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Increase of cGMP, cADP-ribose and inositol 1,4,5-trisphosphate preceding Ca^{2+} transients in fertilization of sea urchin eggs

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SUMMARY

Transient increases, or oscillations, of cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, occur during fertilization of animal egg cells. In sea urchin eggs, the increased Ca^{2+} is derived from intracellular stores, but the principal signaling and release system involved has not yet been agreed upon. Possible candidates are the inositol 1,4,5-trisphosphate receptor/channel (IP_3R) and the ryanodine receptor/channel (RyR) which is activated by cGMP or cyclic ADP-ribose (cADPR). Thus, it seemed that direct measurements of the likely second messenger candidates during sea urchin fertilization would be essential to an understanding of the Ca^{2+} signaling pathway. We therefore measured the cGMP, cADPR and inositol 1,4,5-trisphosphate (IP_3) contents of sea urchin eggs during the early stages of fertilization and compared these with the $[Ca^{2+}]_i$ rise in the presence or absence of an inhibitor against soluble guanylate cyclase. We obtained three major experimental results: (1) cytosolic cGMP levels began to rise first, followed by cADPR and IP_3 levels, all almost doubling before the explosive increase of $[Ca^{2+}]_i$; (2) most of the rise in IP_3 occurred after the Ca^{2+} peak; IP_3 production could also be induced by the artificial elevation of $[Ca^{2+}]_i$, suggesting the large increase in IP_3 is a consequence, rather than a cause, of the Ca^{2+} transient; (3) the measured increase in cGMP was produced by the soluble guanylate cyclase of eggs, and inhibition of soluble guanylate cyclase of eggs diminished the production of both cADPR and IP_3 and the $[Ca^{2+}]_i$ increase without the delay of Ca^{2+} transients. Taken together, these results suggest that the RyR pathway involving cGMP and cADPR is not solely responsible for the initiating event, but contributes to the Ca^{2+} transients by stimulating IP_3 production during fertilization of sea urchin eggs.

Key words: cGMP, cADP-ribose, IP_3, Ca^{2+} transient, Fertilization, Sea urchin eggs

INTRODUCTION

A transient increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ (Ca^{2+} transient), or oscillation of $[Ca^{2+}]_i$ is a characteristic feature of animal eggs at fertilization, from invertebrates to mammals (Jaffe, 1983; Jaffe, 1985; Miyazaki et al., 1993; Stricker, 1999). The transient increase or oscillation of $[Ca^{2+}]_i$ propagates across the egg as a Ca^{2+} wave, which is thought to be the necessary event in the egg activation (Jaffe, 1985; Whitaker and Swann, 1993). In many cell types, including sea urchin eggs, the $[Ca^{2+}]_i$ increase results from a rapid release from cytoplasmic stores, mediated by one or both of two discrete signaling pathways: (1) IP_3 and IP_3R, or (2) cGMP and/or cADPR and RyR (Berridge, 1993; Clapham, 1995). The IP_3 system is certainly present in eggs or oocytes of almost all species of animals, and is considered to be functional in fertilization (Miyazaki et al., 1993). The RyR system also exists in mammalian eggs (Ayabe et al., 1995; Yue et al., 1995), ascidian eggs (Albrieux et al., 1997) and sea urchin eggs (Galione et al., 1991; McPherson et al., 1992), but data are generally less clear concerning the behavior of the RyR system at fertilization. The RyR is thought to be modulated by cADPR (Galione et al., 1991; Takasawa et al., 1993; Albrieux et al., 1997) and, secondarily, by cGMP, which activates ADP-ribosyl cyclase through the action of a cGMP-dependent protein kinase (PKG) to produce cADPR (Galione et al., 1993b).

Partly because sea urchin eggs do contain both the RyR (McPherson et al., 1992) and the IP_3R (Parys et al., 1994), it was supposed that both must be important in fertilization, perhaps being independently responsible for the Ca^{2+} transient (Galione et al., 1993a; Lee et al., 1993). Restricting evidence, however, has come from several experiments that suggest a

primary role for only the IP_3 system. First, in eggs preloaded with heparin, a potent competitive inhibitor of the IP_3-induced Ca^{2+} release (Ghosh et al., 1988), the fertilization-induced Ca^{2+} transient was delayed and reduced in both velocity and amplitude (Mohri et al., 1995). Furthermore, intracellular injection of the tandem SH2 domains of bovine PLCγ produced a concentration-dependent inhibition of the Ca^{2+} release (Carroll et al., 1999; Shearer et al., 1999). As for the RyR system, inhibition of either PKG or ADP-ribosyl cyclase failed to block the Ca^{2+} transient in heparin-loaded eggs (Lee et al., 1996). These discoveries were interpreted to mean that the IP_3 system must be the primary mechanism underlying the Ca^{2+} transient at fertilization of sea urchin eggs. However, another line of evidence has recently shown that nitric oxide (NO) was produced in sperm during their acrosome reaction and in eggs during fertilization, and intracellular injection of NO synthase caused the egg activation (Kuo et al., 2000). NO is expected to stimulate the production of cGMP by activating soluble guanylate cyclase (Garthewaite, 1991), and then cGMP is expected to stimulate the production of cADPR (Galione et al., 1993b).

Thus, the roles of the IP_3 system and RyR pathway in the Ca^{2+} transient, especially in its initiation, are still controversial. As the signal transduction process at fertilization must have a spatial structure, the simultaneous measurements of cGMP, cADPR and IP_3 with micrometer spatial resolution inside the eggs are the best way for precise analysis of regulatory pathways. However, a detailed description of the time courses of changes in cGMP, cADPR and IP_3 contents compared with that of $[Ca^{2+}]_i$ during fertilization would be still needed for revealing the initiating event of the Ca^{2+} transient at fertilization, although biochemical analysis on the contents of second messenger candidates using population of eggs cannot give any spatial information. No one has simultaneously measured the contents of these second messenger candidates in the eggs during fertilization, except for the observations that the cGMP concentration in eggs was higher 20-60 seconds after fertilization than it was before insemination (Ciapa and Epel, 1996), and that the IP_3 content increased slowly after insemination relative to the $[Ca^{2+}]_i$ rise (Lee and Shen, 1998). In the experiments described below, we have carried out these critical measurements, assaying cGMP, cADPR and IP_3 simultaneously throughout the first 150 seconds after fertilization under the presence or absence of a specific inhibitor of soluble guanylate cyclase, 6-anilino-5, 8-quinolinedione (LY83583) (Pandol and Schoeffield-Payne, 1990) and compared these time courses with those of the $[Ca^{2+}]_i$ change to reveal the sequential relationship among them.

