Inositol-1,4,5-Trisphosphate Receptor-1 and -3 and Ryanodine Receptor-3 May Increase Ooplasmic Ca\textsuperscript{2+} During Quail Egg Activation

Shusei Mizushima\textsuperscript{1}, Tomohiro Sasanami\textsuperscript{2}, Tamao Ono\textsuperscript{3,4}, Norio Kansaku\textsuperscript{5} and Asato Kuroiwa\textsuperscript{1}

\textsuperscript{1} Department of Biological Sciences, Faculty of Science, Hokkaido University, Kita 10 Nishi 8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan
\textsuperscript{2} Faculty of Agriculture, Shizuoka University, 836 Ohy, Shizuoka 422–8529, Japan
\textsuperscript{3} Matsumoto Dental University, 1780 Gobara, Hiro-oka, Shiojiri, Nagano 399–0781, Japan
\textsuperscript{4} Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399–4598, Japan
\textsuperscript{5} Laboratory of Animal Genetics and Breeding, Azabu University, Fuchinobe, Sagamihara 252–5201, Japan

Introduction

In all vertebrates examined to date, the fertilizing sperm immediately induces an increase in intracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]i) after sperm-egg fusion (Stricker, 1999; Runft \textit{et al}., 2002). This [Ca\textsuperscript{2+}]i increase evokes a series of events to cause egg activation, such as the resumption of egg meiosis, exocytosis of cortical granules, maternal protein synthesis, and pronuclear formation, thereby leading to initiation of the first zygotic cell cycle program (Miyazaki \textit{et al}., 1993; Stricker, 1999; Ducibella \textit{et al}., 2002; Runft \textit{et al}., 2002).

Although an intracellular [Ca\textsuperscript{2+}]i increase in fertilizing eggs is a universally conserved phenomenon in animals, the shape and pattern of the [Ca\textsuperscript{2+}]i response vary widely among species (Stricker, 1999). A technique used for \textit{in vitro} fertilization in sea urchin and frog revealed a single [Ca\textsuperscript{2+}]i increase from the sperm entry site that propagated throughout the egg within 5 min (Stricker, 1999). In mammalian eggs, the initial elevation in [Ca\textsuperscript{2+}]i is followed by periodic oscillatory increases that spike every 5–15 min and repetitive oscillations that continue until at least the pronuclei are formed (Ca\textsuperscript{2+} oscillation; Miyazaki \textit{et al}., 1993; Jones \textit{et al}., 1995; Nakada \textit{et al}., 1995). In physiologically polyspermic species such as the newt, 2–20 sperms successively enter at different points on the egg surface, with sequential increases in [Ca\textsuperscript{2+}]i occurring at each sperm entry site as small waves; however, each Ca\textsuperscript{2+} wave does not reach the opposite site of the egg (Harada \textit{et al}., 2007, 2011; Iwao, 2012). Therefore, multiple
Ca\textsuperscript{2+} waves induced by all fertilizing sperm appear to be important for propagation over the entire egg to result in complete egg activation.

Birds also exhibit physiological polyspermy during fertilization (Harper, 1904; Patterson, 1910; Fofanova, 1965; Nakanishi et al., 1990; Waddington et al., 1998). In the Japanese quail, 100–200 sperm successively enter the egg cytoplasm during fertilization, a number that is markedly higher than that in newt and even other avian species (Mizushima, 2017). We previously reported a unique pattern of increase in [Ca\textsuperscript{2+}]i in quail eggs following microinjection of 2 ng of sperm protein extract, which is equivalent to 200 sperm (SE) (Mizushima et al., 2014). SE evoked two phases of [Ca\textsuperscript{2+}]i changes: an initial transient increase in [Ca\textsuperscript{2+}]i followed by multiple long-lasting spiral-like signals. A transient Ca\textsuperscript{2+} wave was initiated at the injection site of the germinal disc immediately after SE injection and spread concentrically into the egg cytoplasm. A spiral-like Ca\textsuperscript{2+} signal then occurred at the injection site 10–15 min after microinjection and continued for at least 1 h. We also demonstrated that the initial transient Ca\textsuperscript{2+} wave was required for the resumption of second meiosis, whereas induction of the spiral-like Ca\textsuperscript{2+} signal appeared to be necessary for ensuring the completion of all events to accelerate the cell cycle progression of initial and early cleavage (Mizushima et al., 2014). Furthermore, removing extracellular Ca\textsuperscript{2+} by adding a Ca\textsuperscript{2+} chelator did not affect the induction of an increase in [Ca\textsuperscript{2+}]i, indicating that the main sources of Ca\textsuperscript{2+} during egg activation are egg organelles. Although we reported, for the first time, an increase in [Ca\textsuperscript{2+}]i in avian eggs during fertilization, the underlying cellular and molecular mechanisms have not yet been elucidated in detail.

Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) is mainly responsible for the [Ca\textsuperscript{2+}]i increase associated with fertilization in mammals (Miyazaki et al., 1992; Fissore et al., 1995; Jones and Whittingham, 1996; Lee et al., 2010; Ito et al., 2011). The essential role of ITPR1 in fertilization was confirmed using functional-blocking antibodies, with antibody injection precluding the [Ca\textsuperscript{2+}]i increase triggered by sperm penetration. Following fertilization, ITPR1 was progressively degraded, which corresponded to the termination of sperm-initiated Ca\textsuperscript{2+} oscillations at the interlephase stage (He et al., 1997; Brind et al., 2000; Jellerette et al., 2000; Malcuit et al., 2005). This down-regulation was induced by ubiquitination and subsequent degradation by the proteasome, which desensitized ITPR1 (Brind et al., 2000). Gating of ITPR1 and Ca\textsuperscript{2+} release requires binding of IP\textsubscript{3}, a product of the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC). Phospholipase C\textsubscript{zeta1} (PLC\textsubscript{Z1}) was originally isolated as a sperm-specific isoform from mice, and microinjection of its complementary RNA (cRNA) or recombinant protein into the mouse egg elicited Ca\textsuperscript{2+} oscillations similar to those observed during fertilization. Therefore, PLC\textsubscript{Z1} may be responsible for the production of IP\textsubscript{3} (Saunders et al., 2002). Previous studies on PLC\textsubscript{Z1} demonstrated its ability to induce [Ca\textsuperscript{2+}]i increases across mammalian, chicken, and fish species (Cox et al., 2002; Kouchi et al., 2005; Yoneda et al., 2006; Yoon and Fissore, 2007; Coward et al., 2005, 2011).

However, we discovered that PLC\textsubscript{Z1} was responsible for the induction of the initial transient Ca\textsuperscript{2+} increase in Japanese quail, whereas citrate synthase (CS) andaconit hydratase 2 (ACO2) were needed for long-lasting spiral-like Ca\textsuperscript{2+} oscillations. Heparin and 2-aminoethoxydiphenyl borate, antagonists of ITPRs, precluded the initial Ca\textsuperscript{2+} wave but not the long-lasting spiral-like Ca\textsuperscript{2+} signal (Mizushima et al., 2014). These findings suggest that CS- and ACO2-induced spiral-like Ca\textsuperscript{2+} oscillations are generated by cellular events that differ from the PLC\textsubscript{Z1}-induced IP\textsubscript{3} production pathway. In addition, microinjection of cyclic adenosine diphosphate-ribose, an activator of ryanodine receptors (RYRs), induced similar spiral-like Ca\textsuperscript{2+} signal patterns in eggs, which may be mediated via RYRs. Although three separate isoforms of ITPRs (ITPR1, ITPR2, and ITPR3) and RYRs (RYR1, RYR2, and RYR3), which are encoded by different genes, have been isolated from birds (Percival et al., 1994; Guillemette et al., 2005), the type of receptor responsible for the [Ca\textsuperscript{2+}]i increase during egg activation in Japanese quail remains unclear. Therefore, this study was conducted to identify the maternal isoforms of ITPRs and RYRs expressed in eggs and investigate the downregulation of ITPRs and RYRs after microinjections of sperm-borne egg-activating factors to reveal their involvement in quail egg activation.

Materials and Methods

Animals

Male and female Japanese quail, Coturnix japonica, 8–20 weeks of age (Motoki Corporation, Saitama, Japan), were maintained individually under a photoperiod of 14 h light:10 h dark (lights on at 05:00) with ad libitum access to water and a commercial diet (Muroran Uzuraen, Muroran, Japan). All experimental procedures for the care and use of animals were approved by the Animal Care and Use Committee of Hokkaido University (approval number 14-0135).

