 0 sec, InsP₃ mass was 0.812 ± 0.071 pmol/mg protein. The first significant change in InsP₃ mass was at T = 30 sec, where InsP₃ mass was 1.098 ± 0.133 pmol/mg protein (p < 0.05). InsP₃ mass increased slightly until T = 50 sec, where InsP₃ mass was 1.318 ± 0.125 pmol/mg protein (p < 0.01). After that, a burst of InsP₃ production, which reached 5.012 ± 0.622 pmol/mg protein (p < 0.01) at T = 2 min was observed. In contrast, eggs treated with 20 μM U73122 failed to exhibit any significant change in InsP₃ mass until 2 min post insemination (1.33 ± 0.27 pmol/mg protein, p < 0.05, N = 4). The change in InsP₃ mass at each time point was compared to the respective InsP₃ mass at T = 0 by using the student t test, with "*" for p < 0.05 and "**" for p < 0.01. For simplicity of the graph, only the high or low error bars are shown from T = 0 to 50 sec for the control and 20 μM U73122 treatments, respectively. (B) A representative pairs of experiments from Fig. 4A are shown. The filled and open circles are the change in InsP₃ mass for the control and U73122-treated eggs, respectively. In addition, the formation of FE for the control eggs (cross mark) was also recorded to correlate the time sequence between InsP₃ production and cortical reaction.
Na\(^{+}\)-H\(^{+}\) antiporter, which results in cytoplasmic alkalinization in sea urchin eggs during fertilization (Shen, 1989). To further examine U73122 inhibition of PLC activity during fertilization, we monitored the change in pH\(_i\) during fertilization. Eggs were treated with 15-25 \(\mu\)M U73122 or U73343 and injected with 20 \(\mu\)g/mL BCECF-dextran to monitor the change in 490/440 fluorescence ratio during fertilization. Fertilization caused a rise in the 490/440 fluorescence ratio from 15.3 \(\pm\) 0.4 to 19.5 \(\pm\) 1.0 \((n = 8)\) in U73343-treated eggs, but only a slight rise from 15.6 \(\pm\) 0.4 to 16.5 \(\pm\) 1.0 \((n = 5)\) was observed in U73122-treated eggs. A pair of recordings for eggs treated with 20 \(\mu\)M U73122 or U73343 are shown in Fig. 5.

**U73122 Blocked the GTP\(\gamma\)S-Induced Ca\(^{2+}\) Release and Cytoplasmic Alkalization**

U73122 has generally been reported to block PLC\(\beta\)-dependent pathways in other cell systems. We examined this possibility in sea urchin eggs by monitoring the effects of U73122 on the GTP\(\gamma\)S-induced Ca\(^{2+}\) release. Injection of GTP\(\gamma\)S, an activator of G-protein, triggers a fertilization-like rise in [Ca\(^{2+}\)]\(_i\) in eggs (Swann et al., 1987; Rakow and Shen, 1990; Crossley et al., 1991). In control experiments \((n = 5)\), the injection of 200 \(\mu\)M GTP\(\gamma\)S caused a brief injection damage rise in [Ca\(^{2+}\)]\(_i\) from 77 \(\pm\) 22 to 272 \(\pm\) 83 nM, which quickly returned to 97 \(\pm\) 28 nM. After a delay of 80 \(\pm\) 8 sec, the [Ca\(^{2+}\)]\(_i\) rose to 1.0 \(\pm\) 0.2 \(\mu\)M and one such recording is shown in Fig. 6A. The injection of 200 \(\mu\)M GTP\(\gamma\)S in 20 \(\mu\)M U73122-treated eggs \((n = 4)\) also caused a brief injection damage rise in [Ca\(^{2+}\)]\(_i\) from 75 \(\pm\) 3.2 to 230 \(\pm\) 53 nM, which quickly returned to 85 \(\pm\) 11 nM. However, no further change in [Ca\(^{2+}\)]\(_i\) was observed and one such recording is shown in Fig. 6A. If the injection of GTP\(\gamma\)S stimulates PIP\(_2\) hydrolysis, then a rise in pH\(_i\) due to the DAG production would also be expected. Therefore, we investigated
Fig. 5. U73122 inhibited cytoplasmic alkalization during fertilization. Eggs were pretreated with 20 μM U73122 or U73343. BCECF-dextran (Mₐ, 40,000) were injected into the eggs at 20 μg/mL to monitor the change in pHᵢ, which is measured as the fluorescence ratio to excitation at 490 and 440 nm. In this record the estimated change in pHᵢ (Shen and Buck, 1990) was from 7.0 to 7.6. In contrast, the 490/440 fluorescence ratio remained unchanged upon insemination of the U73122-treated egg. These recordings are representatives of at least 5 experiments for each treatment.
the GTPyS-induced pH changes in the absence or presence of 20 μM U73122. Injection of 200 μM GTPyS triggered a shift of pH from 14.0 ± 0.9 to 18.6 ± 1.3 (n = 3) in control eggs and one example is shown in Fig. 6B. In contrast, 200 μM GTPyS injection did not stimulate any sudden rise in pH (14.7 ± 0.4 to 15.5 ± 0.3, n = 3) in the 20 μM-U73122 treated eggs.