MATERIALS AND METHODS

Preparation of gametes

Three Japanese species of sea urchin were used: the winter species *Hemicentrotus pulcherrimus*; the summer species *Anthocidaris crassispina*; and the autumn species *Pseudocentrotus depressus*. Spawning of eggs was induced by injection of 0.5 M KCl into the coelomic cavity, and the shed eggs were washed three times with sea water filtered through two layers of filter paper (No. 1, Toyo Roshi, Tokyo, Japan) (FSW). Only batches of eggs with a percentage of healthy eggs that exceeded 99%, judged by symmetrical elevation of the fertilization envelope, were used. The eggs were stripped of their jelly coats by washing twice with Ca^{2+}- and Mg^{2+}-free artificial sea water (CaMgFASW; 520 mM NaCl, 10 mM KCl, 10 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl]-propanesulfonic acid (Epps), titrated to pH 8.2 with tris(hydroxymethyl)aminomethane (Tris), and 2 mM ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA)), then were rinsed twice more with standard artificial sea water (stdASW; 430 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM Epps, ~12.5 mM Tris, pH 8.2) or with nominal zero-Ca^{2+} artificial sea water (0CaASW; 445 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM Epps, ~12.5 mM Tris, pH8.2), and were finally resuspended in fresh stdASW or 0CaASW at a density of $3\text{-}5\times10^4$ eggs/ml. Sperm were likewise shed by injecting 0.5 M KCl, collected directly from the genital pores, stored at 4°C until use, and diluted 5000-fold into stdASW just before use. For maintenance and experimental analyses, the suspension temperature was adjusted to ~17°C for *H. pulcherrimus* and to ~20°C for *A. crassispina* and *P. depressus*.

Assays for cGMP, IP_3 and cADPR

Each egg suspension was mixed with an equal volume of sperm suspension, and when necessary, eggs was preincubated in stdASW containing 200 μM LY83583 (Research Biochemicals International, Natick, MA) for 15-30 minutes at ~20°C (*A. crassispina*) or ~17°C (*H. pulcherrimus*) before insemination. For assays of ionophore-triggered eggs, each suspension was diluted with an equal volume of ASW containing 20 μg/ml of a Ca^{2+} ionophore, A23187 (Wako Pure Chemicals, Osaka, Japan). At appropriate intervals thereafter, aliquots (0.2-0.5 ml for experiments on cGMP and IP_3; 10 ml for experiments on all the three) of the egg suspension were withdrawn and injected into one-fifth the volume of 21% (w/v) ice-cold perchloric acid. Immediately after each series of sampling, the appearance of the fertilization envelope was examined, and the batch to be processed further was selected only if the envelope formation was greater than 95%, except for the experiments in 0CaASW containing A23187. The stopped mixture was kept on ice for 30 minutes with occasional mixing, and then centrifuged at $>10^4$ ***g*** and 4°C for 15 minutes. The precipitate (acid-insoluble fraction) from each aliquot was assayed for the total protein via the Coomassie Blue reaction (Coomassie Protein Assay Reagent, Pierce Chemical, Rockford, IL). From this measurement, the number of eggs in each aliquot was calculated, using the value for the protein content of single egg cells determined on the same batch of eggs (0.02-0.04 μg/egg). The supernatant fluid (acid-soluble fraction) was neutralized with a mixture of 2.4 M KOH, 240 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes), and 6 or 40 mM ethylene glycol bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), and the precipitates of potassium perchlorate were removed by centrifugation. Aliquots of this neutralized acid-soluble fraction were used to assay cGMP and IP_3.

IP_3 levels were determined by isotope dilution, using a Biotrak assay system TRK 1000 (Amersham Pharmacia Biotech, Buckinghamshire, UK). cGMP levels were determined by enzyme immunoassay via a Biotrak RPN 226 kit (Amersham Pharmacia Biotech). In a few experiments, cGMP was determined by radioimmunoassay using a Biotrak TRK 500 kit (Amersham Pharmacia Biotech). Because radioassays routinely gave values 4.5±0.8 times (mean±s.d., n=21) as large as the cGMP values obtained by enzyme assays on the same samples, we normalized the values obtained by radioassays to the values calculated by the enzyme assays, in order to avoid the confusion between both assays. Because salt concentrations in the IP_3 assay and the presence or absence of A23187 in the cGMP radioassay significantly affected the assay values, standard solutions of IP_3 and cGMP were prepared in the same media as the experimental samples, which were processed in parallel with the experimental samples but without eggs or sperm.

For cADPR measurements, large amounts of gametes were required. Our measurements of cADPR, which required to mix large volumes of egg and sperm suspension, lead to uncertainty in

synchrony of fertilization, especially in *A. crassispina*. cADPR in each acid-soluble fraction was concentrated by adsorbing to acid-washed charcoal (Norit X Plus, American Norit Company, Amersfoort, The Netherlands), and eluting from charcoal with pyridine water (Tsuboi and Price, 1959). By this method, more than 60% of cADPR was recovered except samples containing sperm alone (~45%), as checked in preliminary experiments using [^{32}P]cADPR. Each eluate was subsequently lyophilized, then dissolved in ultra-pure water, and incubated with bovine alkaline phosphatase (Type II-G, Sigma Chemical Co., St. Louis, MO) and venom phosphodiesterase (*Crotalus adamanteus* venom, Worthington Biochemical, NJ), in order to destroy the nucleotides that crossreacted with the anti-cADPR antiserum. cADPR was then quantified by radioimmunoassay with anti-cADPR antiserum, as described previously (Takahashi et al., 1995). By ratioing the total amounts of cGMP, cADPR and IP_3 in each experimental sample to the total egg number, the individual contents per egg were obtained. However, the contents of each compound per egg were probably not absolute values. Averaged intracellular concentrations of the individuals can be obtained by dividing the contents per egg by the egg volume (4×10^{-10} l for *A. crassispina* or 5×10^{-10} l for *H. pulcherrimus*) estimated from the measured diameter.

Guanylate cyclase assay

The method of Harumi et al. (Harumi et al., 1992) was used with slight modifications. Briefly, dry sperm and wet-packed dejellied unfertilized eggs were homogenized in a medium containing 1% (w/v) Nonidet-P40 (BDH Laboratory, Poole, UK), 0.5 M glycerol, 1 mM EGTA, 1 mM dithiothreitol (DTT; Wako), 0.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 10 μM leupeptin (Peptide Institute, Osaka, Japan), 10 μM pepstatin A (Peptide Institute) and 20 mM Hepes buffered to pH 7.0 with Tris, with 10 strokes of a glass-Teflon homogenizer on ice, and then stirred for 60 minutes at 0°C. For fertilized eggs, the dejellied eggs were inseminated in the same way as for the experiments on cGMP, cADPR and IP_3 measurements, and after 30 seconds the suspension was poured into a fivefold volume of ice-cold stdASW, and centrifuged immediately at 50 ***g*** and 4°C for 20 seconds. The resultant pellet was homogenized as for sperm and unfertilized eggs.

The assay medium contains 0.5% Nonidet-P40, 0.25 M glycerol, 5 mg/ml bovine serum albumin (fraction V, Sigma), 4 mM $MnCl_2$, 15 mM sodium phosphocreatine (Sigma), 30 units/ml creatine phosphokinase (Sigma), 1 mM 3-isobutyl-1-methylxanthine (Research Biochemicals International, Natick, MA), 1 mM DTT, 0.5 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A and 40 mM Hepes buffered to pH 7.5 with Tris, with or without 200 μM LY83583. The reaction was started by the addition of 5′-GTP (Sigma), incubated at 20°C for 10 minutes, and then product cGMP was quantified by radioimmunoassay in the same way as for the experiments on cGMP during fertilization.

