Regulation of mouse egg activation: presence of ryanodine receptors and effects of microinjected ryanodine and cyclic ADP ribose on uninseminated and inseminated eggs

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Sperm-induced activation of mammalian eggs is associated with a transient increase in Ca2+ concentrations thought to be derived from inositol 1,4,5-trisphosphate-sensitive and -insensitive intracellular stores. Whereas the importance of inositol 1,4,5-trisphosphate-sensitive Ca2+ stores has been evaluated, the identity and role of inositol 1,4,5-trisphosphate-insensitive stores are poorly understood. To explore the role of the ryanodine-sensitive Ca2+ store, we first used reverse transcription-polymerase chain reaction to identify transcripts of the ryanodine receptor in eggs and determined that transcripts for the type 2 and 3 receptor were present. Immunoprecipitation of radioiodinated egg extracts with an antibody that recognizes both type 2 and 3 receptors detected specifically a band of Mr=520,000. Immunolocalization of the receptor(s) using laser-scanning confocal microscopy revealed that the receptor(s) was uniformly distributed in the cortex of the germinal vesicle-intact oocyte, but became asymmetrically localized to the cortex in a region apposed to the meiotic spindle in the metaphase II-arrested egg; this asymmetrical localization developed by metaphase I. The role of the ryanodine receptor in mouse egg activation was examined by determining the effects of microinjected ryanodine or cyclic ADP ribose on endpoints of egg activation in either uninseminated or inseminated eggs. Ryanodine induced the conversion of the zona pellucida glycoprotein ZP2 to its postfertilization form ZP2f in a biphasic concentration-dependent manner; nanomolar concentrations stimulated this conversion, whereas micromolar concentrations had no stimulatory effect. Cyclic ADP ribose also promoted the ZP2 conversion, but with a hyperbolic concentration dependence. Neither of these compounds induced cell cycle resumption. Inhibiting the inositol 1,4,5-trisphosphate-sensitive Ca2+ store did not inhibit the ryanodine-induced ZP2 conversion and, reciprocally, inhibiting the ryanodine-sensitive Ca2+ store did not inhibit the inositol 1,4,5-trisphosphate-induced ZP2 conversion. Last, treatment of eggs under conditions that would block the release of Ca2+ from the ryanodine-sensitive store had no effect on any event of egg activation following fertilization. Results of these experiments suggest that although ryanodine receptors are present and functional, release of Ca2+ from this store is not essential for sperm-induced egg activation.

Key words: mouse egg, zona pellucida, cyclic ADP ribose, ryanodine, ryanodine receptor, fertilization, egg activation, cell cycle

INTRODUCTION

In eggs of all species studied to date, with the exception of Pleurodeles waltii (Grandin and Charbonneau, 1992), a transient increase in the intracellular Ca2+ concentration occurs following fertilization and is essential for egg activation (Nucitelli, 1991; Miyazaki et al., 1993). In lower species, such as echinoderms and Xenopus laevis, only a single transient occurs that traverses the egg in a wave-like fashion and that originates at the point of sperm-egg fusion (Eisen et al., 1984; Nucitelli, 1991). The source of the Ca2+ for this initial transient, which is independent of extracellular Ca2+, originates from intracellular stores (Miyazaki et al., 1993). In mammals and ascidians, an initial Ca2+ transient also originates from intracellular stores, occurs independently of extracellular Ca2+, and spreads in a similar wave-like fashion (Miyazaki et al., 1986). Moreover, mammalian and ascidian eggs also display a series of subsequent oscillations in intracellular Ca2+. For example, in the hamster, the initial transient is followed by several subsequent transients that are shorter in duration and that occur at relatively constant intervals spaced 2-4 minutes apart (Miyazaki et al., 1993). In contrast to the initial Ca2+ wave, each of these subsequent Ca2+ transients is preceded by a slow increase in intracellular Ca2+ concentration that is followed by an abrupt increase once an apparent threshold in intracellular Ca2+ is reached. These oscillations, which arise simultaneously throughout the egg cytoplasm, can occur for several hours following fertilization (mouse, Kline and Kline, 1992; cow,
Fissore et al., 1992; pig, Sun et al., 1992) and are dependent on extracellular Ca²⁺ (Igusa and Miyazaki, 1983).

In somatic cells, the mobilization of intracellular Ca²⁺ from non-mitochondrial stores is mediated by both inositol 1,4,5-trisphosphate (IP₃) receptor-sensitive and IP₃ receptor-insensitive mechanisms (Fleischer and Inui, 1989; Berridge, 1993a,b). Regulation of Ca²⁺-release through ryanodine receptors likely represents the major source of IP₃-sensitive Ca²⁺ release (McPherson and Campbell, 1993a), and it is postulated that cyclic ADP ribose (cADPR) is the endogenous intracellular ligand that regulates Ca²⁺ release from the ryanodine-sensitive store (Lee, 1994). In sea urchin eggs, both IP₃-sensitive and -insensitive stores are present; the IP₃-insensitive store is thought to be a ryanodine receptor-sensitive store. It is inferred that both stores are mobilized and independently regulated following fertilization since inhibiting Ca²⁺ release from one store does not inhibit the release from the other store (Galione et al., 1993; Lee et al., 1993; Buck et al., 1994). In mammalian eggs, it is clear that an IP₃ receptor is present (Miyazaki et al., 1992; Xu, Kopf, Schultz, unpublished observations) and an IP₃-sensitive store is recruited following fertilization (Miyazaki et al., 1992; Xu et al., 1994). In contrast, there are conflicting reports regarding the presence of functional ryanodine receptors in mammalian eggs. The results of one study reported that microinjection of ryanodine (micromolar final intracellular concentrations) into mouse eggs results in an increase in intracellular Ca²⁺ concentrations (Swann, 1992), whereas in another study no such increase is observed (Kline and Kline, 1994). Moreover, in the mammal the role of Ca²⁺ mobilized from ryanodine-sensitive stores in events of egg activation following fertilization (e.g., cortical granule (CG) exocytosis and zona pellucida (ZP) modifications, recruitment of maternal mRNAs, and cell cycle resumption) is unresolved.