**U73122 Inhibition Was Reversible and Specific**

The effect U73122 on sperm-induced Ca²⁺ release was reversible. Eggs were pretreated with 15-25 μM U73122. No rise in [Ca²⁺]i during fertilization was recorded in these eggs. Subsequently, U73122 was removed by extensive perfusion of ASW. Readdition of sperm was able to trigger a rise in [Ca²⁺]i from 107.2 ± 14.6 nM to 1.1 ± 0.2 μM (n = 6) and a typical recording is shown in Fig. 7.

The inhibition of U73122 may be due to its alkylation of the sulphydryl group (or other nucleophiles) of G proteins required for the signaling pathway and the activity of U73122 can be suppressed by treating with sulphydryl reducing agents (Smrcka et al., 1991; Yule and Williams, 1992). To examine this possibility, eggs were treated with dithiothreitol (DTT), a reducing agent, to block thio linkages by U73122. Eggs treated with 10 mM DTT still showed a sperm-induced rise in [Ca²⁺]i from 93.2 ± 9.6 nM to 1.1 ± 0.2 μM (n = 5). No inhibition of Ca²⁺ response (94.5 ± 8.2 nM to 1.3 ± 0.2 μM, n = 5) during fertilization was observed in eggs treated with 20 μM U73122 in the presence of 10 mM DTT and one such recording is shown in Fig. 8. In contrast, the subsequent addition of 10 mM DTT and refertilization only partially reversed the inhibition of sperm-induced Ca²⁺ release by preincubation of U73122 in 6 of 10 eggs (140.8 ± 35.8 nM to 474.7 ± 141.4 nM, n = 6). A
Fig. 6. U73122 blocked the GTPγS-induced Ca\textsuperscript{2+} release and cytoplasmic alkalinization. Eggs were injected with 200 μM GTPγS (arrow) in the absence or presence of 20 μM U73122. (A) For the control egg, GTPγS injection caused an increase in [Ca\textsuperscript{2+}]\textsubscript{i} to 1.3 μM at T = 126 sec. In contrast, the U73122-treated egg did not show any significant rise in [Ca\textsuperscript{2+}]\textsubscript{i} in response to the GTPγS injection. The injection procedure caused a brief small rise in [Ca\textsuperscript{2+}]\textsubscript{i}, which quickly returned to basal levels in both eggs. (B) In this record the injection of GTPγS triggered an estimated increase in pH\textsubscript{i} from 6.8 to 7.4 in the control egg. In 20 μM U73122-treated egg, injection of GTPγS had no significant effect on pH\textsubscript{i} in the egg.
Fig. 7. U73122 inhibition was reversible. Eggs were treated with 25 μM U73122 and inseminated. No significant change in $[Ca^{2+}]_i$ was observed and subsequently U73122 was washed out by perfusing with ASW. Acrosome-reacted sperm was readded to the dish and the $[Ca^{2+}]_i$ increased from 112 nM to 1.3 μM. This recording is a representative of 3 experiments at 25 μM and 3 other experiments at 15 μM.
Fig. 8. U73122 inhibition was partially reversible by dithiothreitol (DTT). Eggs were pretreated by 20 μM U73122 with (dotted line) or without (solid line) 10 mM DTT for 10 min. In eggs pretreated with 20 μM U73122 and 10 mM DTT, fertilization triggered a rise in 
$\left[\text{Ca}^{2+}\right]_i$ such as that in this recording from 98 nM to 1.17 μM. In eggs pretreated with 20 μM U73122 alone, no Ca$^{2+}$ response during fertilization was observed. The subsequent addition of 10 mM DTT and refertilization partially reversed the U73122 inhibition. In this record, 10 mM DTT was added ~ 3 min post insemination and allowed to incubated for ~ 15 min. For the clarity of graph, 10 min of the record during the DTT incubation period was truncated. Subsequent refertilization resulted in a transient rise in $\left[\text{Ca}^{2+}\right]_i$ to a peak of 522 nM.
sample attenuated transient rise in \([\text{Ca}^{2+}]_i\) during refertilization is also shown in Fig. 8. The other eggs that failed to show any \(\text{Ca}^{2+}\) response during refertilization after DTT addition, showed a transient rise in \([\text{Ca}^{2+}]_i\) (228 ± 100 nM to 848.8 ± 321.8 nM, \(n = 4\)) during refertilization subsequent to the removal of U73122 and DTT by perfusion with ASW.