DNA sequencing

Total RNA, which had been extracted from the unfertilized eggs of *A. crassispina* according to Seimiya et al. (Seimiya et al., 1997), was used as the template to synthesize the first strand of cDNA using an oligo (dT) primer according to the manufacturer's protocol (SuperScript Preamplification System for First Strand cDNA Synthesis, Gibco BRL, Grand Island, NY). The cDNA fragments encoding guanylate cyclase(s) were amplified using PCR with the following degenerate oligonucleotide primers: P2, 5′-GAYATHGTNGGNTTYAC-3′; P6, 5′-GTRTTNACNGTRTCNCC-3′; and P7, 5′-ARRCARTANCKNGGCAT-3′, which were synthesized based on the amino acid sequences of three conserved regions (DIVGFT, DTVNTA and MPRYCL) in known membrane guanylate cyclases as described previously (Seimiya et al., 1997). The cDNA fragments in pBluescript vectors (Stratagene, La Jolla, CA) were used directly to determine the nucleotide sequences as a template by the dideoxy chain termination procedure (Sanger et al., 1977) with an Applied Biosystems 373A sequencer, and analyzed on a DNASIS software (Hitachi Software Engineering, Tokyo, Japan).

Microinjection and measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured with a Ca^{2+}-sensitive fluorescent dye, indo-1 (Dojindo, Kumamoto, Japan). Each egg cell was fastened to a poly-L-lysine (M_r=1-4×10^3; Sigma) coated glass coverslip at the bottom of 150 μl of stdASW in a Lucite-frame chamber, mounted on the stage of a TMD epifluorescence microscope (Nikon, Tokyo, Japan). The dye was pressure-injected into eggs at a final concentration of ~100 μM, from a 10-mM stock solution containing 100 mM potassium aspartate and 10 mM Hepes, titrated to pH 7.0 with Tris, according to the method of Hiramoto (Hiramoto, 1974). Then, 150 μl 5000-fold diluted sperm suspension or 20 μg/ml Ca^{2+}-ionophore, bromo-A23187 (Molecular Probes, Eugene, OR), was added into the chamber. Experiments on *H. pulcherrimus* were performed at 16-20°C and on *A. crassispina* at 20-25°C. For measuring the fluorescence of indo-1, selected wave lengths were 355 nm (bandpass) at the excitation filter, 380 nm (cutoff) at the dichroic mirror, and simultaneously 405 nm (bandpass) and 485 nm (bandpass) at the emission detectors, which were Hamamatsu R647-01 photomultipliers (Hamamatsu Photonics, Hamamatsu, Japan). Fluorescence from the whole egg was measured, and $[Ca^{2+}]_i$ was calculated from the ratio of 405 nm fluorescence intensity to the corresponding 485 nm intensity (Grynkiewicz et al., 1985).

RESULTS

Time courses of cGMP, IP_3 and $[Ca^{2+}]_i$ changes

In order to determine whether any of the putative signal compounds could actually serve as messengers for the sperm-induced $[Ca^{2+}]_i$ increase, we explicitly measured the time courses of changes in cGMP and IP_3 contents of egg cells early in fertilization, and compared those with that of $[Ca^{2+}]_i$ change. Fig. 1 shows the representative results for cGMP and IP_3 simultaneously measured on *A. crassispina* eggs (Fig. 1A) and *H. pulcherrimus* eggs (Fig. 1B), superimposed on the averaged time courses of $[Ca^{2+}]_i$ change (n=5 for Fig. 1A; n=9 for Fig. 1B). Fig. 1 shows that in both species, cGMP levels began to rise immediately after insemination, and that IP_3 levels did not change quickly and reached its peak after the Ca^{2+} peak. The fertilization potential (FP), which could be known from the initial small stepwise $[Ca^{2+}]_i$ increase (arrow), emerged about 5 seconds after insemination, and from the independent observations, the fertilization envelope became visible at ~40 seconds in *A. crassispina* and at ~50 seconds in *H. pulcherrimus*.

Fig. 2 compares the time courses of the cGMP and IP_3 changes averaged from three independent experiments with that of the Ca^{2+} change. The cGMP contents of unfertilized eggs (mean and s.d., n=3) were 4.0±0.7 (*A. crassispina*) to 6.6±1.4 (*H. pulcherrimus*) amoles/egg, and cGMP levels began to rise within 10 seconds of insemination, reaching an initial peak (9.4±1.5 amoles/egg in *A. crassispina*; 12±1 amoles/egg in *H. pulcherrimus*) 30 seconds after insemination, almost doubling in this 30 second period. These changes very clearly precede the corresponding changes of $[Ca^{2+}]_i$, which did not rise detectably until 30 seconds (*A. crassispina*) or 40 seconds (*H. pulcherrimus*) after insemination and peaked only after another 20 seconds (*A. crassispina*) or 25 seconds (*H. pulcherrimus*) (Fig. 2C,F). A second peak in cGMP levels occurred 80-90 seconds in *A. crassispina* or 100-110 seconds

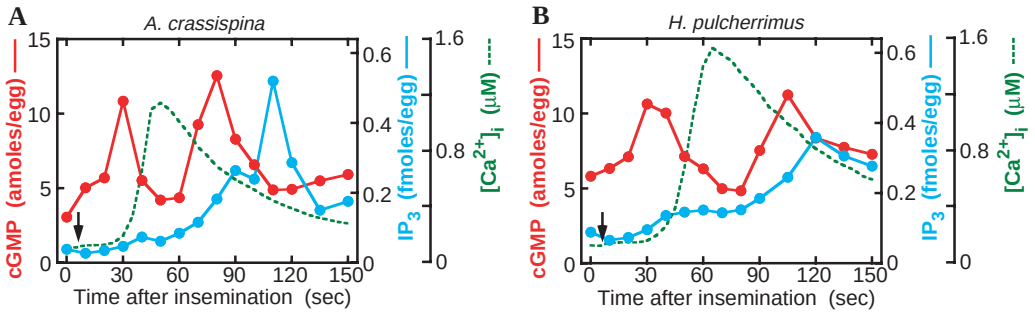

Fig. 1. Simultaneous measurements of the changes in cGMP and IP_3 contents of *A. crassispina* (A) and *H. pulcherrimus* (B) eggs, compared with $[Ca^{2+}]_i$, after insemination. cGMP (red) and IP_3 (blue) values at each point after insemination were obtained on the same aliquot of egg suspension, and $[Ca^{2+}]_i$ (broken line) is an average over five independent experiments for *A. crassispina* or nine for *H. pulcherrimus*. Arrows indicate the onset of the fertilization potential. Scales for cGMP are on the left axis in each panel, and scales for $InsP_3$ and $[Ca^{2+}]_i$ are on the right axis.

in *H. pulcherrimus* after insemination, but its significance is discussed later. In contrast to cGMP, IP_3, of which contents in unfertilized eggs were 29±8 amoles/egg (mean and s.d., n=3) in *A. crassispina* and 64±1 amoles/egg in *H. pulcherrimus*, did not respond to fertilization until 10 seconds (*A. crassispina*: Fig. 2B) or 20 seconds (*H. pulcherrimus*: Fig. 2E), and after it approximately doubled, the level of IP_3 remained elevated for 20-30 seconds. This slow or 'creep' phase of IP_3 production continued during the steep rising phase of $[Ca^{2+}]_i$ in both species. Thus, after the Ca^{2+} peak, IP_3 levels climbed more steeply to its maximum. Actual peaks of the IP_3 content occurred at 110 seconds in *A. crassispina* (0.42±0.13 fmoles/egg) and 120 seconds in *H. pulcherrimus* (0.44±0.16 fmoles/egg), about 1 minute after the Ca^{2+} peaks. This finding, contrary to our expectation, suggests the very opposite relationship between IP_3 and $[Ca^{2+}]_i$, i.e., that the rise in $[Ca^{2+}]_i$ in turn causes IP_3 to rise.