Microinjections of PLC\textsubscript{Z1}, CS, and ACO2 cRNAs

To prepare PLC\textsubscript{Z1}, CS, and ACO2 cRNAs, the PCR products of quail PLC\textsubscript{Z1}, CS, and ACO2 cloned into the pGEM-T easy vector (Mizushima et al., 2014) were subcloned into pTNT plasmids (Promega, Madison, WA, USA), which were then subjected to RNA synthesis using a Ribomax RNA synthesis system (Promega). RNA concentrations were measured using a spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific, Waltham, MA, USA).

Unfertilized eggs were recovered from the anterior magnum within 2 h of egg oviposition (Mizushima et al., 2014), and each egg was microinjected with PLC\textsubscript{Z1} (60 ng/µL), CS (100 ng/µL), and ACO2 (100 ng/µL) cRNAs and then cultured in vitro for 30 min or 3 h. All procedures used for microinjections and in vitro cultures were performed as described by Mizushima et al. (2014) and Ono et al. (1994), respectively.

mRNA Expression Analysis of ITPRs and RYRs

Total RNA was extracted from tissues such as the ovary, whole brain, heart, liver, stage X blastoderms isolated from freshly laid eggs, or the germinal discs of eggs collected from the infundibulum 30 min after predicted ovulation; 0.2 µg of
Table 1. **Oligonucleotide primers used for RT-PCR**

| Gene | Forward primer, 5' → 3' | Reverse primer, 5' → 3' | Accession number |
|------|-------------------------|-------------------------|------------------|
| **ITPR1** | GGTAAACCCGTAGATCATGAGG | GTAATCTCGTGAAGGATGCC | AB_839359 |
| **ITPR2** | GCTCAGATATCCTAGGGATCTC | ACTTCCCTCCTCATCACTGTC | AB_839360 |
| **ITPR3** | AAGGACGTAGACCCAGAAG | ACCTCCCTCAGTGATGCTC | AB_839361 |
| **RYR1** | GCTGACCGAGAAGAAGAGAGA | TCGAGAACCTACAGACCCCA | XM_032441838.1 |
| **RYR2** | AAGTCAAGATCAGGAAACGC | TGAAGCGACCTGAGTTGTTA | XM_03244314.1 |
| **RYR3** | TCAGTAGGAGAAGAACTGCT | AGTCTGCTGCAAGAGGGGC | XM_032444977.1 |
| **TUBG** | ATGCCGCGGGAGATCATCAC | GCTGACCGAGAAGAGCAAGT | XM_032443314.1 |

Total RNA was reverse-transcribed using a ReverTra Ace kit (TOYOBO, Osaka, Japan). The sample volume was 10 μL. One microliter of cDNA from germinal disc samples was amplified using gene-specific primers for the **ITPR1**, 2, and 3 and **RYR1**, 2, and 3 genes, and the γ-tubulin (TUBG) gene was amplified as an internal control for cDNA (Table 1). The specificity of PCR was confirmed by sequence analysis.

**Western Blot Analysis of ITPRs and RYRs**

Germinal discs were collected according to the method described by Mizushima et al. (2009) and dissolved in intracellular-like medium (120 mM KCl, 0.1 mM EGTA, 10 mM Na-β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, and 20 mM HEPES–NaOH, pH 7.5) by homogenization and sonication. The supernatant was collected by centrifugation at 10,000 ×g for 10 min. Each extract was heated at 70°C for 5 min, and 20 μg protein per lane was resolved by SDS-PAGE (Laemmli, 1970) on a 6% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Following transfer and blocking for 30 min using a detector block (SeraCare Life Sciences, Milford, MA, USA), the membrane was incubated at 4°C overnight with a rabbit anti-rat ITPR1 antibody (Alomone Labs Ltd., Jerusalem, Israel), rabbit anti-human ITPR3 antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA), or mouse anti-chicken RYR antibody (GenTex, Inc., Irvine, CA). The membrane was then incubated at 4°C for 1 h with a goat anti-rabbit or a donkey anti-mouse secondary antibody coupled with horseradish peroxidase (Millipore). An anti-chicken γ-tubulin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) was used to detect TUBG after separating the proteins on a 12% polyacrylamide gel. Immunoreactivity was detected using Immobilon Western Detection Reagent (Millipore) and LAS 3000 (GE Healthcare, Little Chalfont, UK). Visualized blots were digitized using ImageJ 1.48v software (NIH, Bethesda, MD, USA).

**Statistical Analysis**

Protein expression levels were normalized relative to those of the TUBG protein as an internal control. Data were expressed as the mean±standard deviation and analyzed for significant differences by ANOVA. Means were compared using Tukey’s test. Differences were considered to be significant at P<0.05.