U73122 might block the sperm-induced \(\text{Ca}^{2+}\) release by directly interfering with the intracellular \(\text{Ca}^{2+}\) release pathways, including \(\text{InsP}_3\) and cADPR-mediated \(\text{Ca}^{2+}\) release (Willems et al., 1994). This possibility was tested by monitoring agonist-mediated \(\text{Ca}^{2+}\) release in U73122-treated eggs. Injection of \(\text{InsP}_3\) (400 nM) or cADPR (100 nM) triggered a rise in \([\text{Ca}^{2+}]_i\) to a peak of 1.9 ± 0.5 \(\mu\text{M}\) (\(n = 5\)) and 1.8 ± 0.3 \(\mu\text{M}\) (\(n = 3\)) in 25 \(\mu\text{M}\) U73122-treated eggs, respectively. Examples of such recordings are shown in Fig. 9 A and B for \(\text{InsP}_3\) and cADPR-induced \(\text{Ca}^{2+}\) release, respectively. These data were comparable to \(\text{InsP}_3\) and cADPR-induced \(\text{Ca}^{2+}\) release in untreated control eggs (data not shown).

**DISCUSSION**

In this study we provide direct evidence showing that the production of \(\text{InsP}_3\) is absolutely required for the rise in \([\text{Ca}^{2+}]_i\) in sea urchin eggs during fertilization by blocking the activity of PLC. We demonstrated that U73122 inhibited the sperm-induced \(\text{Ca}^{2+}\) release and cytoplasmic alkalinization by preventing the PLC-dependent \(\text{InsP}_3\) and presumably DAG production. It has been suggested that \(\text{InsP}_3\) may play a central role during fertilization by mediating the sperm-induced \(\text{Ca}^{2+}\) release. A rapid turnover of phosphatidylinositolides has been observed in sea urchin eggs (Turner et al., 1984, Kamel et al., 1985; Swann et al., 1987;
Fig. 9. U73122 did not affect the agonist-induced Ca\(^{2+}\) release. Eggs were pretreated with 25 μM U73122. No significant change in [Ca\(^{2+}\)]\(_i\) during fertilization was observed. Subsequently, injection of (A) 400 nM InsP\(_3\) caused a rise in [Ca\(^{2+}\)]\(_i\) from 83 nM to 1.8 μM. This recording is a representative of 5 experiments. (B) 100 nM cADPR caused a rise in [Ca\(^{2+}\)]\(_i\) from 103 nM to 2.0 μM. This recording is a representative of 3 experiments.
Ciapa et al., 1992) and the relative amounts of InsP₃ and DAG increased during fertilization (Ciapa and Whitaker, 1986). Microinjection of InsP₃ triggered responses that mimic the egg activating processes, including the fertilization potential (Slack et al., 1986), rise in [Ca²⁺]; (Swann and Whitaker, 1986; Swann et al., 1987) and cortical reaction in sea urchin eggs (Whitaker and Irvine, 1984). However, early studies failed to block the sperm-induced Ca²⁺ release by 0.3 mg/mL heparin (Rakow and Shen, 1990; Crossley et al., 1991) or InsP₃ receptor antibodies (Swann et al., 1994; Shen, 1995), which led to the possibility that an InsP₃-independent pathway may also exist in sea urchin eggs. Demonstration of a ryanodine-mediated Ca²⁺ release mechanism (Galione et al., 1991; Buck et al., 1992), which may be regulated by cADPR (Galione et al., 1991; Galione, 1992, 1993), led to the proposal of redundant Ca²⁺ release mechanisms during fertilization. This redundant Ca²⁺ release mechanisms hypothesis was supported by the observations that sperm-induced Ca²⁺ release was blocked by the combination of heparin and ruthenium red, a ryanodine receptor antagonist (Galione et al., 1993), or heparin and 8-amino-cADPR, a cADPR antagonist (Lee et al., 1993). However, questions were raised against this hypothesis because of the possible toxicity of ruthenium red to the eggs and the high amount (4.7 mg/mL) of heparin used in the latter study (Shen, 1995). More recently, Mohri et al., (1995) showed a dose-dependent inhibition of the sperm-induced Ca²⁺ release in the heparin or pentosan polysulfates-loaded eggs. By blocking the purported production pathway for cADPR, we further demonstrated that the ryanodine receptor Ca²⁺ release mechanism is unnecessary during fertilization (Lee et al., 1996). It seems that InsP₃-dependent Ca²⁺ release is the primary pathway utilized during sea urchin fertilization, which is similar to the fertilization response of mammalian (Miyazaki
et al., 1992; Kline and Kline, 1994) and Xenopus (Nuccitelli et al., 1993) eggs. Since antagonists of Ca\(^{2+}\) releasing mechanisms have pleiotropic effects on other cellular functions (Shen, 1995), instead of blocking the binding of InsP\(_3\) to its receptor, we have shown here that the sperm-induced Ca\(^{2+}\) release could be prevented by inhibiting the production of InsP\(_3\). Our results correlate with the findings of another study reporting U73122 block of Ca\(^{2+}\) oscillation in mouse eggs during fertilization (Dupont et al., 1996).