However, when the initial 50 seconds of Fig. 2 were redrawn by normalizing the cGMP and IP_3 contents, and $[Ca^{2+}]_i$ to the individual values of unfertilized eggs (Fig. 3), it was revealed that both of cGMP and IP_3 levels increased to a very similar extents, i.e. two- to threefold, within 30 seconds (Fig. 3A,B,D,E), and both increases occurred during the latent periods for the $[Ca^{2+}]_i$ rise (Fig. 3C,F).

Egg contents of cGMP and IP_3 before fertilization and at their peaks were compared with previously reported values. Ciapa and Epel (Ciapa and Epel, 1996) have reported for cGMP content of *Paracentrotus lividus* eggs to be 0.41±0.05 pmoles/mg protein (n=11) via radioimmunoassay before fertilization, and to double within 20 seconds of fertilization. Lee and Shen (Lee and Shen, 1998) have reported for IP_3 contents of *Lytechinus pictus* eggs to be 0.812±0.071 pmoles/mg protein (n=4) before fertilization, and 5.012±0.622 pmoles/mg protein (n=4) 2 minutes after insemination. Our obtained cGMP contents were approximately double those reported by Ciapa and Epel (Ciapa and Epel, 1996), and the IP_3 contents were two- to fourfold of those reported by Lee and Shen (Lee and Shen, 1998). The reason for these differences is probably a difference in the species used.

Demonstration of IP_3 production under artificial $[Ca^{2+}]_i$ elevation

The temporal relationship between IP_3 and $[Ca^{2+}]_i$ (Fig. 1, Fig. 2) suggested the large increase of IP_3 to be secondary to the $[Ca^{2+}]_i$ increase rather than causal to it. Several reports (Ciapa and Whitaker, 1986; Swann and Whitaker, 1986; Ciapa et al., 1992) have already presented the hypothesis that Ca^{2+} causes the IP_3 production. Therefore, that inference was examined by determining IP_3 and cGMP contents in unfertilized *H. pulcherrimus* eggs in which $[Ca^{2+}]_i$ had been elevated artificially by a Ca^{2+} ionophore, A23187. Fig. 4 displays the resultant rise in $[Ca^{2+}]_i$, the corresponding effect on IP_3 and, as a control, the lack of effect on cGMP. $[Ca^{2+}]_i$

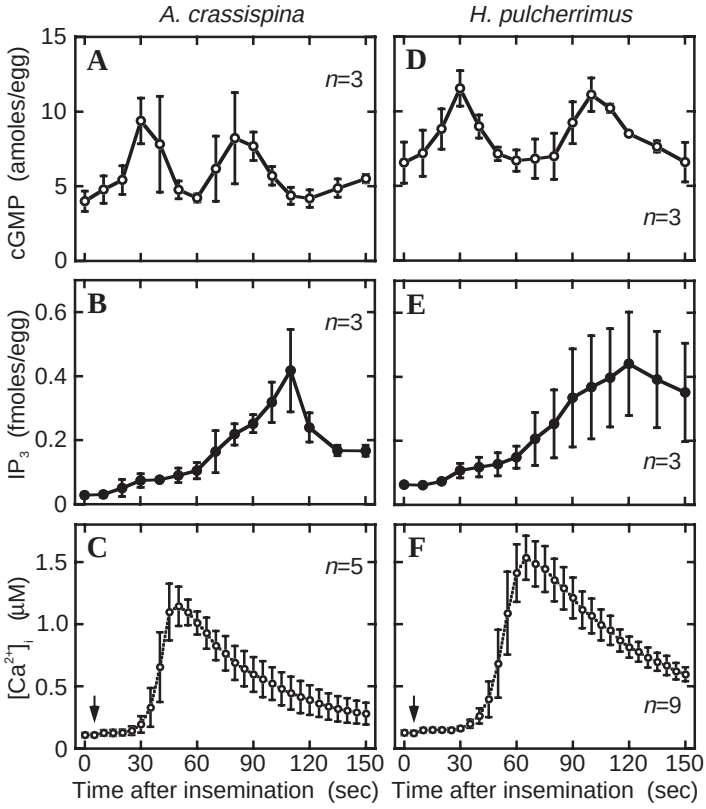

Fig. 2. Averaged time courses of the changes in cGMP and IP_3 contents of sea urchin eggs, compared with $[Ca^{2+}]_i$, after insemination. (A-C) *A. crassispina* and (D-F) *H. pulcherrimus*. cGMP (A,D) and IP_3 (B,E) values are averages from three independent experiments. Error bars represent s.d. Arrows indicate the onset of fertilization potential.

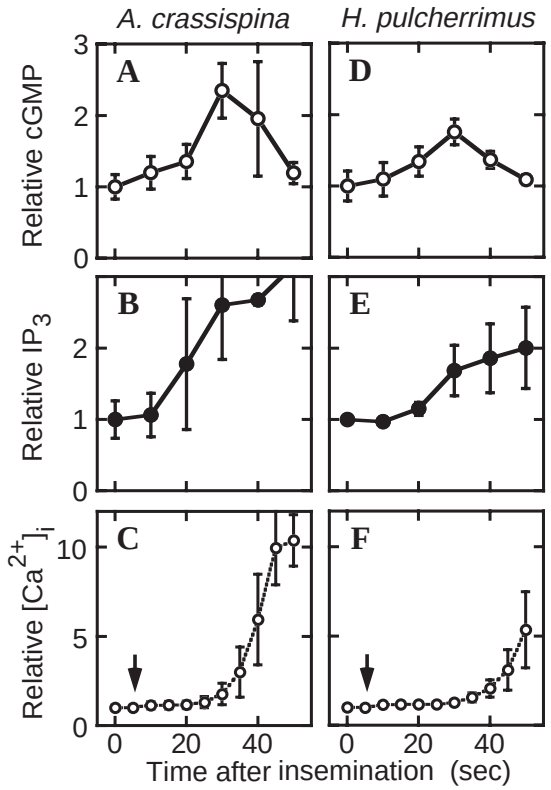

Fig. 3. Increases in cGMP (A,D) and IP_3 (B,E) contents during the latent periods of Ca^{2+} transients (C,F) in *A. crassispina* (A-C) and *H. pulcherrimus* eggs (D-F). Data for the initial 50 seconds of Fig. 2 were normalized to the cGMP content, IP_3 content or $[Ca^{2+}]_i$ of the unfertilized eggs. Arrows indicate the onset of fertilization potential.

began to increase a couple of seconds after addition of A23187 into the medium, but the rate and extent of rise depended on the extracellular Ca^{2+} ion concentration. Clearly, the artificial elevation of $[Ca^{2+}]_i$ was accompanied by the production of a large amount of IP_3 for eggs in stdASW (Fig. 4A), although for eggs in 0CaASW, A23187 caused only a very small increase of $[Ca^{2+}]_i$ and the production of a small amount of IP_3 (Fig. 4B). In *P. depressus*, also, IP_3 production was induced with a longer lag period (data not shown).