We report here that mouse eggs contain transcripts for ryanodine receptors types 2 and 3, as well as immunoreactive protein, as assessed by immunoprecipitation and laser-scanning confocal microscopy. This receptor(s) appears functional since microinjection of ryanodine-sensitive stores are present; the IP₃-insensitive store is thought to be a ryanodine receptor-sensitive store. It is inferred that both stores are mobilized and independently regulated following fertilization since inhibiting Ca²⁺ release from one store does not inhibit the release from the other store (Galione et al., 1993; Lee et al., 1993; Buck et al., 1994). In mammalian eggs, it is clear that an IP₃ receptor is present (Miyazaki et al., 1992; Xu, Kopf, Schultz, unpublished observations) and an IP₃-sensitive store is recruited following fertilization (Miyazaki et al., 1992; Xu et al., 1994). In contrast, there are conflicting reports regarding the presence of functional ryanodine receptors in mammalian eggs. The results of one study reported that microinjection of ryanodine (micromolar final intracellular concentrations) into mouse eggs results in an increase in intracellular Ca²⁺ concentrations (Swann, 1992), whereas in another study no such increase is observed (Kline and Kline, 1994). Moreover, in the mammal the role of Ca²⁺ mobilized from ryanodine-sensitive stores in events of egg activation following fertilization (e.g., cortical granule (CG) exocytosis and zona pellucida (ZP) modifications, recruitment of maternal mRNAs, and cell cycle resumption) is unresolved.

We report here that mouse eggs contain transcripts for ryanodine receptors types 2 and 3, as well as immunoreactive protein, as assessed by immunoprecipitation and laser-scanning confocal microscopy. This receptor(s) appears functional since microinjection of ryanodine or cADPR under conditions in which Ca²⁺ release from the IP₃-sensitive store is inhibited can result in cortical granule exocytosis, but not cell cycle resumption. Last, treatment of eggs under conditions that would block the release of Ca²⁺ from the ryanodine-sensitive store has no effect on any event of egg activation following fertilization.

**MATERIALS AND METHODS**

**Collection of gametes and in vitro fertilization**

Fully grown, germinal vesicle (GV)-intact oocytes and ovulated metaphase-II arrested eggs were collected from gonadotropin-primed 6-week-old female CF-1 mice (Harlan) and freed of attached cumulus cells as previously described (Schultz et al., 1983; Moore et al., 1993); the collection medium was bicarbonate-free minimal essential medium (Earle’s salt) supplemented with pyruvate (100 µg/ml), gentamicin (10 µg/ml), polyvinylpyrrolidone (3 mg/ml) (MEM/PVP) and 25 mM Hepes, pH 7.3 (MEM/Hepes). Oocytes and eggs were cultured in MEM/PVP containing 25 mM bicarbonate (MEM/Bicarbonate) at 37°C in a humidified atmosphere of 5% CO₂ in air. GV breakdown was inhibited by including 0.2 mM 3-isobutyl-1-methyl xanthine (IBMX) in the culture medium.

For in vitro fertilization (IVF), histone H1 kinase assays and morphological observations, eggs were collected and freed of cumulus cells in bicarbonate-free Waymouth medium (Gibco-BRL Life Technologies, Inc.) supplemented with 10% fetal calf serum and 20 mM Hepes, pH 7.2 (Waymouth/FCS/Hepes), and then cultured in Waymouth medium supplemented with 10% fetal calf serum and 25 mM bicarbonate (Waymouth/FCS/Bicarbonate).

Following microinjection, the eggs were cultured in Waymouth/FCS/Bicarbonate for 1 hour prior to in vitro fertilization, which was conducted as previously described (Moore et al., 1994).

**Microinjection of mouse eggs and oocytes**

Eggs or oocytes were microinjected with ~10 pl of the appropriate solution as previously described (Kurasawa et al., 1989; Williams et al., 1992). Final intracellular concentrations are indicated in the figure legends and are based on an egg volume of 200 pl. For ZP analysis, eggs or oocytes were microinjected in MEM/Hepes. IBMX was added to the medium when oocytes were microinjected. For IVF, histone H1 kinase assays, and morphological observations, eggs were microinjected in Waymouth/FCS/Hepes.

**Quantification of ZP2 to ZP2ᵢ conversion**

The ZP2 to ZP2ᵢ conversion was detected and the extent quantified as previously described (Moos et al., 1994).

**Antibodies**

A goat polyclonal antibody against purified rabbit brain ryanodine receptor, goat 43 antibody, (McPherson and Campbell, 1993b), was a generous gift of Dr Kevin Campbell (University of Iowa). Monoclonal antibody (mAb) C3-33, which is against purified rabbit cardiac ryanodine receptor (Lai et al., 1992), was generously provided by Dr Gerhard Meissner (University of North Carolina, Chapel Hill). Monoclonal antibodies 18A10 and 4C11, which are against the inositol 1,4,5-trisphosphate receptor, were generous gifts of Dr Katsuhiko Mikoshiba (University of Tokyo).

