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Maturation-associated increase in IP_3 receptor type 1: role in conferring increased IP_3 sensitivity and Ca^{2+} oscillatory behavior in mouse eggs

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Abstract

Maturation of mouse oocytes is accompanied by an increase in sensitivity to inositol 1,4,5-trisphosphate (IP_3)-mediated release of intracellular calcium. To test the hypothesis that the maturation-associated 1.5- to 2.0-fold increase in the mass of the type 1 IP_3 receptor (IP_3R-1) confers this increase in IP_3 sensitivity, we employed RNA interference to prevent this change in IP_3R-1 protein level. Microinjection into germinal vesicle (GV)-intact oocytes of dsRNA corresponding to the IP_3R-1 sequence resulted in a >90% reduction in the amount of maternal IP_3R-1 mRNA and prevented the maturation-associated increase in the mass of the IP_3R-1 protein. These injected oocytes matured to metaphase II, and there was no effect on the maturation-associated increases in $p34^{cdc2}$/cyclin B kinase and MAP kinase activities or the global pattern of protein synthesis. IP_3-induced cortical granule exocytosis was significantly decreased in these eggs when compared with controls previously injected with enhanced green fluorescent protein (EGFP) dsRNA. Following insemination, the IP_3R-1 dsRNA-injected eggs displayed significantly fewer Ca^{2+} transients than controls, and the duration of the first Ca^{2+} transient was about half that of controls. These results support the hypothesis that the maturation-associated increase in the mass of IP_3R-1 confers the increase in IP_3-sensitivity that is observed following oocyte maturation and is necessary for the proper Ca^{2+} oscillatory pattern following insemination.

Keywords: IP_3 receptor; Egg activation; Cortical granule exocytosis; RNAi

Introduction

Fertilization of mouse eggs induces a series of Ca^{2+} oscillations in the egg cytoplasm that are critical for sperm-induced egg activation, since restricting their number by either BAPTA (Kline and Kline, 1992) or the heavy metal chelator TPEN (Lawrence et al., 1998) inhibits cortical granule (CG) exocytosis, second polar body emission, and pronucleus (PN) formation. Furthermore, activating eggs by precise manipulation of the number of Ca^{2+} transients with electric field pulses has demonstrated that different events of egg activation require different numbers of Ca^{2+} oscillations (Ducibella et al., 2002). For example, CG exocytosis requires fewer Ca^{2+} oscillations than reinitiation of meiosis, while more Ca^{2+} oscillations are required for PN formation. This relationship ensures that the correct temporal sequence of events comprising egg activation occurs.

Intracellular Ca^{2+} stores are the source of Ca^{2+} for these oscillations, although Ca^{2+} oscillations following the initial Ca^{2+} transient require replenishment of the internal Ca^{2+} stores by external Ca^{2+} (Igusa and Miyazaki, 1983). Mouse eggs contain both IP_3 receptors and ryanodine receptors that can mediate Ca^{2+} release from internal stores (Ayabe et al., 1995; Mehlmann et al., 1996; Parrington et al., 1998). The ryanodine receptor-mediated pathway does not appear to be involved in mouse egg activation (Ayabe et al., 1995). The IP_3 receptor-mediated pathway, utilizing the type 1 IP_3 receptor (IP_3R-1), however, is required for sperm-induced egg activation, because inhibition of IP_3R-1 function blocks both the Ca^{2+} oscillations (Miyazaki et al., 1992) and all the events of egg activation (Xu et al., 1994).

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The sensitivity of Ca^{2+} release induced by IP_3 is greater in metaphase II-arrested eggs than in germinal vesicle (GV)-intact oocytes. For example, GV-intact mouse oocytes display a reduced sensitivity to agents that release Ca^{2+} and a reduced ability to undergo CG exocytosis, when compared with metaphase II-arrested eggs (Abbott et al., 1999; Fujiwara et al., 1993; Mehlmann and Kline, 1994; Mehlmann et al., 1996). The reduced ability to undergo CG exocytosis is not due to a deficiency in the CG machinery itself, since activators of protein kinase C induce CG exocytosis in GV-intact oocytes (Ducibella et al., 1993). This change in IP_3-sensitivity could result in an egg that responds to sperm with robust changes in Ca^{2+} release that, in turn, lead to the multiple responses comprising egg activation. Nevertheless, the molecular basis for the acquisition of this increase in IP_3-sensitivity is unresolved.