Initially we were unable to detect a consistent rise in InsP\(_3\) mass during the first 2 min of fertilization by adapting the protocols of Ciapa and Maggio (1993), using a commercial InsP\(_3\) assay kit from DuPont NEN (NEK-064, Wilmington, DE). We switched to using an InsP\(_3\) assay kit from Amersham (TRK-1000, Arlington Heights, IL) and modified the procedures of Osawa et al., (1997). We found that the TRK-1000, as well as, NEK-064 are sensitive to high Ca\(^{2+}\) and Mg\(^{2+}\) contents in ASW (Worley et al., 1987). It is difficult to precisely remove ASW by centrifugation (Ciapa and Maggio, 1993) from samples at 10 sec interval during the first 2 min of fertilization. Although sea urchin eggs can be fertilized in a Ca\(^{2+}\)-free medium by using acrosome-reacted sperm (Schmidt et al., 1982), there is a requirement for Mg\(^{2+}\) in ASW during sea urchin fertilization (Sano and Mohri, 1976; Mohri et al., 1994). Preliminary trials showed a low and inconsistent fertilization rate using ASW with low amounts of Ca\(^{2+}\) and Mg\(^{2+}\). Thereafter we determined a reduction of Ca\(^{2+}\) and Mg\(^{2+}\) contents in ASW in half was sufficient for high fertilization rate with minimal interference of the assay by chelating the residual Ca\(^{2+}\) and Mg\(^{2+}\) with additions of 10 mM EGTA and 40 mM EDTA in 2 N KOH for neutralization of phosphoric acid. The InsP\(_3\) mass in unfertilized eggs measured using NEK-064 was 1.33 ± 0.35 pmol/mg protein (n = 17), which was similar to the
published value (1.1 ± 0.3 pmol/mg protein) using the same kit (Ciapa et al., 1994), but significantly (p < 0.01) higher than the TRK-1000 determined value of 0.81 ± 0.07 pmol/mg protein, (n = 4). It appeared that the binding of InsP₃ was quenched more severely using the NEK-064 assay compared to the TRK-1000 assay under these experimental conditions. This would reduce the sensitivity of the NEK-064 assay kit to detect the rise in InsP₃ mass during the first 2 min of fertilization.

A rise in InsP₃ mass was observed using NEK-064; however, the kinetics were inconsistent among trials and a lower maximum rise (2.0 ± 0.4 fold, n = 12) was measured during the first 2 min of fertilization. In contrast, we consistently detected a two-phased increase in InsP₃ mass during the first 2 min of fertilization using TRK-1000. The rise in InsP₃ mass was first detected at 30 sec, which preceded the initiation of cortical reaction and the bulk increase in InsP₃ mass started at 50 sec. InsP₃ mass continued to rise and reached ~ 6-fold increase by T = 2 min. By measuring the incorporation of [³H]-inositol into InsP₃, Kamel et al. (1985) showed a 5-fold increase in InsP₃ labelling in eggs during sea urchin fertilization; however, Ciapa and Whitaker (1986), only reported a 66% increase in InsP₃ labelling by using the same method. The discrepancy may be due to the intrinsic difficulty in incorporating [³H]-inositol to an equilibrium state in sea urchin eggs (Ciapa et al., 1992), which is avoided by measurement of InsP₃ mass. The increase in InsP₃ mass during Xenopus fertilization has also been reported by two laboratories, which showed an increase of 6 (Stith et al., 1993) and 3.2-fold (Snow et al., 1996). The increase in InsP₃ mass during fertilization measured in this study was higher than a 1.9-fold increase in sea urchin zygotes exposed to sperm extract (Osawa et al., 1997), which may reflect the differences between zygotes and eggs or the
different stimulating methods of live sperm versus sperm extract. More importantly, the increase of InsP₃ mass was blocked in 20 μM U73122-treated eggs using either NEK-064 or TRK-1000 assay kits. Although the inhibition of InsP₃ production by U73122 might partly be due to the failure of fertilization (~ 70 % fertilization rate); nonetheless, the InsP₃ production was almost completely blocked.