**Radioiodination, immunoprecipitation and SDS-PAGE**

Ovulated metaphase II-arrested eggs (5,000) were lysed in 100 mM NaCl, 20 mM Tris-Cl, pH 7.5, 0.5% Triton X-100, 0.5% Nonidet P-40, containing 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. Radioiodination of egg extracts and immunoprecipitation were carried out as previously described (Evans et al., 1995). In these experiments, 5 µl of goat 43 antibody and normal goat serum were used. The immune complexes were solubilized in sample buffer and subjected to SDS-PAGE in a 5% gel (Laemmli, 1970). Following electrophoresis, the gel was fixed in 10% methanol/10% acetic acid and dried. The gel was scanned with a phosphorimager (Molecular Dynamics).

**Immunofluorescence and laser-scanning confocal microscopy**

The fixation and processing of oocytes or eggs for laser-scanning confocal microscopy was conducted as previously described (Worrad et al., 1994). In these experiments, goat 43 antibody or normal goat serum was used at a 1:10 dilution. To determine the specificity of the primary antibody, the goat 43 antibody was initially incubated with a mouse cardiac extract at 4°C overnight prior to use. The secondary antibody used in these studies was Texas Red-conjugated rabbit anti-IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Staining of DNA with YOYO-1 (Molecular Probes, Inc) was also performed as previously described (Worrad et al., 1994). Fluorescence was detected on a laser-scanning confocal microscope equipped with Bio-Rad MRC-600 software. The images were printed on a Sony Model UP-5000 Color Video Printer. For each experimental series, eggs and oocytes at different stages were processed together under the same conditions and images were captured using the same filter sets.

**Reverse transcription and polymerase chain reaction**

Total RNA was extracted from oocytes/eggs as previously described.
(Temeles et al., 1994). Reverse transcription was performed on 200 oocyte/egg equivalents in a Gene Amp thin-walled tube. The reaction was carried out in 20 µl of a solution containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 unit/µl of RNasin, 10 ng/µl of oligo(dT) (Pharmacia), and 1 mM each of dATP, dGTP, dCTP and dTTP. The reaction mixture was incubated at 37°C for 2 minutes, 200 units of reverse transcriptase (Superscript II, Gibco-BRL Life Technologies, Inc.) were then added and the tube was transferred to a Perkin Elmer Cetus 9600 thermocycler. Reverse transcription was conducted for 1 hour at 42°C; the sample was then heated for 5 minutes at 99°C and kept at 4°C.

Polymerase chain reaction (PCR) was performed as previously described (Temeles et al., 1994) with the following modifications. The reaction was performed in 100 µl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.25 mM MgCl₂, 0.15 mM each of the four dNTPs, 2.5 units AmpliTaq polymerase (Cetus-Perkin Elmer), 0.4 µM each of the appropriate 3' and 5' primers, and a volume of the reverse transcription reaction that was equivalent to five oocytes/eggs.

The basic PCR program was a 94°C soak for 1 minute, followed by 60 cycles of the following programs: 94°C for 15 seconds, 53°C (type 1 receptor) or 57°C (type 2 receptor) or 47°C (type 3 receptor) for 30 seconds, and 72°C for 30 seconds; the last cycle was followed by a 5 minute extension at 72°C.

Since the mouse sequence was not available, the 3' primer used in these studies was based on an amino acid sequence common to all three cloned rabbit receptor types (amino acids 5009 to 5016); 5'-TA(C/T)GT(A/T/G/C)TGGAA(A/G)ATGA(C/T)CA(A/G)GA-3'. Two sets of 5' primers for the rabbit receptor type 1 were synthesized, 5'-CTGGTACCTGGCTCATGTG-3' and 5'-TGGGCC-TGGTACATGGTGATGT-3', which correspond to nucleotides 1421-14440 and 14506-14525, respectively (Takeshima et al., 1989). The 5' primer for the rabbit receptor type 2 (5'-GTGAAAGTGGTACATGGTGATGT-3') corresponds to bases 14693 to 14713 of the cloned rabbit cardiac muscle ryanodine receptor (Nakai et al., 1990; Otsu et al., 1990). The 5' primer for ryanodine receptor type 3 (5'-GA(T/C)ATGAA(G/A)ATGA(C/T)AACCCCG(A/T)-AT(C/A/T)TGGA-3') corresponds to bases 13888-13910 of the cloned rabbit brain ryanodine receptor (Hakamata et al., 1992), and also takes into account the amino acid sequence of the human (Sorrentino et al., 1993) and mink (Giannini et al., 1992) ryanodine receptor type 3.

DNA sequencing

Following PCR amplification, 12.5 µl of the product was removed and treated with 1 µl of a solution of 20 µg/ml of RNase A (Worthington) for 10 minutes at room temperature. After addition of 2.7 µl of 6x loading buffer (0.25% bromophenol blue in 40% sucrose), the entire sample was subjected to electrophoresis in a 2% agarose gel. The bands were located under UV light and excised with a razor blade. The amplicons were purified using GENECLEAN Kit (Bio 101 Inc.) and ligated to a plasmid (pCR II) according to the manufacturer’s instructions. Competent cells were transformed using the TA Cloning Kit (InNitrogen), spread on LB agar plates containing 60 µg/ml of ampicillin and 40 µg/ml of X-gal, and then incubated at 37°C overnight. The plasmid-containing colonies were picked and cultured in LB medium containing 60 µg/ml of ampicillin at 37°C overnight with shaking. Plasmid DNA was purified using PERFECTprep Plasmid DNA Kit (5 Prim-em3 Prime, Inc.) according to the manufacturer’s instructions. After digesting with EcoRI and NcoI, the cloned DNA samples were screened by electrophoresis on a 1.5% agarose gel. The inserts were then sequenced using an ABI Taq DyeDeoxy Terminator Cycle Sequencing kit and an ABI model 373A DNA sequencer according to the manufacturer’s instructions.