The 1.5- to 2-fold increase in the mass of IP_3R-1 that occurs during oocyte maturation (Fissore et al., 1999; Mehlmann et al., 1996) could confer on eggs the increased ability to respond to IP_3, as well as the ability of sperm to induce Ca^{2+} oscillations. The amount of the IP_3R-1 has been experimentally down-regulated by adenophostin, an IP_3 analog, treatment during oocyte maturation, and as anticipated, these eggs do not exhibit Ca^{2+} oscillations following insemination (Brind et al., 2000). Furthermore, correlated with the cessation of Ca^{2+} oscillations following insemination is a decrease in the mass of IP_3R-1 protein to levels similar to those in the oocyte (Brind et al., 2000; He et al., 1999; Jellerette et al., 2000); this decrease is completed around the time of PN formation. While these results confirm the importance of the IP_3-R-1 in Ca^{2+} oscillations, they do not address the biological significance of the maturation-associated increase in the IP_3R-1 mass.

RNA interference (RNAi) is a powerful method to target the selective destruction of any mRNA (Hammond et al., 2001; Hutvagner and Zamore, 2002; Zamore, 2001), and the pathway operates in the oocyte (Svoboda et al., 2000). We employed RNAi to prevent the maturation-associated increase in the mass of the IP_3R-1 protein so that the resulting eggs have similar amounts of IP_3R-1 as GV oocytes. We report that, although these oocytes mature to metaphase II, they display a reduced sensitivity to IP_3-mediated CG exocytosis. Moreover, following insemination, the first Ca^{2+} transient is of reduced duration, and the subsequent Ca^{2+} oscillations are either absent or significantly reduced in number.

Materials and methods

Collection of gametes

Sperm were collected from 12- to 24-week-old (C57BL6/J $\times$ SJL/J) F_1 males (Jackson Laboratories) as previously described (Moore et al., 1993). Fully grown GV-stage oocytes were collected from 6-week-old CF-1 females (Harlan) 44–48 h after intraperitoneal injection of 5 IU per mouse of pregnant mare's serum gonadotropin (PMSG) as previously described (Schultz et al., 1983). Cumulus cells were removed by repeated pipetting of oocytes through a fine-bore pipette. Denuded oocytes were maintained under paraffin oil in CZB medium (Chatot et al., 1989) supplemented with 0.2 μM IBMX (to inhibit meiotic maturation) at 37°C in an atmosphere of 5% CO_2 in humidified air until further use. *Zona pellucida* (ZP)-free eggs were obtained by removing ZP with acidic Tyrode's solution (Bornslaeger and Schultz, 1985).

Preparation of dsRNA

Double-stranded RNAs were prepared as previously described (Svoboda et al., 2000). For the IP_3R-1 dsRNA, mouse brain cDNA primed with oligo-dT was used as a template for PCR amplification of a 680-bp fragment near the 5′ end of IP_3R-1's open reading frame. The sequence for the forward primer was 5′-TAGGCGACAAGGTAGTTTTGAAT-3′, and for the reverse primer was 5′-GATGGGGATGTTTGTGCTGTGTA-3′. PCR cycle conditions were as follows: initial denaturation at 94°C for 4 min was followed by 36 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, finishing with a final extension at 72°C for 7 min. The PCR product was cloned into the pCRII plasmid by TA cloning (Invitrogen, CA). Positive clones were used as a template for in vitro transcription of sense and antisense RNAs with SP6 and T7 RNA polymerases. For enhanced green fluorescent protein (EGFP) dsRNA, 450-bp sense and antisense templates for reverse transcription were generated by PCR, and sense and antisense RNAs were generated by using SP6 RNA polymerase. Equimolar amounts of sense and antisense RNAs were annealed. The dsRNA was purified by phenol/chloroform extraction and ethanol precipitation. Purified dsRNAs were resuspended in water and stored at -80°C.

Microinjection of mouse oocytes and eggs

Denuded GV-intact oocytes were microinjected with $\sim$10 pl of the appropriate dsRNA as previously described (Kurasawa et al., 1989). The final concentration of dsRNA was 1–5 $\times$ 10^6 molecules per oocyte. Microinjected oocytes were cultured under paraffin oil in CZB medium supplemented with 0.2 μM IBMX (to prevent maturation) at 37°C in an atmosphere of 5% CO_2 in humidified air for 20 h to allow the destruction of homologous RNA. Oocytes were then washed and cultured in IBMX-free CZB medium for 14–16 h to allow meiotic maturation to metaphase II. MII-arrested eggs were either microinjected with $\sim$10 pl of IP_3 with the indicated final concentration or processed for the analysis of other effects of microinjected dsRNA.