InsP₃ can be generated from PIP₂ hydrolysis by different isozymes of PLC, including PLCβ, PLCγ and PLCδ (Lee and Rhee, 1995). The activation mechanism for PLCδ is unclear at present. PLCβ and PLCγ are generally accepted to be activated through heterotrimeric G-protein and tyrosine phosphorylation, respectively. GTPγS, an activator of G-protein, triggered fertilization-like responses, including the rise in [Ca²⁺], and cytoplasmic alkalization in sea urchin eggs (Crossley et al., 1991). It has been reported that U73122 inhibited the GTPγS-induced phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells (Thompson et al., 1991). On the other hand, U73122 did not affect the β₂ integrin-induced tyrosine phosphorylation of PLCγ2 or the formation of InsP₃ in human neutrophils (Hellberg et al., 1996). These results suggested that inhibition by U73122 might be specific to PLCβ (Bleasdale et al., 1990; Thompson et al., 1991; Yule and Williams, 1992). We found that the GTPγS-mediated responses in sea urchin eggs were abolished by U73122 at a dosage similar to inhibition of fertilization responses, which would suggest the production of InsP₃ during fertilization is PLCβ-mediated.

Dupont et al., (1996) reported that U73122 inhibited sperm-egg fusion during fertilization in mouse eggs. A lesser extent of inhibition of sperm-egg fusion was found in this study; however, high concentrations (≥ 15 μM) of U73122 did block fertilization (Table 2).
Roldan et al., (1994) demonstrated that progesterone and zona pellucida could activate sperm PIP$_2$ hydrolysis, which generated InsP$_3$ and DAG. DAG can activate phospholipase A$_2$ (PLA$_2$), which produces fusogenic free fatty acids and lysophosphatides (Roldan and Harrison, 1993). U73122 might interfere with the hydrolysis of PIP$_2$ in sperm and inhibit the activation of PLA$_2$ by DAG that resulted in the failure of sperm-egg fusion. In this study, acrosome-reacted sperm were used to avoid the possible effect of U73122 on sperm. To definitely show that U73122 blocked the rise in [Ca$^{2+}$], during fertilization, we monitored the change in [Ca$^{2+}$], and sperm incorporation in the same eggs. For cases when Ca$^{2+}$ release was blocked and sperm incorporation was observed, the decondensation of sperm nuclei was inhibited. This observation was consistent with the requirement for cytoplasmic alkalinization for male pronuclear development in sea urchin zygotes (Carron and Longo, 1980), since the rise in pH$_i$ was also inhibited in U73122-treated eggs. Furthermore, continued PLC activity is required for later fertilization events, such as nuclear envelope break down, which has been shown to be driven by an increase in [Ca$^{2+}$], (Steinhardt and Alderton, 1988) that is InsP$_3$-dependent (Twigg et al., 1988; Ciapa et al., 1994). In addition, pronucleus formation in mouse eggs was also blocked by a InsP$_3$ receptor monoclonal antibody (Xu et al., 1994) or phosducin, a protein that binds tightly to free G protein $\beta\gamma$ subunits (Moore et al., 1994).

The inhibition of sperm nuclei decondensation and migration by 20 $\mu$M U73122 (Fig. 2B) raises a possibility that the sperm might not have fused with the egg. Hoechst No. 33342 is a cell membrane permeant dye and it is possible that the dye might diffuse across both egg and sperm membranes to stain attached but not fused sperm. However, we observed many other attached sperm, which were not stained, and it seems unlikely that diffused dye only
selectively stained a few sperm. Lawrence et al. (1997) also used the same staining method and found no staining of attached sperm on the mouse eggs, which were treated to prevent sperm-egg fusion. Therefore, it seems unlikely that attached but unfused sperm would incorporate Hoechst No 33342 due to dye diffusion during the 10 min incubation period used in this study. The sperm nuclei decondensed in eggs treated with 10 μM U73122 where a Ca^{2+} transient occurred during fertilization and in eggs treated with 10 μM U73122 and 1 mg/mL heparin where a Ca^{2+} transient was inhibited (Fig. 3). These results suggested that 10 μM U73122 was not enough to completely block PIP2 hydrolysis, which caused partial production of DAG to elevate pH and allow the decondensation of sperm nucleus (Fig. 2B).