This finding indicates that an earlier $[Ca^{2+}]_i$ increase, caused by other pathways, could induce the IP_3 production, being consistent with the above-described hypothesis (Ciapa and Whitaker, 1986; Swann and Whitaker, 1986; Ciapa et al., 1992), but inconsistent with the result indicating that the chelation of intracellular Ca^{2+} did not affect the IP_3 production in fertilization of *Xenopus laevis* oocytes (Stith et al., 1994).

cGMP is produced by egg soluble guanylate cyclase

Fig. 1, Fig. 2 provide no information on the source of cGMP for the first rise. That source could be either the egg itself or the sperm's acrosomal reaction, as sperm-activating peptides such as speract and resact residing in the jelly coat of eggs are known to induce the cGMP production in sperm (Hansbrough and Garbers, 1981). In order to test the latter possibility, we measured cGMP production by sperm inseminated into a suspension of egg jelly coats at the same density as into egg suspensions. The result showed that in both species of *A. crassispin*a and *H. pulcherrimus*, cGMP produced by sperm was below the limit of detection (data not shown). It indicates that the initial rise in cGMP normally observed in sea urchin eggs at fertilization (Fig. 1, Fig. 2) represents an egg-cell reaction rather than a sperm reaction.

In order to determine which of the two main types of guanylate cyclase, soluble or membrane bound, was functional immediately after fertilization, effects of a specific inhibitor of soluble guanylate cyclase, LY83583 (Pandol and Schoeffield-Payne, 1990), on guanylate cyclase activity was examined on separate homogenates of sperm, unfertilized eggs and fertilized eggs of *P. depressus*. The results are summarized in Table 1. Sea urchin sperm are known to possess abundant membrane-bound guanylate cyclase (Radany et al., 1983; Harumi et al., 1992), and in these experiments (Table 1) the guanylate cyclase activity of sperm homogenates was indeed high, and proved almost insensitive to LY83583. Homogenates of unfertilized eggs, on the other hand, showed a very low guanylate cyclase activity, and equivalent fertilized egg homogenates displayed a reliable level about 10-fold higher that of unfertilized eggs. For both types of egg homogenates, the guanylate cyclase activity was ~65% inhibited by 200 μM LY83583. The possibility that contaminating sperm enzyme might vitiate the measurements on fertilized eggs is unlikely, because the sperm guanylate cyclase activity is insensitive to LY83583. These results suggest

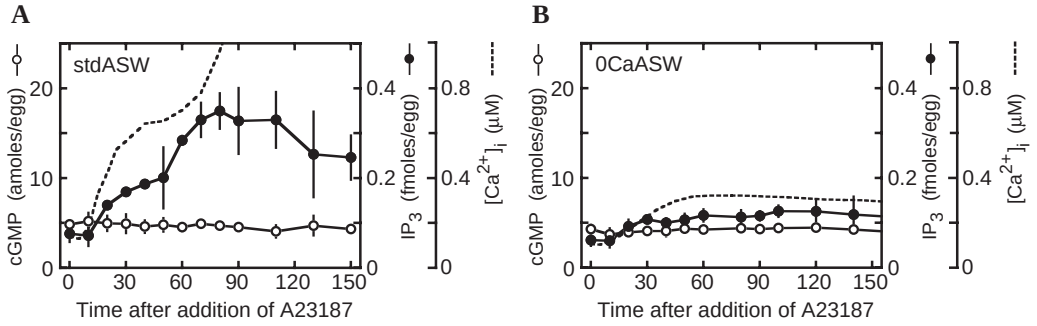

Fig. 4. Effect of artificial $[Ca^{2+}]_i$ elevation on IP_3 production. A Ca^{2+}-ionophore A23187 was applied to unfertilized *H. pulcherrimus* eggs in stdASW (A) or 0CaASW (B) to a final concentration of 10 μg/ml. Data for cGMP (white circles) and IP_3 (black circles) are averages of two experiments. Bars indicate the highest and lowest values. $[Ca^{2+}]_i$ time courses (broken line) are typical ones. $[Ca^{2+}]_i$ in stdASW (A) rose far higher than 3 μM (impossible to measure with indo-1), and 7-8 minutes later began to descend very slowly. More than 90% eggs were observed to form the fertilization envelope. Note that IP_3 levels rose along with the $[Ca^{2+}]_i$ increase, but cGMP levels did not change significantly.

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Sea urchin     1  DIVGFTTICSRIPPMAVVSMLNGMYTKFDNLSELYEVYKV  40
Rat kidney   413  DVVTFTNICAACEPIQIVNMLNSMYSKFDRLTSVHDVYKV 452
                  * * ** **    *   * *** ** *** *     ****

Sea urchin    41  ETIGDAYMVVSGAPTTTKYHAVRIAEMSLGMRESMNDLRD  80
Rat kidney   453  ETIGDAYMVVGGVPVPVESHAQRVANFALGMRISAKEVMN 492
                  ********** * *     ** * *   **** *

Sea urchin    81  PSSNNEIVKIRVGIHSGMVVAGVVGLKMPSYC 112
Rat kidney   493  PVT-GEPIQIRVGIHTGPVLAGVVGDKMPRYC 523
                  *    *   ****** * * ***** *** **
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Fig. 5. Comparison of the amino acid sequence deduced from the nucleotide sequence of a cDNA fragment from unfertilized *A. crassispina* eggs with the deduced sequence of soluble guanylate cyclase of rat kidney. The deduced amino acid sequences are indicated by single-letter code. Sequenced *A. crassispina* cDNA fragment was 338 base pairs in length. The amino acid sequence of soluble guanylate cyclase (EC 4.6.1.2) of rat kidney is taken from Yuen et al. (Yuen et al., 1990), and its catalytic domain is from residue 360 to 584 in the full length of 682 amino acid residues. The identical amino acids are indicated by asterisks below the sequences (55% match). Note the highly conservative homology in their deduced amino acid sequences.

that soluble guanylate cyclase within unfertilized eggs is probably activated upon fertilization. The existence of soluble guanylate cyclase within the eggs before fertilization is supported by the finding that the deduced amino acid sequence for one of cDNA fragments obtained from unfertilized *A. crassispina* eggs was highly homologous to the amino acid sequence of the catalytic domain of the soluble guanylate cyclase of rat kidney (Yuen et al., 1990) as shown in Fig. 5.