In vitro fertilization and Ca^{2+} imaging

ZP-free eggs were incubated in Whitten's medium (Whitten, 1971) containing 0.01% PVA (average MW 30,000–70,000), 10 μM fura-2-AM (Molecular Probes Inc., Eugene, OR), and 0.025% Pluronic F-127 (Poenie et al., 1986) at 37°C in an atmosphere of 5% CO_2 in humidified air for 20 min. The fura-2-loaded eggs from both control and experimental groups were transferred to the same 10-μl drop of Whitten's medium containing no BSA and placed on a temperature-controlled microscope stage under laminar flow of 5% CO_2 in air. After the eggs settled down, 10 μl of Whitten's medium containing 30 mg/ml BSA and 2×10^5 sperm was gently added. For Ca^{2+} imaging, the cells were illuminated by using a 100-watt xenon are lamp; light output was passed through a Lambda 10-2 filter wheel (Sutter Instrument Co., Novato, CA) to alternate excitation wavelengths between 340 and 380 nm. Emitted light passed through a fura-2 bandpass filter cube and was recorded by using a Princeton Instruments MicroMAX CCD camera (Roper Scientific, Trenton, NJ). The emitted fluorescence was averaged for each egg, and the 340/380 emission ratios were analyzed to determine alterations in intracellular calcium by using MetaFluor software (Universal Imaging Corp., West Chester, PA).

RNA isolation and RT-PCR

Poly(A)-containing RNAs were isolated from 25 in vitro matured eggs by using magnetic beads (Dynal Biotech, NY) according to the manufacturer's microscale protocol. Prior to RNA isolation, 0.125 pg of rabbit α-globin mRNA per egg was added to the sample as an external standard (Svoboda et al., 2000; Temeles et al., 1994). Oligo-dT-primed reverse transcription was performed with Superscript II (Life Technologies, MA) according to the manufacturer's protocol. Semiquantitative PCR was performed as previously described (Svoboda et al., 2000) by using one egg equivalent for the external standard globin and the internal standard tissue plasminogen activator (*Plat*), and four egg equivalents for IP_3R-1. PCR cycle conditions for IP_3R-1 were: initial denaturation at 94°C for 4 min was followed by 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, finishing with a final extension at 72°C for 7 min. The sequence for the forward primer of IP_3R-1 was 5′-CAGGCAACCAGCAGAATCAA-3′ and for the reverse primer was 5′-TCTCCACCTCCGTATCCACA-3′. The [α-^{32}P]-dCTP-labeled PCR products were subjected to electrophoresis in an 8% polyacrylamide gel, and the signal was quantified by using the Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunoblotting

Samples of 20 eggs in vitro matured as described above or 20 oocytes cultured in the presence of IBMX for the same period of time were lysed in SDS sample buffer (Laemmli, 1970). Immunoblotting was performed as previously described (Jellerette et al., 2000), and only a single band was detected. The IP_3R-1 antibody was the generous gift of Jan Parys, and the secondary antibody was conjugated with alkaline phosphatase (Jackson Laboratory, West Grove, PA). The immunoblot was developed by using an ECF substrate (Amersham, Piscataway, NJ), and the signal was quantified by using the Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

CG staining and quantification

Three hours after IP_3 injection, the ZP was removed with acidic Tyrode's solution. ZP-free eggs were fixed, labeled with biotinylated *Lens culinaris* agglutinin (LCA; Polysciences, Warrington, PA), and detected with Texas red streptavidin (Gibco BRL, Gaitherburg, MD) as previously described (Ducibella et al., 1988). LCA-labeled CGs were quantified as previously described (Ducibella et al., 1988, 1990) by using MetaMorph software (Universal Imaging Corp., West Chester, PA), and the data are expressed as mean CG density.

Histone H1 and MAP kinase assay

Histone H1 kinase activity and MAP kinase activity in single eggs were measured as previously described (Svoboda et al., 2000)

[^{35}S]Methionine radiolabeling of eggs and two-dimensional gel electrophoresis

Radiolabeling with [^{35}S]methionine (1 mCi/ml, specific activity ~1500 Ci/mmol; Amersham) was performed as previously described (Abbott et al., 1998; Xu et al., 1994). In vitro matured eggs and oocytes cultured in the presence of IBMX for the same period of time were radiolabeled during the last 3 h of maturation/culture. About 25 eggs/oocytes from each group were transferred to lysis buffer containing 9.9 M urea, 4% NP-40, 2.2% ampholytes (pH 3–10; Genomic Solutions, Chelmsford, MA), and 100 mM dithiothreitol. Two-dimensional gel electrophoresis was performed by using the Investigator 2-D Electrophoresis System (Millipore) according to the manufacturer's instructions. Radiolabeled proteins were detected by using the Storm 860 PhosphorImager.