**Results**

**Expression Profiles of ITPRs and RYRs in Quail Tissues**

To investigate the presence of all ITPR and RYR transcripts in quail, we initially analyzed mRNAs isolated from various tissues, as tissue-specific expression of each ITPR and RYR isoform has been reported in mammals (Newton et al., 1994; Giannini et al., 1995). Fig. 1 shows the mRNA expression levels of ITPRs and RYRs in the ovary, brain, heart, liver, and blastoderm of quail determined by RT-PCR. The PCR products of ITPR1 and ITPR2 were detected in all tissues, except for ITPR1 in the heart. In contrast, the PCR products of ITPR3 were only detected in the ovary, brain, and blastoderm. Analyses of the mRNA distribution of RYR1 and RYR2 revealed the expression of the former in the ovary, brain, and liver and of the latter in only the brain and heart. In contrast, the mRNA expression of RYR3 was detected in all tissues. These results demonstrate that multiple types of ITPRs and RYRs were co-expressed in most tissues, and their expression profiles were similar to those of murine tissues (Newton et al., 1994; Giannini et al., 1995). Collectively, these results indicate the presence of all transcripts encoding three distinct types of ITPRs and RYRs.
isoforms of bona fide ITPRs and RYRs in quail.

**Expression of ITPRs and RYRs in Quail Eggs**

Figure 2A shows the mRNA expression of the ITPR and RYR isoforms in the germinal discs of ovulated eggs detected by RT-PCR. The PCR products of ITPR1, ITPR3, and RYR3 showed the predicted sizes, whereas those of ITPR2, RYR1, and RYR2 were below the detection limit. Western blot analysis showed that anti-ITPR1, anti-ITPR3, and anti-RYR3 reacted with bands at approximately 270, 250, and 500 kDa, respectively (Fig. 2B). The bands did not react with the normal rabbit or mouse IgG bands. These results indicate that the ITPR1, ITPR3, and RYR3 proteins are present in ovulated eggs.

**Down-regulation of ITPRs and RYRs After a Microinjection of Sperm-borne Egg-activating Factors**

To investigate the involvement of ITPR1, ITPR3, and RYR3 in the generation of the initial Ca\(^{2+}\) wave and spiral-like Ca\(^{2+}\) oscillations, we examined the degradation of these proteins after microinjections of sperm-borne egg-activating factors. As shown in Fig. 3A and 3C, the intensities of the bands for the ITPR1 and ITPR3 proteins were both significantly weaker following triple injection of PLCZ1, CS, and ACO2 cRNA than those observed in eggs injected with solvent 30 min after microinjection. However, RYR3 was not degraded (Fig. 3A and 3C). In addition, similar degradation of the ITPR1 and ITPR3 proteins was observed 30 min after single injection of PLCZ1 cRNA (Fig. 3B and 3C). In contrast, when the eggs were microinjected with CS and ACO2 cRNAs without PLCZ1 cRNA, RYR3 protein expression as well as ITPR1 and ITPR3 protein levels did not decrease after 30 min of incubation (Fig. 3B and 3E). Time-course studies indicated that RYR3 was degraded by CS and ACO2 cRNAs with or without PLCZ1 cRNA 3 h after injection (Fig. 3D–F). In contrast, neither ITPR1 nor ITPR3 protein degradation occurred following microinjections of CS and ACO2 cRNAs, even after 3 h (Fig. 3E and 3F).

**Discussion**

In the present study, we demonstrated for the first time that ITPR1 and ITPR3 are expressed at both the mRNA and protein levels in quail eggs. In mice, although all ITPR isoform mRNAs were expressed in the egg, the ITPR1 and ITPR2 proteins were predominant (Fissore et al., 1999). The ITPR3 protein was present to a lesser extent, if any, because it was not observed by immunochemistry and its detection by Western blotting required more than 1000 eggs (Fissore et al., 1999). ITPR1 was localized in the periphery of ovulated MII mouse eggs, whereas ITPR2 was restricted to the cortical vesicle. The cortical vesicle plays a pivotal role in responding to the polyspermy block by releasing its contents outwards to make the zona pellucida refractory to the binding and fusion of a second sperm (the zona reaction). Therefore, ITPR2 may not release the large amount of Ca\(^{2+}\) required to trigger Ca\(^{2+}\) oscillations but may amplify the signaling events required for the polyspermy block in mouse eggs. In contrast, neither a membrane block nor intracellular organelles similar to the cortical vesicle have been detected in polyspermic eggs, such as in birds (Mizushima, 2017). In addition, an interesting feature of the ITPR2 mRNA expression pattern in the present study is its absence in quail eggs, although ITPR2 mRNA was expressed in all tissues studied. These results support the hypothesis that ITPR2 may be involved in the polyspermy
block, a process that does not occur in avian eggs in which polyspermic fertilization takes place.