cADPR increases rapidly in parallel with cGMP

cGMP is claimed to stimulate Ca^{2+} release from the endoplasmic reticulum (ER) via PKG (Galione et al., 1993b) operating to phosphorylate ADP-ribosyl cyclase in sea urchin eggs, which in turn elevates the rate of synthesis of cADPR. Fig. 6 shows the representative time courses of changes in cADPR contents measured simultaneously with cGMP and IP_3 from three experiments on *A. crassispina* eggs and four on *H. pulcherrimus* eggs. In both species (*A. crassispina*: Fig. 6A,B and *H. pulcherrimus*: Fig. 6C,D), the cADPR contents rose, peaked and subsided essentially in parallel with cGMP, at least during the first minute after fertilization, although somewhat uncertain in synchrony of fertilization because of the need to mix large volumes of eggs and sperm suspension as mentioned in Materials and Methods. In addition, cADPR began to increase after cGMP (Fig. 6A,B) or reached the peak after that of cGMP (Fig. 6C). These results agrees with the above-mentioned hypothesis that cGMP stimulates the cADPR synthesis (Galione et al., 1993b; Willmott et al., 1996). The cADPR content of unfertilized eggs and its peak value were estimated in *A. crassispina* to be 5.3±1.1 amoles/egg (n=3) and 18.3±8.6 amoles/egg (n=3), respectively, and in *H. pulcherrimus* to be 4.3±6.3 amoles/egg (n=4) and 10.6±8.8 amoles/egg (n=4) respectively.

Table 1. Guanylate cyclase activity in *P. depressus* egg and sperm homogenates

Homogenates	LY83583	Guanylate cyclase activity (nmoles cGMP/min/mg protein)	n
Sperm	–	28±11	3
	+	26	2
Unfertilized eggs	–	0.20±0.09	3
	+	0.07	2
Fertilized eggs	–	2.1±0.2	3
	+	0.7	2

cGMP produced by each homogenate in the presence or absence of the inhibitor of soluble guanylate cyclase, LY83583 (200 μM), was quantitated as described in Materials and Methods, and values are averages of data at two or three different protein concentrations (mean±s.d.).

Inhibition of soluble guanylate cyclase diminishes second messengers and $[Ca^{2+}]_i$

The possibility that cGMP is involved in the induction of Ca^{2+} transients was further tested by examining the effects of

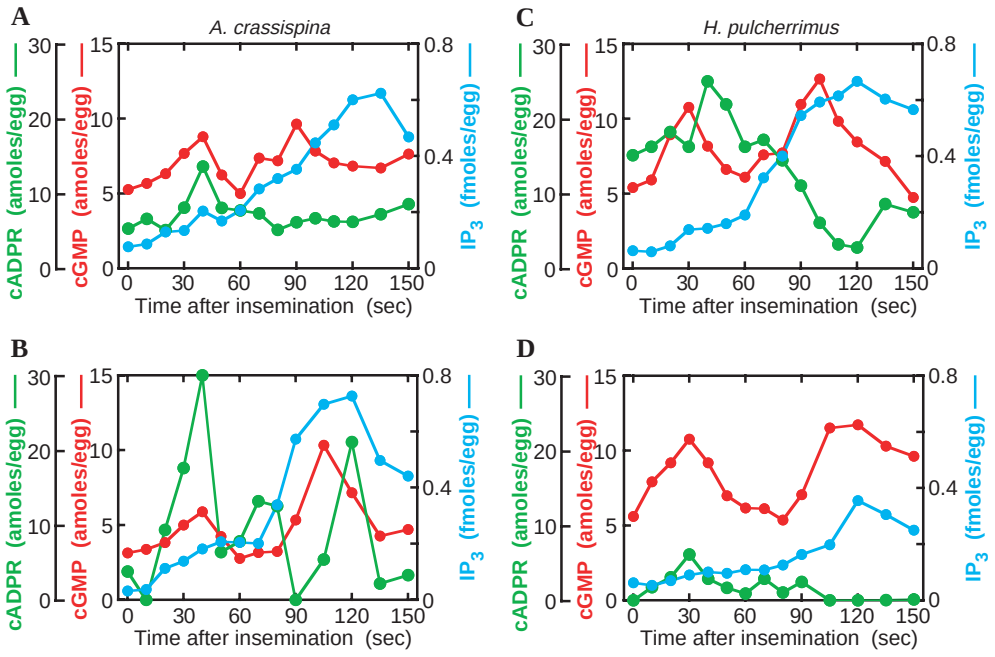

Fig. 6. Simultaneous measurements of cADPR change with the cGMP and IP_3 changes in eggs after insemination. Two representative data from three experiments on *A. crassispina* (A,B) and two from four experiments on *H. pulcherrimus* (C,D) are shown. cGMP (red), cADPR (green) and IP_3 (blue) at each sampling point were measured on the same aliquot of egg suspension. Scales for cGMP and cADPR are on the left axis in each panel and scales for $InsP_3$ are on the right axis.

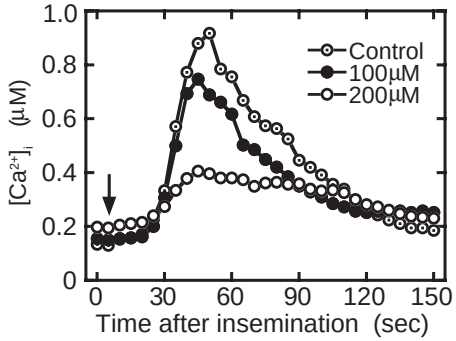

Fig. 7. Effect of preincubation of eggs in LY83583 on the Ca^{2+} transients at fertilization. *A. crassispina* eggs were incubated in stdASW containing the indicated concentration of LY83583 for 30 minutes, and then an equal volume of sperm suspension in stdASW was added. An arrow indicates the onset of fertilization potential. Each trace is a typical one.

LY83583 on the Ca^{2+} transients and the levels of the second messenger candidates. Eggs preincubated in 200 μM LY83583 showed a fertilization envelope formation, but the magnitude of elevation was reduced. These eggs did not divide. LY83583 gave the concentration-dependent inhibitory effect on the peak value of Ca^{2+} transients, but almost no effect on the length of the latent period from the action potential to the onset of explosive increase of $[Ca^{2+}]_i$ (Fig. 7). LY83583, also, did not affect on the RyR signaling pathway, as injected cGMP induced equivalent Ca^{2+} transients in LY83583-preincubated and control eggs (data not shown). Effects of LY83583 on changes in cGMP, cADPR and IP_3 contents were examined by measuring all the three second messenger candidates from the LY83583-preincubated eggs and unincubated control eggs of *A. crassispina* (Fig. 8A-C) or *H. pulcherrimus* (Fig. 8D-F). As shown in Fig. 8A,D, the increase in cGMP was considerably diminished in LY83583-preincubated eggs, compared with the control eggs, as had been expected. This lack of cGMP increase (Fig. 8A,D) accompanied the reduction of net synthesis of cADPR at least for the first 1 minute (Fig. 8B,E), being