Results

Effects of IP_3R-1 dsRNA on the abundance of IP_3R-1 mRNA and maturation-associated increase in the mass of the IP_3R-1 protein

Since there is no new transcription during meiotic maturation, the increase in IP_3R-1 mass solely depends on

translation of stored maternal mRNA. Destruction of maternally stored IP_3R-1 mRNA should abolish the maturation-associated increase in IP_3R-1 but should not affect protein levels if the protein is stable during the time course of the experiment. We employed RNAi that has been shown to effectively reduce the level of a targeted mRNA in a variety of organisms (Bosher and Labouesse, 2000; Svoboda et al., 2000). A 680-bp dsRNA homologous to mouse brain IP_3R-1 was microinjected into GV-stage oocytes. To control for nonspecific effects of microinjecting dsRNA, a 450-bp dsRNA homologous to enhanced green fluorescence protein (EGFP) was used. In addition, it should be noted that there is no significant sequence homology as deduced from BLAST analysis between the IP_3R-1 dsRNA and the IP_3R-2 (data not shown), which is also expressed in oocytes (Parrington et al., 1998). Because RNAi requires virtually total sequence homology (Elbashir et al., 2001), it is most unlikely that other isoforms of the IP_3 receptor would be targeted.

Oocytes microinjected with dsRNAs were cultured and matured to metaphase II. RT-PCR analysis revealed that injection of IP_3R-1 dsRNA resulted in a >90% decrease in the amount of IP_3R-1 mRNA as compared with the controls (Fig. 1). Targeting also was specific, since there was no apparent decrease in the amount of the nontargeted *Plat* mRNA (Fig. 1). These results are consistent with our previous finding that dsRNA can target the destruction of a specific RNA with high efficiency and high specificity in mouse oocytes (Svoboda et al., 2000, 2001).

To determine whether the maturation-associated increase in the mass of the IP_3R-1 protein was prevented by the RNAi approach, in vitro matured metaphase II-arrested eggs previously injected with either IP_3R-1 dsRNA or EGFP dsRNA, and noninjected GV oocytes that had been cultured in the presence of IBMX for the same period of time, were subjected to gel electrophoresis and immunoblotting. Eggs that had been injected with IP_3R-1 dsRNA had similar amounts of IP_3R-1 protein as the noninjected GV oocytes (Fig. 2), suggesting that IP_3R-1 protein was stable over this time period. In contrast, the control eggs previously injected with EGFP dsRNA had the expected maturation-associated increase in IP_3R-1 protein. These experiments demonstrated that injection of IP_3R-1 dsRNA effectively eliminated the maturation-associated increase in the mass of IP_3R-1 protein (Fig. 2).

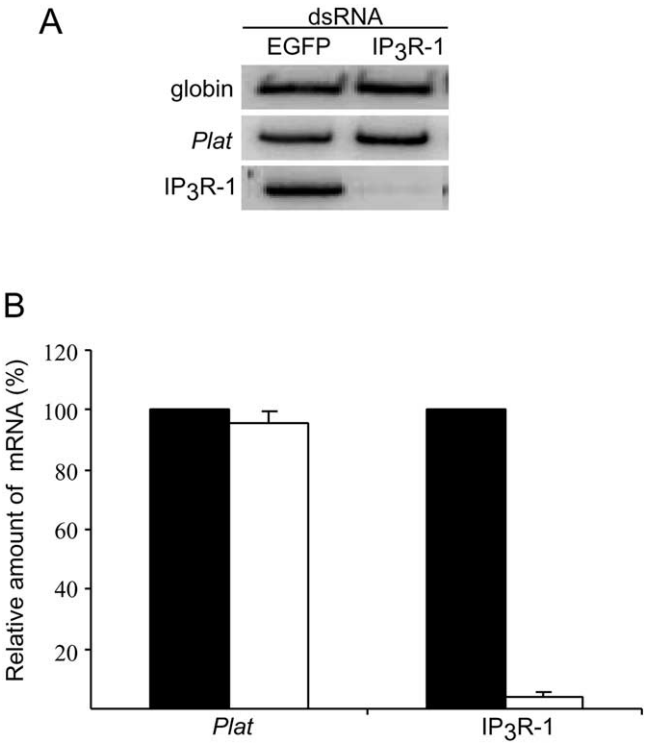

Fig. 1. Effect of IP_3R-1 dsRNA injection on IP_3R-1 mRNA. (A) RT-PCR analysis of the effect of injecting either EGFP dsRNA or IP_3R-1 dsRNA on the targeted endogenous IP_3R-1 mRNA or the nontargeted *Plat* mRNA. RNA was isolated and the relative amount of IP_3R-1 and *Plat* transcripts was determined by RT-PCR as described in Materials and methods. The intensity of the globin band permits comparison of the different lanes, since it normalizes for RNA recovery and efficiency of the RT-PCR portion of the assay. (B) Quantification of the RT-PCR analyses. The experiment was performed six times, and the results are expressed as mean ± S.E.M. Solid bars, oocytes injected with EGFP dsRNA; open bars, oocytes injected with IP_3R-1 mRNA.