Histone H1 kinase assay

Histone H1 kinase activity in egg extracts was determined as previously described (Xu et al., 1994). Following SDS-PAGE, the 12% gel was fixed in 10% methanol/10% acetic acid and dried. Following autoradiography at ~80°C using Kodak XAR5 X-ray film the bands were quantified using image processor (Image-1/AT, Universal Imaging Corporation). For each experiment, the mean value of the H1 kinase activity of un injected eggs was arbitrarily set as 1.0 and the values obtained for eggs subjected to the appropriate experimental treatments were expressed relative to this value.

Materials

Ryanodine (>99% pure) was obtained from Calbiochem. Anhydroryanodine was from LC Laboratories. Cyclic ADPR was generously provided by Dr Hon Cheung Lee (University of Minnesota). The compounds were dissolved in 10 mM Hepes, pH 7.0. Other chemicals were from Sigma unless otherwise stated.

RESULTS

Detection of ryanodine receptor type 2 and 3 transcripts in mouse eggs

To date, three types of ryanodine receptors (RyR) have been found, namely, RyR1, RyR2 and RyR3. These receptors are encoded by different genes (McPherson and Campbell, 1993a). Prior to undertaking experiments to examine the effect of ryanodine on events of egg activation, we used reverse transcription-PCR (RT-PCR) to detect which forms of the receptor were present in mouse eggs.

Amplicons of the correct size were observed using primers designed to detect either RyR2 or RyR3 (data not shown). Sequence analysis of these amplicons revealed that they were highly homologous to the rabbit RyR 2 and RyR3, being 89% and 90%, respectively (Fig. 1A). At the amino acid level, only three differences were found for RyR2, and complete identity was found for RyR3 (Fig. 1B).

RyR1, however, was not detected in mouse eggs using two different 5' primers and a common 3' primer under several different conditions in which an amplicon of the anticipated size was detected when mouse brain RNA was used as a control (data not shown). Sequence analysis of the mouse brain amplicon revealed that it had a high degree of homology (89%) to the rabbit RyR1 (Fig. 1A); at the amino acid level, the sequences were identical (Fig. 1B). Thus, the primers used to detect RyR1 in mouse eggs were capable of amplifying RyR1 mRNA. Since no amplicon for RyR1 was observed following RT-PCR using the same amount of egg RNA in which amplicons for RyR2 and RyR3 were detected, it is likely that RyR1 was not present or was present at very low levels in the mouse egg.

Detection of ryanodine receptor protein in mouse eggs and oocytes

Using monoclonal antibody C3-33, which is against purified rabbit cardiac ryanodine receptor (Lai et al., 1992) and recognizes RyR2, immunoblotting and ECL detected an immunospecific band of the appropriate size when a mouse cardiac protein extract (25 µg) was examined, but no band corresponding to the RyR was observed when an egg protein extract (25 µg) was examined (data not shown). Since we have previously found that immunoprecipitation of radioiodinated egg extracts can be more sensitive than immunoblotting (Evans et al., 1995), radioiodinated extracts were immunoprecipitated with goat 43 antibody, which recognizes RyR2 and RyR3.
Fig. 1. (A) Nucleotide sequences for mouse (mou) and rabbit (rab) ryanodine receptor types 1-3. The numbers refer to the nucleotide number in the GenBank (accession numbers RyR1, X15209; RyR2, M59743; RyR3, X68650). The sequences of the primers used in the RT-PCR are underlined. (McPherson and Campbell, 1993b). Results of this experiment revealed the presence of a band of $M_r$=52,000 that was not present when normal goat serum was used instead of goat 43 antibody (Fig. 2).

Using laser-scanning confocal microscopy and the goat 43 antibody, a fluorescent signal was asymmetrically localized to the cortex of the egg (Fig. 3A). This signal was likely to be specific since no signal was observed when normal goat serum was used instead of the primary antibody (Fig. 3B). In addition, if the goat 43 antibody was initially incubated with a mouse cardiac protein extract, the intensity of the signal was substantially reduced (Fig. 3C). This loss of signal intensity was likely due to the adsorption of the antibodies by RyRs present in the cardiac extract, since the asymmetric fluorescent signal was still observed following incubation of the goat 43 antibody with a similar concentration of BSA (Fig. 3D).

The asymmetric distribution of the RyR in metaphase II arrested eggs prompted us to examine its distribution in the GV-intact oocyte in order to determine whether this asymmetry existed in the GV-intact oocyte or whether it developed during meiotic maturation. The receptor was distributed symmetrically in the cortex of the GV-intact oocyte (Fig. 4A) and fluorescence was also detected in the cytoplasm. Following removal of IBMX, which permits meiosis to resume (Schultz et al., 1983), ~50% of the eggs manifested the asymmetry at 7 hours (Fig. 4B); at this time the eggs are in, or close to, metaphase I (Fig. 4F). Following 14 hours of maturation in vitro, the eggs were arrested in metaphase II (Fig. 4G) and >80% of them displayed this asymmetry (Fig. 4C); this staining pattern was the thickening of the cortical staining and the loss of cytoplasmic staining. Moreover, staining for DNA in these samples revealed that the region of reduced staining intensity corresponded to the region in which the meiotic...
spindle was located (Fig. 4D,H). Last, the development of this asymmetric staining pattern depended on resumption of meiosis, since it did not appear when the oocytes were cultured 7 hours in the presence of IBMX, which prevents germinal vesicle breakdown (data not shown).