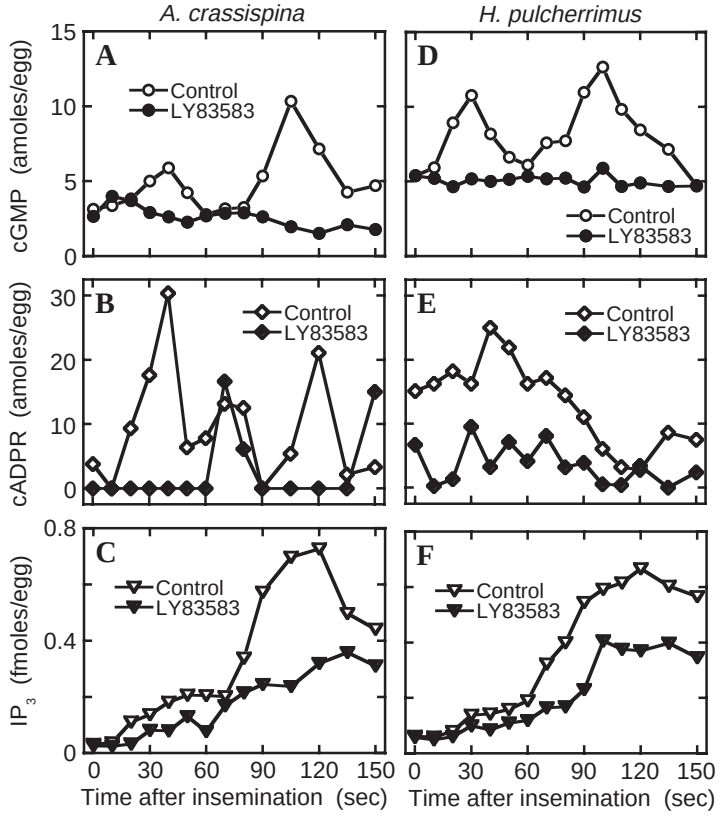

Fig. 8. Comparison of time courses of changes in cGMP (A,D), cADPR (B,E) and IP_3 (C,F) contents of LY83583 preincubated eggs with those of unincubated control eggs after insemination. *A. crassispina* eggs for LY83583-preincubation (black symbols) and control (white symbols) (A-C) were prepared from the same batch of sea urchins and *H. pulcherrimus* eggs for both experiments (D-F) were obtained from the same batch of eggs, and each set of eggs was processed in parallel. Data on *H. pulcherrimus* control eggs are the same with Fig. 6B. Note the inhibition of cADPR increase for the first minute in LY83583-preincubated eggs of both species. The reason for the cADPR spikes at 70 and 150 seconds in the presence of LY83583 is unknown.

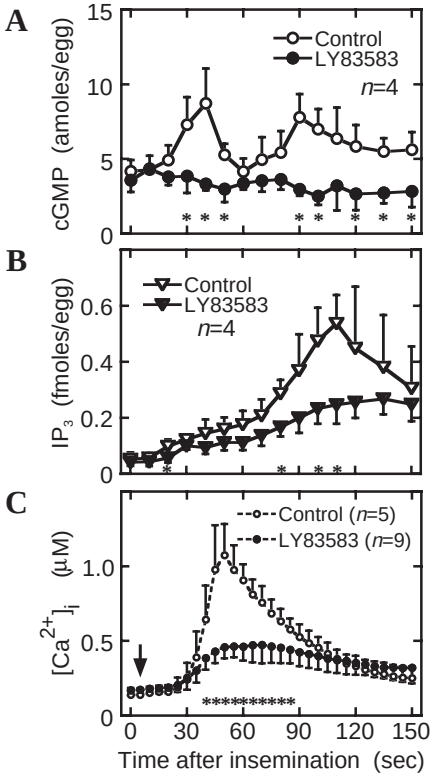

Fig. 9. Averaged time courses of the changes in cGMP (A), IP_3 (B) contents and $[Ca^{2+}]_i$ (C) in LY83583 preincubated *A. crassispina* eggs. cGMP and IP_3 contents are averages of four independent experiments and $[Ca^{2+}]_i$ data are averages of five and nine experiments for control and 200 μM LY83583, respectively. Error bars represent s.d. An arrow in C indicates the onset of fertilization potential. Asterisks indicate the points showing significant difference between LY83583 preincubated eggs (black symbols) and unincubated control eggs (white symbols) ($P<0.05$, *t*-test). Note almost no increase in cGMP and the diminution both of IP_3 increase and Ca^{2+} transients.

consistent with the contention that cGMP stimulates the cADPR production via PKG operating to phosphorylate ADP-ribosyl cyclase (Galione et al., 1993b). LY83583 reduced considerably the increase in IP_3 level (Fig. 8C,F), suggesting the cGMP-induced Ca^{2+} release affects the net synthesis of IP_3. To examine the effects of inhibition of soluble guanylate cyclase on IP_3 and Ca^{2+} transients more explicitly, the cGMP and IP_3 changes, and the $[Ca^{2+}]_i$ changes were averaged over four and nine experiments, respectively (Fig. 9). Inhibition of cGMP production diminished significantly the $[Ca^{2+}]_i$ increase, with the peak value reduced to ~45% of the control (Fig. 9C) but gave no effect on the length of the latent periods. The same effect on $[Ca^{2+}]_i$ was seen in *H. pulcherrimus* (data not shown). Inhibition of the cGMP production, also, was confirmed to reduce the IP_3 production by half (Fig. 9B), which coincided with the reduction in the $[Ca^{2+}]_i$ peak. These results strongly suggest that cGMP induces the Ca^{2+} release through RyR of ER via elevating the cADPR concentration, and released Ca^{2+} stimulates the IP_3 production, which in turn induces more Ca^{2+} release from ER.

DISCUSSION

The experiments described above were designed to identify the major chemical events that underlie initiation of the Ca^{2+} transient on fertilization of sea urchin eggs. The experiments were conducted on several species of Japanese sea urchin, and focussed on changes in cytoplasmic concentrations of Ca^{2+} itself and of the three putative chemical messengers: cGMP, cADPR and IP_3. An intrinsic problem with chemical measurements of this sort, on populations of cells, is that only average concentrations versus time are obtained. Although analysis on intracellular changes in cGMP, cADPR and IP_3 using single cells is ultimately the best way, analysis using populations of eggs, however, gave enough information to resolve quantitative and temporal differences, as shown above.

A small increase of IP_3 early in fertilization and the IP_3 production induced by the $[Ca^{2+}]_i$ elevation

One of the salient outcomes of the experiments reported above is the finding that the IP_3 peak is reached considerably later than the $[Ca^{2+}]_i$ peak (Fig. 1, Fig. 2). This large increase of IP_3 after the Ca^{2+} peak (Fig. 1, Fig. 2) can be interpreted to indicate that the IP_3 production is secondary to the $[Ca^{2+}]_i$ increase rather than causal to it. Supporting this argument, the artificial $[Ca^{2+}]_i$ elevation by A23187 actually induced IP_3 production (Fig. 4), and the artificial diminution of Ca^{2+} transient by LY83583 accompanied a reduction of IP_3 production (Fig. 8, Fig. 9).