Effects of IP_3R-1 dsRNA on oocyte maturation

Although the IP_3R-1 dsRNA-injected oocytes appeared to undergo normal maturation and arrest at metaphase II, it was possible that this treatment resulted in more subtle perturbations of maturation. Therefore, before examining the effect of IP_3R-1 dsRNA on IP_3 sensitivity, we examined further the specificity of the IP_3R-1 dsRNA on other aspects of oocyte maturation. Oocyte maturation is accompanied by changes in the pattern of protein synthesis that are due both to recruitment of maternal mRNAs and to posttranslation modifications (Schultz et al., 1978). In addition, both $p34^{cdc2}$/cyclin B kinase (MPF) and MAP kinase activities increase during maturation (Verlhac et al., 1994); the increase in MAP kinase is due to the recruitment of Mos mRNA that results in the generation of Mos protein that, in turn, ultimately leads to MAP kinase activation (Verlhac et al., 1996). Analysis of 2D gels revealed similar patterns of protein synthesis in control uninjected, IP_3R-1 dsRNA-injected, and EGFP dsRNA-injected oocytes that were allowed to mature to MII (Fig. 3). Moreover, there was no change in the maturation-associated increase in both MPF and MAP kinase activities (Fig. 4). These results provide further evidence for the specificity of the RNAi approach.

Effects of IP_3R-1 dsRNA on IP_3 sensitivity

Microinjected IP_3 can induce CG exocytosis and ZP modifications in MII eggs (Cran et al., 1988; Ducibella et

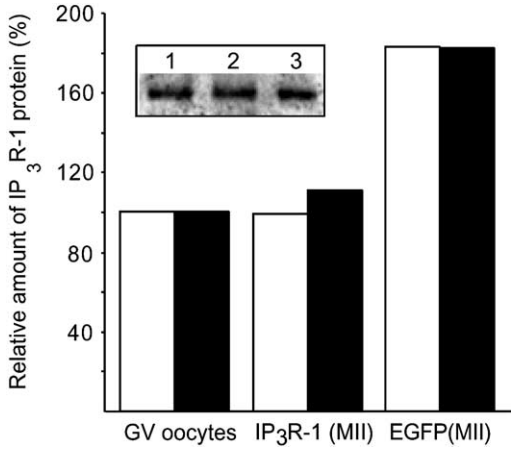

Fig. 2. Effect of IP_3R-1 dsRNA injection on IP_3R-1 protein. Oocytes were injected with either IP_3R-1 dsRNA or EGFP dsRNA, and immunoblot analysis was conducted as described in Materials and methods. Shown are the results of two experiments. Open bars, experiment 1; closed bars, experiment 2. The inset shows the results of one of the quantified immunoblots. Lane 1, GV oocytes; lane 2, oocytes injected with IP3R-1 dsRNA and matured to MII; lane 3, oocytes injected with EGFP dsRNA and matured to MII.

al., 1993). We predicted that preventing the maturation-associated increase in the mass of the IP_3R-1 by RNAi would result in an MII egg that exhibits reduced CG exocytosis in response to microinjected IP_3. To test this prediction, different concentrations of IP_3 were injected into eggs previously injected with dsRNAs, and CGs were stained and quantified 3 h after IP_3 injection. The mean CG density of eggs not injected with IP_3 was 42 CGs/100 μm^2 that was very close to the mean density of 43 CGs/100 μm^2 previously reported (Ducibella et al., 1988). When IP_3 was injected at a final calculated intracellular concentration of 10 nM, the CG density of eggs previously injected with IP_3R-1 dsRNA (29 CGs/100 μm^2) was significantly higher than that of eggs previously injected with EGFP dsRNA (16 CGs/100 μm^2) (Fig. 5). This difference was due to a difference in IP_3 sensitivity because injection of 100 nM IP_3 resulted in a significant further loss of CGs in the IP_3R-1 dsRNA-injected eggs (22 CGs/100 μm^2) but not in the controls (Fig. 5). Moreover, this difference could not be accounted for by differences in the size of the intracellular store of Ca^{2+} in the IP_3R-1 dsRNA-injected egg. There was no difference in the amplitude of the Ca^{2+} transient in response to ionomcyin treatment of IP_3R-1 and EGFP dsRNA-injected eggs in Ca^{2+}-free medium (1.01 ± .09 and 1.09 ± .07, respectively, where $n \geq 12$). Likewise, there was no difference in the duration of the Ca^{2+} transient (255 ± 19 vs 269 ± 34 s, for IP_3R-1 and EGFP dsRNA-injected eggs, respectively). Thus, preventing the maturation-associated increase in the mass of the IP_3R-1 protein resulted in eggs that displayed reduced sensitivity to IP_3, as detected by the ability of microinjected IP_3 to stimulate CG exocytosis.