**Effects of microinjected ryanodine and cyclic ADP ribose on the ZP2 to ZP2f conversion**

Ryanodine has been reported to release Ca\(^{2+}\) from intracellular stores in mouse eggs (Swann, 1992). It was not reported, however, if any events of egg activation occurred in response to the microinjected ryanodine. Accordingly, we examined if microinjected ryanodine could elicit cortical granule (CG) exocytosis, which results in modifications of the ZP, one of which is the proteolytic conversion of ZP2 to ZP2f (Bleil et al., 1981; Moller and Wassarman, 1989).

Microinjection of metaphase II-arrested eggs with ryanodine resulted in a concentration-dependent conversion of ZP2 to ZP2f (Fig. 5A). Moreover, this conversion was biphasic and maximal conversion was observed at about 5 nM. This stimulatory effect of ryanodine on the ZP2 to ZP2f conversion was likely due to ryanodine binding to its receptor, since microinjection of anhydroryanodine, which is structurally similar to ryanodine but binds poorly to the receptor (Pessah et al., 1985), did not promote the conversion of ZP2 to ZP2f (Fig. 5B). It should be noted that the extent of this conversion by ryanodine was significantly less than that obtained following microinjection of IP\(_3\), which also promotes the ZP2 to ZP2f conversion (Kurasawa et al., 1989) (Fig. 5A).

The effect of microinjected cADPR on the ZP2 to ZP2f conversion was also examined, since cADPR has been suggested to be the endogenous ligand for the RyR (Lee, 1994). Microinjection of metaphase II-arrested eggs with cADPR resulted in a concentration-dependent conversion of ZP2 to ZP2f; maximal conversion was observed at about 50 nM (Fig. 6A). Similar to ryanodine, the maximal extent of conversion of ZP2 to ZP2f was about the same, i.e. 50%. In contrast to ryanodine, however, no biphasic response was observed. The response elicited by cADPR was not observed when the cADPR was heat treated prior to microinjection (Fig. 6A); heat treatment hydrolyzes and inactivates cADPR (Lee, 1994).

To examine if the ZP2 conversion observed in response to microinjected cADPR could be attributed to cADPR-mediated Ca\(^{2+}\) release, eggs were microinjected with a solution containing cADPR and EGTA (the final concentrations of cADPR and EGTA were 500 \text{mM} and 5 \text{mM}, respectively). No stimulation of the IP\(_3\)-sensitive store was observed following microinjection of cADPR and EGTA (the final concentrations of cADPR and EGTA were 500 \text{mM} and 5 \text{mM}, respectively). No stimulation of the IP\(_3\)-sensitive store was observed (Fig. 6B).
1989). 18A10 inhibits the sperm-induced rise in intracellular Ca^{2+} in hamster eggs (Miyazaki et al., 1992), and in mouse eggs this antibody inhibits all aspects of sperm-induced egg activation (Xu et al., 1994).

Eggs first microinjected with 18A10 and then with ryanodine exhibited a ZP2 to ZP2 f conversion (Fig. 7A) that was similar in its extent to that observed with ryanodine injected alone (Fig. 5A). As anticipated, the same stimulatory effect was observed when the eggs were first microinjected with monoclonal antibody 4C11 prior to ryanodine microinjection (Fig. 7A); 4C11 recognizes the amino terminus of the IP_{3} receptor but does not block either IP_{3} binding or IP_{3}-induced Ca^{2+} release (Nakade et al., 1991) or events of egg activation (Xu et al., 1994). Thus, ryanodine apparently does not mobilize Ca^{2+} from an IP_{3}-sensitive store.

To provide additional evidence that the IP_{3}-sensitive and ryanodine-sensitive Ca^{2+} stores were independently regulated, eggs were first microinjected with a high concentration of ryanodine and then injected with IP_{3}. As discussed below, high concentrations of ryanodine are believed to inhibit ryanodine-

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Fig. 3. Laser-scanning confocal micrographs of metaphase II-arrested eggs stained with the goat antibody 43. Egg stained with the goat antibody 43 (A), normal goat serum (B), the goat 43 antibody initially incubated with mouse cardiac protein extract (C), or the goat 43 antibody initially incubated with BSA (D). More than 20 eggs were examined in each group and representative images are shown. Similar results were obtained using antibody rabbit 46, which recognizes all three types of ryanodine receptor (data not shown, McPherson and Campbell, 1993b).

Fig. 4. Laser-scanning confocal micrographs of oocytes at different stages of meiotic maturation stained with the goat antibody 43 (upper row) and YOYO-1 (lower row). (A,E), GV-intact oocyte; (B,F) in vitro matured metaphase I oocyte; (C,G), in vitro matured metaphase II-arrested egg; (D,H); in vivo matured metaphase II-arrested eggs. The nuclear staining observed in the GV-intact oocyte in A is not due to goat 43 antibody but to bleed through of the fluorescence due to the YOYO-1 staining. The experiment was performed two times, and more than 20 cells were examined at each time point. Similar results were obtained for each experiment and shown are representative images. It should be noted that the images collected for goat 43 antibody staining (A-D) and those collected for YOYO-1 staining (E-H) were obtained on different optical sections of the same cells.
mediated Ca\(^{2+}\) release (Meissner, 1986; Fleischer and Inui, 1989) and we noted that such concentrations did not result in any stimulation in the conversion of ZP2 to ZP2\(_f\) (Fig. 5A). Microinjection of eggs with IP\(_3\) following an initial injection with either a high concentration of ryanodine (500 \(\mu\)M final concentration) or buffer resulted in similar extents of ZP2 to ZP2\(_f\) conversion (Fig. 7B). Thus, IP\(_3\) did not appear to mobilize Ca\(^{2+}\) from a ryanodine-sensitive store.