It appears that the decondensation of sperm nucleus was independent of rise in [Ca^{2+}]_{i} since it also occurred in heparin and U73122 treated eggs, where the rise in [Ca^{2+}]_{i} was suppressed.

The specificity of U73122 is of concern as with all pharmacological agents. In the present study, an increase in resting [Ca^{2+}]_{i} was observed in eggs treated with high level (≥ 15 μM) of U73122 and in some eggs a sustained rise in [Ca^{2+}]_{i} was observed, which led to egg lysis. InsP_{3} receptor sensitivity is modulated by Ca^{2+} concentration (Bezprozvanny and Ehrlich, 1995). Since U73122 inhibited the production of InsP_{3} if the InsP_{3} receptor sensitivity is increased by the elevated [Ca^{2+}]_{i}, the sperm-induced Ca^{2+} response should have been increased. Instead the Ca^{2+} response was inhibited in U73122-treated eggs. U73122 did not appear any effect on InsP_{3} receptor sensitivity since the exogenous InsP_{3}-induced Ca^{2+} response was not affected in the U73122-treated egg (Fig 9A).

The rise in resting [Ca^{2+}]_{i} in U73122-treated eggs might be due to the release of Ca^{2+} from internal stores (Willems et al., 1994) or Ca^{2+} entry caused by the plasma membrane
leakage. U73122 at 25 µM did not affect the Ca²⁺ homeostasis in sea urchin egg microsomes (data not shown), which was unlike the rat liver microsomes, where U73122 stimulated the release of actively stored Ca²⁺ via the inhibition of internal Ca²⁺ pump (de Moel et al., 1995). In intact eggs, it is more likely that the rise in resting [Ca²⁺]ᵢ was due to membrane leakage, since it could be alleviated by lowering the Ca²⁺ concentration in the bath (data not shown). This suggested that high levels (> 25 µM) of U73122 might permeabilize the plasma membrane and cause a sustained Ca²⁺ influx. Due to the problems of U73122 at high concentration, we tested the specificity of U73122 at 10 µM, which showed no rise in basal [Ca²⁺]ᵢ and no inhibition of sperm-egg fusion. To prevent the activity of InsP₃, which might be generated by the residual PLC activity, 1 mg/mL heparin was also loaded to block the binding of InsP₃ to its receptors. The Ca²⁺ release was blocked in these U73122 and heparin-treated eggs without hampering sperm incorporation or disturbing resting Ca²⁺ homeostasis in all eggs examined. This finding further suggested that Ca²⁺ release during fertilization is not due to redundant Ca²⁺ release mechanisms but requires InsP₃-dependent Ca²⁺ release.

Hellberg et al., (1996) reported that U73122 reduced the InsP₃-induced Ca²⁺ release in permeabilized human neutrophils presumably via the direct inhibition on the Ca²⁺ channels. This possibility was minimized in this study by showing Ca²⁺ responses to InsP₃ and cADPR injections in eggs incubated with U73122, which are comparable to the controls. U73122 may act as a strong sulfhydryl reagent by attacking the sulfhydryl groups of Gₛ protein with its electrophilic maleimide side chain (Thompson et al., 1991; Yule and Williams, 1992). The maleimide group of U73122 can be inactivated by reacting with mercaptoethanol (Bleasdale et al., 1990) or DTT (Smrcka et al., 1991; Thompson et al., 1991). We showed
here that DTT also abolished the U73122 inhibition of the sperm-induced Ca^{2+} response when it was present during the U73122 preincubation period. However, when DTT was added subsequent to the U73122 treatment, it could only partially reverse the inhibition. Finally, U73122 at 25 μM was not cytotoxic and its inhibition of the rise in [Ca^{2+}]_i during fertilization was reversible, which is similar to that reported in mouse eggs (DuPont et al., 1996).