However, the closer inspection of time courses of IP_3 changes in the early phase of fertilization (Fig. 1, Fig. 2, Fig. 9) proves a small increase of IP_3 (0.2–0.3 μM) before the onset of the explosive $[Ca^{2+}]_i$ increase in both species of *A. crassispina* and *H. pulcherrimus*. Such a concentration of IP_3 is enough to induce a $[Ca^{2+}]_i$ increase comparable with the sperm-induced Ca^{2+} transient (Whalley et al., 1992) and enough to induce an elevation of the fertilization envelope (Whitaker and Irvine, 1984). We also confirmed those. Fig. 3 clearly shows that the early increase of IP_3 was comparable with that of cGMP, although the onset of its increase was slightly later than cGMP. Thus, this early small increase in IP_3 could induce the Ca^{2+} transients at fertilization, while the significance of the large increase of IP_3 after the Ca^{2+} peak, in the egg activation, is unclear at present. Lee and Shen (Lee and Shen, 1998) have reported that the inhibition of IP_3 production with an inhibitor against phospholipase C, U73122, completely abolished the Ca^{2+} transient. However, they later reported that U73122 blocked cGMP-induced Ca^{2+} release in sea urchin eggs (Lee et al., 1998). De Nadai et al. (De Nadai et al., 1998) have shown the presence of a Ca^{2+}-sensitive phospholipase activity and PLCγ protein in sea urchin eggs. Carroll et al. (Carroll et al., 1999) and Shearer et al. (Shearer et al., 1999) have shown that the microinjection of the tandem SH2 domains of bovine PLCγ, which is linked to a receptor or cytoplasmic tyrosine kinase, inhibits the Ca^{2+} release after fertilization of sea urchin eggs. These results could indicate that the early increase in IP_3 results from PLC action and results in release of Ca^{2+} from the ER. IP_3 production induced by the artificial $[Ca^{2+}]_i$ elevation by A23187 in unfertilized *H. pulcherrimus* eggs (Fig. 4) is also probably due to this PLC action. In *Xenopus laevis* oocytes, however, the level of $[Ca^{2+}]_i$ does not affect the IP_3 production at fertilization (Stith et al., 1994), suggesting a species difference in the mechanism of the signal transduction at fertilization.

The RyR pathway involving cGMP and cADPR interacts with the IP_3 system in the fertilization-induced Ca^{2+} transients

As for the RyR pathway via cGMP and cADPR, on the other hand, the above-mentioned experiments showed that the fertilization-induced increase in cGMP preceded the explosive $[Ca^{2+}]_i$ rise in both species of *A. crassispina* and *H. pulcherrimus* (Fig. 1, Fig. 2, Fig. 3), that cADPR increases and decreases in parallel with cGMP at least during the first minute (Fig. 6), and that inhibition of cGMP production had a large effect on net synthesis of cADPR (Fig. 8), the IP_3 production (Fig. 8, Fig. 9) and the $[Ca^{2+}]_i$ increase (Fig. 7, Fig. 9). These findings suggest that cGMP could play a key role in the Ca^{2+} transients at fertilization of sea urchin eggs. cADPR also seems to contribute to the fertilization-induced Ca^{2+} transients, because cADPR increased in fertilized eggs to the averaged concentration of ~35 nM in *H. pulcherrimus* and ~80 nM in *A. crassispina*, estimated from the recovery of charcoal procedure (~60%) and the cell volume. Several investigators have reported that 10-150 nM cADPR induces the Ca^{2+} release in *Lytechinus pictus* eggs (Dargie et al., 1990; Galione et al., 1993a; Lee et al., 1993; Buck et al., 1994). Dargie et al. (Dargie et al., 1990) have also mentioned that cADPR induced the cortical reaction above 45 nM. Incidentally, the increase in cADPR at fertilization is remarkable in being one of a very few examples to show net synthesis of cADPR in response to extracellular stimuli (Clementi et al., 1996; Takasawa et al., 1998; Guse et al., 1999). However, cGMP injection will not induce the $[Ca^{2+}]_i$ increase in the unfertilized eggs when injected at the peak concentration (~25 nM by enzyme assay and ~110 nM by radioassay), which was estimated from the values, 9.4±1.5 amoles/egg (n=3) in *A. crassispina* or 12±1 amoles/egg (n=3) in *H. pulcherrimus*. Whalley et al. (Whalley et al., 1992) have reported that the final concentrations of more than 5 μM are required for cGMP to induce the Ca^{2+} transients, and we could barely induce at 10 μM. The above cGMP

concentrations are averaged for the entire cytoplasm of the egg. Therefore, if cGMP were to play some role in the initiation of Ca^{2+} release from the ER, cGMP production is required to be localized strictly perhaps around the sperm entry site. Such a possibility is suggested by the recent results of Kuo et al. (Kuo et al., 2000), who showed that NO was produced in sperm and eggs during fertilization, that intracellular injection of NO synthase caused the egg activation, and that NO synthase accounted for 0.4% of sperm soluble proteins. As NO is expected to activate soluble guanylate cyclase in eggs (Garthewaite, 1991), the cGMP production is likely to begin around the sperm entry site, which in turn could induce the cADPR production so that Ca^{2+} is finally released from the ER, as suggested by Willmott et al. (Willmott et al., 1996). Released Ca^{2+} could stimulate the Ca^{2+}-sensitive PLCγ, which in turn accelerates the Ca^{2+} release from the ER via the IP_3 increase. A second peak of the cGMP production after fertilization (Fig. 1, Fig. 2, Fig. 6, Fig. 8, Fig. 9) is probably related to NO synthesis by egg itself (Kuo et al., 2000).

Other evidence against the role of RyR pathway is that the inhibition of soluble guanylate cyclase, such that the cGMP increase did not occur (Fig. 8, Fig. 9), did not abolish the $[Ca^{2+}]_i$ increase (Fig. 7, Fig. 9), and did not lengthen the latent periods for the $[Ca^{2+}]_i$ rise (Fig. 7, Fig. 9). These findings imply that the RyR pathway is not solely responsible for the induction of Ca^{2+} transients. An essential point for the initiation of the $[Ca^{2+}]_i$ increase must exist elsewhere. One possibility is a putative receptor that directly links to the IP_3 system, including Src tyrosine kinase and PLCγ. An intriguing alternative hypothesis has been suggested by Kuo et al. (Kuo et al., 2000): NO activates Src tyrosine kinase in addition to guanylate cyclase and in turn PLCγ is activated. Thus, our results could not reveal the initiating event of the Ca^{2+} transients at fertilization of sea urchin eggs, but revealed the interaction of the RyR pathway with the IP_3R pathway.

Conclusion

The experiments described above showed that cGMP began to increase first, followed by cADPR and IP_3, all before the Ca^{2+} transients, and suggest that the RyR pathway involving cGMP and cADPR contributes to the Ca^{2+} transients by stimulating the IP_3 production during fertilization of sea urchin eggs.

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