In mammalian eggs, 70–80% of the ITPR1 protein was degraded when the eggs were microinjected with PLCZ1 cRNA or sperm (Malcuit et al., 2005). Additionally, microinjection of the anti-ITPR1 antibody completely inhibited fertilization-associated increases in \([Ca^{2+}]_i\), indicating the role of ITPR1 in the release of most of the \(Ca^{2+}\) from intracellular \(Ca^{2+}\) stores during egg activation. In contrast to mouse eggs, ITPR3 was expressed at significant levels in quail eggs, and ITPR1 and ITPR3 were both progressively degraded 30 min after the microinjection of PLCZ1 cRNA. It is important to note that the timing of ITPR degradation synchronized with that of the termination of the initial transient \([Ca^{2+}]_i\) increase (Mizushima et al., 2014). These results strongly support that gating of both ITPR1 and ITPR3 in response to sperm entry is involved in inducing the initial \([Ca^{2+}]_i\) increase. Microinjection of mouse PLCZ1 cRNA, which triggered \(Ca^{2+}\) oscillations in mouse eggs, only generated a \(Ca^{2+}\) wave and not mammalian-like \(Ca^{2+}\) oscillations in quail eggs (data not shown). A previous study reported that IP3-activated \(Ca^{2+}\) signals differ in somatic cells expressing different ITPR isoforms (Ehrlich and Watras, 1988; Khodakhah and Ogden, 1993; Hajnoczky and Thomas, 1994). This result implies that the different ITPR isoforms contribute to the distinct regulation of \(Ca^{2+}\) release from cells and may have different affinities for IP3 (Newton et al., 1994; Joseph et al., 1995). In addition, ITPR isoforms assemble as homo- or heterotetramers to form functional channels; thus, it is reasonable to postulate that the different binding affinities of IP3 to each channel in mouse and quail eggs contribute to the species-specific patterns of \([Ca^{2+}]_i\) increases (Nucifora et al., 1996).

Interestingly, RYR3 was downregulated during egg activation in quail. We previously reported that an RYR agonist generated a \(Ca^{2+}\) spike similar to that of CS- and ACO2-induced spiral-like oscillations (Mizushima et al., 2014).
Collectively, these findings and the present results suggest that the periodic and long-lasting $\text{Ca}^{2+}$ spikes are mediated via RYR3. Although the duration of CS- and ACO2-generated spiral-like oscillations has not been examined, intracytoplasmic sperm injection-treated quail eggs initiated the first cleavage 3 h after microinjections, indicating the completion of quail egg activation within 3 h of these injections (Mizushima et al., 2014). This assumption is supported by the present results showing that RYR3 was degraded 3 h after microinjection.

RYRs have not been detected in frog and hamster eggs using RYR-specific antibodies (Miyazaki et al., 1992; Parys et al., 1994). Furthermore, RYR agonists did not induce changes in $[\text{Ca}^{2+}]_i$, suggesting that RYRs do not play a major role in $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores during egg activation in these species (Miyazaki et al., 1992; Nuccitelli et al., 1993). Although conflicting findings on the presence of RYR2 and RYR3 in mouse and bovine eggs have been reported by immunological studies (Carroll and Swann, 1992; Swann, 1992; Kline and Kline, 1994; Yue et al., 1995 and 1998), evidence suggests that a functional disturbance in ITPR1 inhibits all aspects of egg activation in mice, hamsters, and humans (Miyazaki et al., 1992; Xu et al., 1994; Goud et al., 2002).

In summary, our results suggest that ITPR1, ITPR3, and RYR3 regulate the two distinct $\text{Ca}^{2+}$ signals generated by PLCZ1, CS, and ACO2 in Japanese quail. However, as single injection of CS or ACO2 cRNA did not induce significant release of $\text{Ca}^{2+}$ from quail eggs (Mizushima et al., 2014), further studies are needed to identify the cellular and biochemical components mediating $\text{Ca}^{2+}$ release from RYR3 channels.

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Conflicts of Interest

The authors declare no conflict of interest.

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