Effects of IP_3R-1 dsRNA on sperm-induced Ca^{2+} oscillations

To investigate the effect of inhibiting the maturation-associated increase in the mass of the IP_3R-1 protein on sperm-induced Ca^{2+} oscillations, eggs previously injected with dsRNAs were loaded with fura-2 and inseminated after ZP removal. Ca^{2+} oscillations were monitored for 2–3 h after insemination. Different patterns of Ca^{2+} oscillations were observed in both the control and the experimental groups. A large fraction of the control eggs (50%) previously injected with EGFP dsRNA displayed Ca^{2+} oscillation patterns characteristic of monospermic eggs (Faure et al., 1999), 33% showed incomplete Ca^{2+} oscillation patterns, and 18% showed Ca^{2+} oscillation patterns characteristic of polyspermic eggs (Fig. 6A). Eggs previously in-

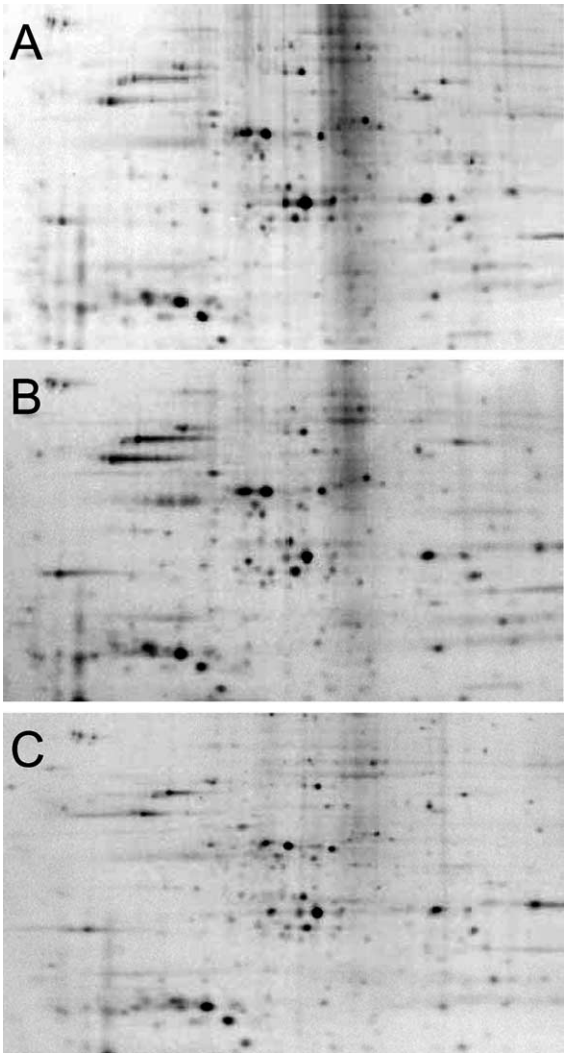

Fig. 3. Effect of IP_3R-1 dsRNA injection on maturation-associated changes in protein synthesis. Oocytes were injected with either EGFP dsRNA or IP_3R-1 dsRNA and, following maturation, were radiolabeled and subjected to 2D gel electrophoresis as described under Materials and methods. (A) Uninjected oocytes. (B) Oocytes injected with EGFP dsRNA. (C) Oocytes injected with IP_3R-1 dsRNA.

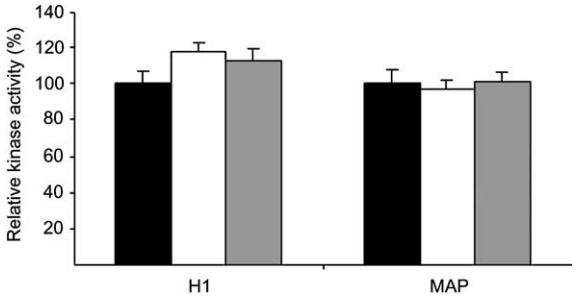

Fig. 4. Effect of IP_3R-1 dsRNA injection on maturation-associated increase in MPF and MAP kinase activities. Oocytes were injected with either EGFP dsRNA or IP_3R-1 dsRNA, and the kinase assays for both MPF and MAP in single eggs were conducted as described under Materials and methods. The experiment was conducted 2 times, and at least 12 eggs were assayed in each experiment. Similar results were obtained in each experiment, and the data, which are pooled, are expressed as the mean ± S.E.M. Solid bars, uninjected oocytes; open bars, EGFP-injected oocytes; gray bars, IP_3R-1-injected oocytes.

jected with IP_3R-1 dsRNA, on the other hand, had a significantly lower number of Ca^{2+} rises (Fig. 6B). The majority of these eggs (58%) exhibited one to three Ca^{2+} rises, 26% showed multiple but incomplete oscillations, and 16% showed normal monospermic Ca^{2+} oscillation patterns. In two separate experiments, we determined that the incidence of polyspermy was similar in the experimental and control groups (21 vs 24%, respectively); this incidence is high because of the prolonged time of incubation with sperm. These experiments suggest that the maturation-associated increase in the mass of the IP_3R-1 confers upon the egg the ability to undergo a normal pattern of Ca^{2+} oscillations in response to the fertilizing sperm.