In a similar fashion, the ability of cADPR to mobilize Ca\(^{2+}\) from a ryanodine-sensitive store was also examined. Consistent with this proposal was the observation that eggs initially injected with a high concentration of ryanodine (500 \(\mu\)M final concentration) and then injected with cADPR (500 nM final concentration) did not undergo the ZP2 to ZP2\(_f\) conversion, whereas eggs initially injected with buffer and then injected with cADPR underwent the conversion to the same extent as when either low concentrations of ryanodine (Fig. 5A) or cADPR (Fig. 6) were injected alone (Fig. 8). Moreover, results of these experiments suggested that micromolar concentrations of ryanodine inhibit Ca\(^{2+}\) release from the ryanodine-sensitive store.
Effects of microinjected ryanodine on cell cycle resumption

As alluded to above, ryanodine at nanomolar concentrations can stimulate the ZP2 to ZP2f conversion, which is one of the events of egg activation. Egg activation also results in cell cycle resumption and this is associated with a drop in histone H1 kinase activity (which is an indicator of p34cdc2/cyclin B kinase activity), emission of the second polar body, and formation of pronuclei. Consequently, the effect of microinjected ryanodine on these processes was examined. None of the concentrations of microinjected ryanodine (pM-µM) induced a drop in histone H1 kinase (Fig. 9) nor resulted in either the emission of the second polar body or pronucleus formation (data not shown). These results represent another example in which cortical granule exocytosis can be dissociated from cell cycle resumption (Endo et al., 1987; Kurasawa et al., 1989; Moore et al., 1994).

Effects of microinjected ryanodine on sperm-induced egg activation

Results of the previous experiments strongly suggested that a functional ryanodine-sensitive store existed in the mouse egg. If Ca2+ was released from this store following fertilization and was critical for events leading to egg activation, inhibiting this release should block events of egg activation following insemination. Such is the case if Ca2+ release from the IP3-sensitive store is inhibited (Xu et al., 1994). Accordingly, eggs were initially microinjected with a high concentration of ryanodine (500 µM final intracellular concentration), inseminated and then examined for endpoints of fertilization. This concentration of ryanodine was previously shown to inhibit the cADPR-induced ZP2 conversion (Fig. 8), an endpoint of fertilization with 18A10 or 4C11 followed by injection with ryanodine, and similarly, there is no significant difference between eggs microinjected with ryanodine or buffer followed by injection with IP3. 18A10-Ry indicates that the eggs were first injected with antibody 18A10 and then with ryanodine, etc.
Mouse egg activation

Utilization that occurs as a consequence of cortical granule exocytosis stimulated by an increase in intracellular Ca\(^{2+}\) occurs as a consequence of cortical granule exocytosis stimulated by an increase in intracellular Ca\(^{2+}\). These ryanodine-microinjected and inseminated eggs underwent the ZP2 to ZP2\(_f\) conversion (Fig. 10), emitted the second polar body, formed pronuclei and cleaved to the 2-cell stage (Table 1) to a similar extent as inseminated uninjected, or inseminated buffer-injected eggs.

**Acquisition of ryanodine sensitivity during oocyte maturation**

The ability of microinjected IP\(_3\) to release Ca\(^{2+}\) from intracellular stores (Fujiwara et al., 1993; Mehlmann and Kline, 1994), as well as to induce the ZP2 to ZP2\(_f\) conversion (Ducibella et al., 1993), is much greater in metaphase II-arrested eggs than in fully grown GV-intact oocytes. Thus, the sensitivity to IP\(_3\) appears to be acquired during oocyte maturation. Since ryanodine or cADPR could induce the ZP2 to ZP2\(_f\) conversion in metaphase II-arrested eggs, we examined if this response developed during oocyte maturation by assessing the ability of these agents to induce this conversion following microinjection into fully grown GV-intact oocytes. Results of these experiments demonstrated that little, if any, conversion was observed following microinjection of oocytes with either ryanodine (Fig. 11A) or cADPR (Fig. 11B). Thus, similar to the IP\(_3\) response, the ability of the metaphase II-arrested egg to respond to microinjected ryanodine or cADPR develops during oocyte maturation.

**DISCUSSION**

We report here that mouse eggs contain a ryanodine receptor (probably RyR2 and/or RyR3) that, in response to either ryanodine or cADPR, promotes the ZP2 to ZP2\(_f\) conversion but not cell cycle resumption. This response develops during oocyte maturation and is associated with a change in the localization of the receptor; in the GV-intact oocyte, it is uniformly distributed in the cortex and throughout the cytoplasm whereas, in the metaphase II-arrested egg, it is preferentially localized to the cortex in a region apposed to the meiotic spindle and appears less dense in the cytoplasm. This Ca\(^{2+}\) store appears to be regulated independently of the IP\(_3\)-sensitive Ca\(^{2+}\) store. Last, if this Ca\(^{2+}\) store is mobilized during sperm-induced egg activation, it apparently is not essential for egg activation.

**Mouse egg ryanodine receptors**

Mouse eggs contain transcripts for RyR2 and RyR3. The nature of our RT-PCR assay, however, does not permit a direct comparison of the amount of these transcripts. RyR1 is not detected under conditions in which the primers are known to work and when using similar amounts of egg RNA in which the RyR2 and RyR3 transcripts are detected. Thus, if RyR1 mRNA is present in the egg, it is likely to be of low abundance.