In summary, we have demonstrated that there is an absolute requirement for InsP_3 production for generating the rise in [Ca^{2+}]_i in sea urchin eggs during fertilization. It is still uncertain that the pathway is activated via PLCβ and/or PLCγ. Both PLCβ and PLCγ can initiate a Ca^{2+} release in several animal egg systems (Kline et al., 1988; Moore et al., 1994; Shilling et al., 1994; Yim et al., 1994; Dupont et al., 1996). Protein tyrosine kinase activity has been detected during the latent period of fertilization in sea urchin eggs (Satoh and Garbers, 1985; Ciapa and Epel 1991) and a egg membrane protein was tyrosine phosphorylated by 5 sec after insemination (Abassi and Foltz, 1994). However, a number of tyrosine kinase inhibitors have no effects on the early fertilization responses in sea urchin eggs (Moore and Kinsey, 1995), which opposes a role of PLCγ for sperm-induced Ca^{2+} release in sea urchin eggs. Therefore, identifications of signaling proteins like GTP binding proteins, PLC isozymes and tyrosine kinases, and more specific pharmacological functional studies are needed to differentiate the roles of PLCβ and PLCγ during sea urchin fertilization. The recent finding of activation of the G_{q11} through tyrosine phosphorylation of the α subunit even provides the possible crosstalk between tyrosine phosphorylation and activation of PLCβ (Umemori et al., 1997).
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GENERAL CONCLUSIONS

In this study, I examined the InsP₃- and ryanodine-mediated pathways and attempted to test the necessity of each pathway during sea urchin fertilization. Results demonstrated that although the ryanodine-mediated pathway exists in sea urchin eggs, it is not utilized during fertilization. Further evidence suggested there is an absolute requirement for the production of InsP₃, which depends on the phospholipase C activity, during fertilization.

cADPR has been proposed to be the endogenous ligand of ryanodine receptor (Lee, 1994; Galione and White, 1994) and its production can be triggered by cGMP in sea urchin eggs (Galione et al., 1993). Galione et al., (1993) hypothesized that the cGMP-induced cADPR production is mediated by the activation ADP-ribosyl cyclase via a PKG-dependent pathway. This postulated pathway was tested with a variety of activators (cGMP analogs and cIMP) and inhibitors (Rp-8-pCPT-cGMPS, 3-aminopyridine NAD, nicotinamide and spermine). Our observations are consistent with Ca²⁺ release by cGMP in the egg is dependent on an isoform of PKG that is distinct from the mammalian enzyme and activation of ADP ribosyl cyclase to produce cADPR. Inhibitors of either PKG or ADP-ribosyl cyclase activities had no significant effect on the rise in [Ca²⁺], during fertilization. Nor did they prevent the sperm-induced [Ca²⁺] transient in heparin-loaded eggs. It appears the activation of the cGMP-dependent Ca²⁺ release pathway was unnecessary during fertilization.
The idea of InsP$_3$-insensitive Ca$^{2+}$ release during fertilization was proposed due to observations of a rise in [Ca$^{2+}$]$_i$ during fertilization in heparin-loaded eggs (Rakow and Shen, 1990; Crossley et al., 1991). This conclusion was based on the observations that injections of 0.1 to 0.3 mg/mL heparin (final intracellular concentration) blocked Ca$^{2+}$ release by injected InsP$_3$ and GTP$_{y}$S. The latter has been shown to activate endogenous InsP$_3$ production (Crossley et al., 1991). Demonstration of a ryanodine-mediated Ca$^{2+}$ release mechanism (Galione et al., 1991; Buck et al., 1992), which may be regulated by cADPR (Galione et al., 1991; Galione, 1992, 1993), led to the proposal of redundant Ca$^{2+}$ release mechanisms during fertilization. This redundant Ca$^{2+}$ release mechanisms hypothesis was supported by the observations that sperm-induced Ca$^{2+}$ release was blocked by the combination of heparin and ruthenium red, a ryanodine receptor antagonist (Galione et al., 1993), or heparin and 8-amino-cADPR, a cADPR antagonist (Lee et al., 1993). However, questions were raised against this hypothesis because of the possible toxicity of ruthenium red to the eggs and the high amount (4.7 mg/mL) of heparin used in the latter study (Shen, 1995).