It has been reported that GV oocytes exhibit Ca^{2+} oscillations when inseminated, and that the duration and amplitude of the first Ca^{2+} transient are significantly lower than

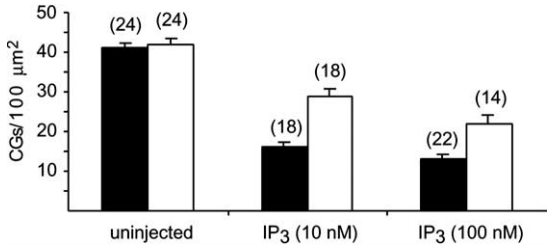

Fig. 5. Effect of microinjected IP_3 on CG exocytosis in IP_3R-1 dsRNA-injected eggs. Oocytes were injected with either EGFP dsRNA or IP_3R-1 dsRNA and, following maturation, were again injected with IP_3 to achieve the final indicated concentration. CG number was quantified as described under Materials and methods. Solid bars, EGFP dsRNA-injected oocytes; open bars, IP_3R-1-injected oocytes. The numbers in parentheses are the number of injected oocytes. The data are expressed as the mean ± S.E.M. The differences between EGFP- and IP_3R-1 dsRNA-injected cells at both IP_3 concentrations are significant ($P < 0.01$, t test). The difference between the 10 and 100 nM IP_3-injected cells was significant for cells injected with IP_3R-1 dsRNA ($P < 0.01$, t test) but did not reach significance in the EGFP dsRNA-injected cells ($P > 0.05$).

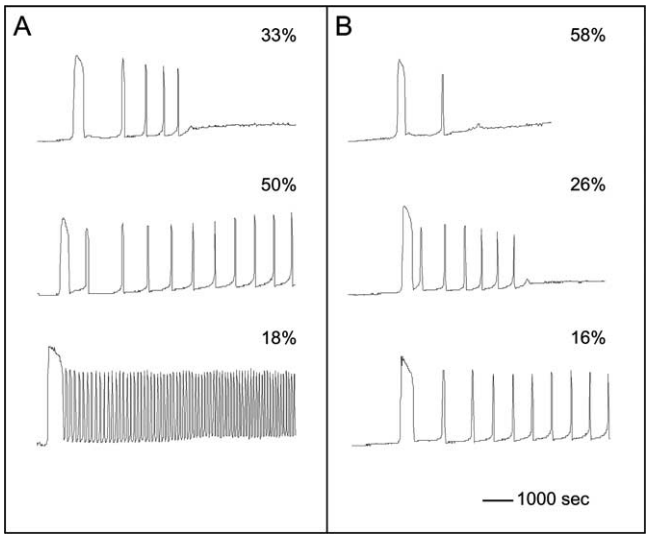

Fig. 6. Effect of IP_3R-1 dsRNA on Ca^{2+} oscillations following insemination. Oocytes were injected with either EGFP dsRNA (A) or IP_3R-1 dsRNA (B) and, following insemination, changes in intracellular Ca^{2+} were monitored as described in Materials and methods. The total number of eggs monitored in (A) was 40 and that in (B) was 38. The percentages refer to the fraction of eggs displaying the shown and representative oscillatory pattern.

those in inseminated metaphase II-arrested eggs (Mehlmann and Kline, 1994). Since eggs previously injected with IP_3R-1 dsRNA had a similar amount of IP_3R-1 protein as oocytes, we also analyzed the first Ca^{2+} transient in our experiments. While the amplitude was not affected by IP_3R-1 dsRNA injection, the duration was significantly lower than the control group (Table 1), suggesting that factors other than the amount of IP_3R-1 protein that control Ca^{2+} oscillations differ between oocytes and eggs.

Discussion

This study further supports our previous report that RNAi is an efficient method for studying gene function in oocytes (Svoboda et al., 2000). More important, however, is that our findings implicate the maturation-associated increase in the mass of the IP_3R-1 in conferring, at least in part, the increased IP_3 sensitivity, as judged by CG exocy-

Table 1
Duration and amplitude of the first Ca^{2+} transient in fertilized eggs previously injected with dsRNA

Injected dsRNA	Mean duration (s)	Mean 340/380 ratio
EGFP	628 ± 70	0.98 ± 0.07
IP_3R-1	325 ± 34	0.87 ± 0.05

Note. The experiment was performed 5 times, and similar results were obtained in each case. The data were pooled and are expressed as the mean ± S.E.M. The number of EGFP- and IP_3R-1-injected eggs analyzed was 40 and 38, respectively.

otsis and the ability of inseminated eggs to display Ca^{2+} oscillations.