Table 1. Effect of microinjected ryanodine on polar body emission, pronucleus formation and cleavage following insemination

<table>
<thead>
<tr>
<th>Injectant</th>
<th>PB</th>
<th>PN</th>
<th>2-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ry 500 μM</td>
<td>31/34</td>
<td>30/34</td>
<td>30/34</td>
</tr>
<tr>
<td>Buffer</td>
<td>27/30</td>
<td>27/30</td>
<td>27/30</td>
</tr>
<tr>
<td>Uninjected</td>
<td>44/46</td>
<td>44/46</td>
<td>44/46</td>
</tr>
</tbody>
</table>

The number of metaphase II-arrested eggs that were analyzed is shown in the denominator.

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**Fig. 10.** Effect of a high concentration of microinjected ryanodine on the sperm-induced ZP2 conversion of metaphase II-arrested eggs. Eggs were injected with either ryanodine (final intracellular concentration, 500 μM) or buffer and then inseminated. The extent of the ZP2 conversion was then determined after 6 hours. The experiment was performed two times and similar results were obtained in each case. The data were pooled and are expressed as the mean ± s.e.m.

**Fig. 11.** Effect of microinjected ryanodine (A) or cyclic ADP ribose (B) on the ZP2 conversion in germinal vesicle-intact oocytes. Oocytes or eggs were injected either buffer, ryanodine (final concentration was 5 nM), or cADPR (final concentration was 50 nM) prior to determining the extent of the ZP2 conversion. Each experiment was performed two times and similar results were obtained in each case. The data were pooled and are expressed as the mean ± s.e.m.
Consistent with the presence of these transcripts is the specific detection of an appropriately sized immunoreactive protein using an antibody that recognizes either RyR2 or RyR3. Although it is possible that only one transcript is translated, it is more likely that both are translated and hence the egg has both types of receptors. This receptor complement contrasts with that of sea urchin eggs in which only RyR1 is detected by immunoblotting (McPherson et al., 1992).

The ryanodine receptor(s) in the mouse egg is preferentially localized to the cortex in the region apposed to the meiotic spindle. It should be noted that the plasma membrane overlying the meiotic spindle is amicrovillar, whereas the rest of the plasma membrane contains microvilli. In contrast, the receptor is uniformly distributed in the cortex of the GV-intact oocyte. The thickness of the cortical staining in the egg also appears greater than that in the oocyte. Moreover, the cytoplasmic staining present in the oocyte is not observed in the egg. These observations suggest that redistribution of receptors in the cortex and recruitment of cytoplasmic receptors may occur during oocyte maturation and give rise to the staining pattern in the metaphase II-arrested egg. It is interesting to note that during oocyte maturation CGs move from the interior of the egg to the cortex (Okada et al., 1986) and that a CG-free domain develops in the region of the spindle (Ducibella et al., 1988, 1990) such that in the metaphase II-arrested egg the CGs are asymmetrically distributed in the cortex, being enriched in the region apposed to the meiotic spindle (Ducibella et al., 1990), which is where the RyRs are localized. These changes in spatial organization may reflect more widespread global changes in oocyte/egg architecture that occur as the symmetrically organized oocyte matures into the asymmetrically organized egg. It should be noted that in the sea urchin, the RyR is not detected histochemically in the oocyte but is detected in the egg and is uniformly distributed in the egg cortex (McPherson et al., 1992).

**Ryanodine- or cADPR-stimulated Ca\(^{2+}\) release in mouse eggs**

Although we did not demonstrate directly that ryanodine or cADPR released Ca\(^{2+}\) in mouse eggs, it is likely that such is the case since ZP2 is converted to ZP\(_2\) following microinjection of these compounds; this conversion is thought to occur as a consequence of Ca\(^{2+}\)-dependent CG exocytosis. Also consistent with these agents releasing Ca\(^{2+}\) is the observation that the concentration-dependence of the ZP2 to ZP\(_2\) conversion for ryanodine is biphasic and that for cADPR is hyperbolic, since ryanodine releases Ca\(^{2+}\) in a biphasic manner (Meissner, 1986) and cADPR releases Ca\(^{2+}\) in sea urchin egg extracts in a hyperbolic manner (Lee, 1993).

The ability of ryanodine and cADPR to stimulate the ZP2 conversion to a similar extent (compare Figs 5A and 6A), and the inhibitory effect of high ryanodine concentrations on the cADPR-induced ZP2 conversion (Fig. 8) are consistent with these compounds mobilizing Ca\(^{2+}\) from the same store (Berridge, 1993b; Lee, 1994). [It should be noted, however, that no evidence exists to date regarding the synthesis and function of cADPR in sperm-induced mammalian egg activation.] Moreover, two lines of evidence suggest that IP\(_3\) and ryanodine/cADPR regulate Ca\(^{2+}\) release by different mechanisms and/or through different stores. First, the extent of the ZP2 conversion in response to IP\(_3\) is greater than that observed for ryanodine/cADPR (compare Figs 5A and 6A). Second, inhibiting IP\(_3\)-mediate Ca\(^{2+}\) release does not prevent the ryanodine/cADPR-induced ZP2 conversion (Fig. 7A), and reciprocally, inhibiting the ryanodine-sensitive store does not inhibit the IP\(_3\)-induced ZP2 conversion (Fig. 7B).