In contrast, Mohri et al., (1995) showed a dose-dependent inhibition of the sperm-induced Ca$^{2+}$ release in the heparin or pentosan polysulfates-loaded eggs. In this study we used 0.7-1.0 mg/mL heparin to reduce InsP$_3$-dependent Ca$^{2+}$ release and treated eggs with inhibitors known to block the PKG or ADP ribosyl cyclase activity. The sperm-induced [Ca$^{2+}$]$_i$ transient still occurred under these circumstances. Several possible explanations may account for our observations that the additional presence of PKG or NADase inhibitors did not prevent the rise in [Ca$^{2+}$]$_i$ in these heparin-loaded eggs during fertilization. The most direct explanation is that InsP$_3$-mediated Ca$^{2+}$ release is indeed the primary prerequisite
mechanism underlying generation of the rise in \([\text{Ca}^{2+}]_i\) during fertilization and at 0.7-1.0 mg/mL heparin, the \(\text{InsP}_3\)-dependent \(\text{Ca}^{2+}\) transient was delayed but not fully suppressed (Mohri et al., 1995). Another possibility is that another heparin-insensitive \(\text{Ca}^{2+}\) release pathway is utilized during fertilization. In sea urchin eggs a new mechanism for \(\text{Ca}^{2+}\) release by nicotinic acid adenine dinucleotide phosphate (NAADP) has been reported (Lee and Aarhus, 1995; Chini et al., 1995a; Perez-Terzic et al., 1995). \(\text{Ca}^{2+}\) release by NAADP is insensitive to heparin and antagonists of cADPR-dependent \(\text{Ca}^{2+}\) release (Chini et al., 1995b; Graeff et al., 1995). Thirdly it may also be possible that compartmentation of the \(\text{Ca}^{2+}\) mobilizing agents or inhibitors might occur, such that local concentrations of natural \(\text{Ca}^{2+}\) release agonists might be sufficient during fertilization. This possibility is particularly intriguing because it otherwise appears that the generation of cADPR is not required during fertilization, since neither 3-AP NAD nor NiAm inhibited the \(\text{Ca}^{2+}\) response during fertilization.

The sperm-induced \(\text{Ca}^{2+}\) release can be inhibited in eggs loaded with heparin at a intracellular amount of > 2 mg/mL (unpublished observation). Heparin, a polyanion, when used at high concentrations, may have pleiotropic effects on other cellular functions (Shen, 1995). To avoid this problem and further understand the role of \(\text{InsP}_3\)-dependent pathway during sea urchin fertilization, we monitored the change in \(\text{InsP}_3\) mass and \([\text{Ca}^{2+}]_i\) during fertilization in the presence or absence of antagonist of \(\text{InsP}_3\) synthesizing enzyme, phospholipase C (PLC). We observed a two-phased increase in \(\text{InsP}_3\) mass, which preceded the cortical reaction, during the first 2 min of fertilization. More importantly, the sperm-induced \(\text{Ca}^{2+}\) transient was suppressed by blocking this increase in \(\text{InsP}_3\) mass in eggs loaded
with the PLC inhibitor, U73122. These results are consistent with the study, which utilized high level of heparin (Mohri et al., 1995) and correlates with the findings of another study reporting U73122 block of Ca^{2+} oscillation in mouse eggs during fertilization (Dupont et al., 1996). It further supports that the utilization of InsP_{3}-mediated pathway as the primary Ca^{2+} releasing mechanism during fertilization is conserved in all species examined, including hamster (Miyazaki et al., 1992), mouse (Kline and Kline, 1994; Dupont et al., 1996), *Xenopus* (Nuccitelli et al., 1993; Galione et al., 1993) and sea urchin (Mohri et al., 1995, Lee et al., 1996).

We also found that the GTPγS-mediated responses in sea urchin eggs were abolished by U73122 at a dosage similar to inhibition of fertilization responses. In contrast, preliminary studies (Shen, W. H. Kinsey and Lee, unpublished results) show that 20 μM U73122 did not inhibit the methyl glyoxal bis-(guanyl hydrazone) dihydrochloride, a tyrosine kinase activator (Stith et al., 1996), -induced Ca^{2+} release in sea urchin eggs. These results suggested that the U73122 inhibition is specific to PLCβ in sea urchin eggs and that sperm-induced Ca^{2+} release is independent of PLCγ activity. However, protein tyrosine kinase activity has been detected during the latent period of fertilization in sea urchin eggs (Satoh and Garbers, 1985; Ciapa and Epel 1991) and a membrane protein was tyrosine phosphorylated by 5 sec after insemination (Abassi and Foltz). Although tyrosine kinase activator and inhibitors studies (Moore and Kinsey, 1995) tend to oppose a role of PLCγ for sperm-induced Ca^{2+} release in sea urchin eggs, the possible involvement of PLCγ during fertilization should not be ignored. Identifications of signaling proteins like GTP binding proteins, PLC isozymes and tyrosine kinases, and more specific pharmacological functional studies are needed to differentiate the
roles of PLCβ and PLCγ during fertilization. The recent finding of activation of the G protein $G_{\gamma 11}$ through tyrosine phosphorylation of the $\alpha$ subunit even provides the possible crosstalk between tyrosine phosphorylation and activation of PLCβ (Umemori et al., 1997).


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