As we predicted, preventing the maturation-associated increase in IP_3R-1 mass significantly decreases the sensitivity of eggs to IP_3-mediated CG exocytosis. It should be noted that eggs previously injected with IP_3R-1 dsRNA are more sensitive to IP_3 than oocytes (data not shown), although they have similar amounts of IP_3R-1 (Fig. 2). This difference implies that other factors are involved in sensitizing CG exocytosis in response to IP_3. Two candidates, which are not mutually exclusive, come to mind. The first is that a pronounced reorganization of the endoplasmic reticulum (ER) occurs during maturation of both mouse (Mehlmann et al., 1995) and hamster (Shiraishi et al., 1995) oocytes. For example, in mouse oocytes, ER accumulations exist in the interior, but not the cortical region. Following meiotic maturation, clusters of the ER exist only in the cortex, the region containing CGs (Mehlmann et al., 1996). Since the IP_3-sensitive Ca^{2+} store resides in the ER (Miyazaki et al., 1993), the reorganization of the ER that results in the intracellular Ca^{2+} stores being closer to the site of CG exocytosis could sensitize IP_3-stimulated CG release. Thus, even though the mass of the IP_3R-1 protein is similar in oocytes and the IP_3R-1 dsRNA-injected eggs, the eggs would display an increase in CG exocytosis in response to IP_3.

The two-fold maturation-associated increase in CaMKII activity (Abbott et al., 2001) could also contribute to this difference in IP_3-stimulated CG exocytosis in oocytes and IP_3R-1 dsRNA-injected eggs. One of the important consequences of the initial sperm-induced rise in Ca^{2+} is the translocation, docking, and fusion of the CGs with the plasma membrane of the egg, resulting in the block to polyspermy (Ducibella, 1996). CaMKII is implicated in regulating secretory vesicle translocation in other systems (Greengard et al., 1993). Thus, the maturation-associated increase in CaMKII that likely occurs in the IP_3R-1 dsRNA-injected eggs could also contribute to the increase in CG exocytosis in response to IP_3, when compared with oocytes.

Adenophostin-induced down-regulation of the IP_3R-1 results in eggs that do not exhibit Ca^{2+} oscillations following insemination (Brind et al., 2000). In fact, in the absence of the receptor, inseminated eggs often display no Ca^{2+} transients. While these results demonstrate the requirement for the IP_3R-1 in the Ca^{2+} oscillatory behavior following insemination, they do not address why inseminated eggs exhibit a robust Ca^{2+} oscillatory pattern while inseminated oocytes do not. Our finding that inhibiting the maturation-associated increase in the mass of the IP_3R-1 is largely responsible for conferring the oscillatory behavior following insemination provides an explanation at the molecular level for the acquisition of this property. For example, many of the IP_3R-1 dsRNA-injected eggs exhibit only 1–3 Ca^{2+} transients following insemination. Moreover, the duration of the first Ca^{2+} transient is significantly shorter than in controls. We did observe, however, that some of the dsRNA-injected eggs exhibit a Ca^{2+} oscillatory pattern similar to that observed following normal monospermic fertilization. This could be due to differences in the RNAi response in individual oocytes. Note that the measurements of the efficiency of the RNAi response to destroy the endogenous IP_3R-1 mRNA and prevent the maturation-associated increase in the mass of the IP_3R-1 protein could not be performed on single eggs. Thus, the maturation-associated increase in the mass of the IP_3R-1 protein may have crossed a threshold level in certain eggs that would then exhibit a normal Ca^{2+} oscillatory pattern. The concept of threshold values has ample precedence. For example, using an RNAi approach, we demonstrated that a critical amount of MAP kinase activity is required to maintain metaphase II arrest in mouse eggs (Svoboda et al., 2000), a result consistent with a recently proposed switch mechanism for MAP kinase activation, as well as other cellular switches (Ferrell, 1999a, 1999b). Alternatively, these eggs could be polyspermic, since inseminated oocytes typically contain 7–10 fused sperm, yet display an oscillation frequency similar to monospermic eggs (Faure et al., 1999; Mehlmann and Kline, 1994). This possibility could not be tested by staining the nuclei in these eggs, since technical difficulties precluded us from removing the eggs intact from the microscope stage.

What could be the biological raison d'etre, if any, for the maturation-associated increase in the mass of the IP_3R-1, as well as for other proteins such as CaMKII? First, the "basal" levels of these proteins present in the oocyte may permit execution of basic cellular processes related to Ca^{2+} homeostatsis and certain Ca^{2+}-dependent processes, but at the same time minimize the risk of parthenogenetic activation. The outcome could confer a selective advantage by minimizing reproductive wastage. Another consequence is that the maturation-associated increase in these proteins, which are involved in critical events of egg activation (e.g., the Ca^{2+} oscillatory behavior, CG exocytosis, and cell cycle resumption), may be an insurance policy that guarantees the production of a fertilization-competent egg.

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