There could be several explanations to account for the difference in the extent of the ZP2 conversion following microinjection of either IP\(_3\) or ryanodine/cADPR. The first is that the IP\(_3\)-sensitive store is larger and hence could release more Ca\(^{2+}\) in response to IP\(_3\) (Fig. 12A). The second is that both stores are of similar size but that the receptor density in the IP\(_3\)-sensitive store is higher (Fig. 12B). A third possibility is that a single store containing both IP\(_3\)- and ryanodine-sensitive receptors exists but that IP\(_3\) receptor density is higher and/or that more Ca\(^{2+}\) is released per occupied IP\(_3\) receptor (Fig. 12C). Experiments addressed at localizing each of these receptors within a single cell and determining their densities may resolve this issue.

The inability of micromolar concentrations of ryanodine to stimulate the ZP2 conversion is consistent with a previous report that micromolar concentrations of ryanodine do not elevate intracellular Ca\(^{2+}\) concentrations in mouse eggs (Kline and Kline, 1994). At first glance, our results appear to conflict with those of Swann (1992), who reported that in mouse eggs micromolar concentrations of ryanodine raise intracellular Ca\(^{2+}\) concentrations in response to IP\(_3\) is greater than that observed
Ca\(^{2+}\) concentrations. It should be noted, however, in the report of Swann (1992) that the absolute changes in Ca\(^{2+}\) concentration were not reported, and if these changes were small, the Ca\(^{2+}\) concentration may not achieve levels critical for cortical granule exocytosis to occur. Such an interpretation is based on unpublished reports from our laboratory, which demonstrate that buffering the intracellular Ca\(^{2+}\) concentration in mouse eggs with Ca\(^{2+}\)-BAPTA result in an essentially all-or-none response in the ZP2 conversion at intracellular free Ca\(^{2+}\) concentrations between 0.5 and 1.0 \(\mu\)M (Xu, Schultz, Kopf, unpublished results).

**Acquisition of sensitivity to ryanodine during oocyte maturation**

Correlated with the maturation-associated changes in the localization of the ryanodine receptor during oocyte maturation is a change in the ability of microinjected ryanodine to elicit the ZP2 conversion. This phenomenon is similar to what we have observed for the IP\(_3\)-induced ZP2 conversion and to what others have observed for IP\(_3\)-induced Ca\(^{2+}\) release (Fujikawa et al., 1993; Mehlmann and Kline, 1994). In the case of IP\(_3\), the inability of this second messenger to induce CG exocytosis in fully grown, GV-intact oocytes correlates with a lower extent of Ca\(^{2+}\) release when compared to metaphase II-arrested eggs (Fujikawa et al., 1993; Mehlmann and Kline, 1994). It should be noted that this maturation-dependent ability of IP\(_3\) to induce CG exocytosis is not due to the competence of the CGs to undergo exocytosis, since activators of protein kinase C release the CGs in GV-intact oocytes to a similar extent as in metaphase II-arrested eggs (Ducibella et al., 1993). A similar explanation may account for the inability of ryanodine to induce CG exocytosis in GV-intact oocytes. This could be examined experimentally by determining the extent of Ca\(^{2+}\) release following microinjection of picomolar to micromolar concentrations of ryanodine into either oocytes or eggs. It is also possible that the maturation-dependent redistribution of the ryanodine receptor underlies the acquisition of this change in response. The apparent enrichment in the density of the receptor in the cortex of the metaphase II-arrested egg (Fig. 4) could result in a more localized cortical release of Ca\(^{2+}\) and thus the critical Ca\(^{2+}\) concentration needed to initiate CG exocytosis may be more readily achieved.

**Role of the ryanodine-sensitive Ca\(^{2+}\) store in sperm-induced egg activation**

A large body of evidence indicates that Ca\(^{2+}\) released following fertilization from intracellular stores is essential for complete egg activation (Nuccitelli, 1991; Kline and Kline, 1992; Miyazaki et al., 1993). The ability of sperm to activate fully mouse eggs that have been microinjected with concentrations of ryanodine that presumably inhibit Ca\(^{2+}\) release from the ryanodine-sensitive store suggests that if this store is mobilized following fertilization the resulting Ca\(^{2+}\) released from this store is not essential for any event of egg activation. This suggests in turn that the Ca\(^{2+}\) mobilized during sperm-induced egg activation originates from an alternatively regulated store. An IP\(_3\)-sensitive store is a likely candidate. Consistent with this suggestion is the observation that inhibiting Ca\(^{2+}\) release from the IP\(_3\)-sensitive store inhibits all aspects of sperm-induced egg activation, e.g., ZP2 conversion, cell cycle resumption, recruitment of maternal mRNAs (Xu et al., 1994). Thus, the mouse egg apparently mobilizes Ca\(^{2+}\) from only one of these two stores, namely, the IP\(_3\)-sensitive store. This is consistent with the observation that hamster eggs microinjected with the IP\(_3\) receptor antibody do not display a sperm-induced Ca\(^{2+}\) transient (Miyazaki et al., 1992).

The apparent use of a single Ca\(^{2+}\) store in the mouse egg following fertilization is in sharp contrast to the sea urchin egg where both stores are present and mobilized following fertilization (Lee et al., 1993; Galiana et al., 1993; Buck et al., 1994). Moreover, following fertilization, each store can, by itself, initiate egg activation (Lee et al., 1993). This could, in principle, provide a compensatory mechanism for Ca\(^{2+}\) release during fertilization in the sea urchin that appears absent in the mouse.

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**REFERENCES**


release underlying calcium waves during fertilization of sea urchin eggs. Science 261, 348-352.


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Note added in proof
For the cDNAs for mouse ryanodine receptor types 1-3 that we present in this communication, the accession numbers are U23754, U23755, U23756, respectively.