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Role of Caspase-3 Cleaved IP₃R1 on Ca²⁺ Homeostasis and Developmental Competence of Mouse Oocytes and Eggs

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Abstract

Apoptosis in most cell types is accompanied by altered Ca²⁺ homeostasis. During apoptosis, caspase-3 mediated cleavage of the type 1 inositol 1,4,5-trisphosphate receptor (IP₃R1) generates a 95-kDa C-terminal fragment (C-IP₃R1), which represents the channel domain of the receptor. Aged mouse eggs display abnormal Ca²⁺ homeostasis and express C-IP₃R1, although whether or not C-IP₃R1 expression contributes to Ca²⁺ misregulation or a decrease in developmental competency is unknown. We sought to answer these questions by injecting in mouse oocytes and eggs cRNAs encoding CIP₃R1. We found that: 1) expression of C-IP₃R1 in eggs lowered the Ca²⁺ content of the endoplasmic reticulum (ER), although, as C-IP₃R1 is quickly degraded at this stage, its expression did not impair pre-implantation embryo development; 2) expression of CIP₃R1 in eggs enhanced fragmentation associated with aging; 3) endogenous IP₃R1 is required for aging associated apoptosis, as its down-regulation prevented fragmentation, and expression of C-IP₃R1 in eggs with downregulated IP₃R1 partly restored fragmentation; 4) C-IP₃R1 expression in GV oocytes resulted in persistent levels of protein, which abolished the increase in the ER releasable Ca²⁺ pool that occurs during maturation, undermined the Ca²⁺ oscillatory ability of matured eggs and their activation potential. Collectively, this study supports a role for IP₃R1 and C-IP₃R1 in regulating Ca²⁺ homeostasis and the ER Ca²⁺ content during oocyte maturation. Nevertheless, the role of C-IP₃R1 on Ca²⁺ homeostasis in aged eggs seems minor, as in MII eggs the majority of endogenous IP₃R1 remains intact and C-IP₃R1 undergoes rapid turnover.

Keywords

IP₃R1; postovulatory egg aging; calcium homeostasis; apoptosis; oocyte quality

INTRODUCTION

In sexually mature mammals, oocytes are arrested for extended periods of time at the prophase stage of the 1st meiotic division (Fair 2010; Mehlmann 2005). Once fully grown and upon hormone stimulation, oocytes resume meiosis while undergoing numerous cellular and biochemical modifications (Azoury et al. 2009; Bhattacharya et al. 2007; Bornslaeger et al. 1988; McDougall and Levasseur 1998; Schindler 2011; Schultz et al. 1983; Skobolina

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1982) that are generally defined as maturation. At the completion of maturation and prior to ovulation, mature oocytes, hereafter referred as eggs, become arrested at the metaphase stage of the 2nd meiotic division (MII), a stage at which they will remain until fertilization. During maturation, oocytes acquire the ability to respond to fertilization with changes in the intracellular concentration of free calcium ($[Ca^{2+}]_i$), also known as $[Ca^{2+}]_i$ oscillations, which are required for egg activation (Fujiwara et al. 1993; Kume et al. 1993).

The Ca^{2+} signal elicited by the sperm displays specific parameters in terms of amplitude, duration and spatio-temporal distribution to maximize egg activation and developmental potential. It is therefore not surprising that aged mouse eggs display disturbed Ca^{2+} homeostasis (Takahashi et al. 2009; Takahashi et al. 2000). After ovulation, a time-dependent aging process starts to set in that compromises the eggs' developmental competence. These changes occur more rapidly in the oviduct (Smith and Lodge 1987; Wilcox et al. 1998; Xu et al. 1997; Zackowski and Leon 1988), although they also occur during *in vitro* culture (Abbott et al. 1998; Trounson et al. 1982). Aged eggs display several phenotypes with some eggs undergoing spontaneous fragmentation prior to fertilization, whereas in others fragmentation occurs after fertilization (Tarin et al. 1999). Fragmented mouse eggs display hallmark features of apoptosis including caspase activity and DNA cleavage (Perez et al. 1999). In these aged eggs, $[Ca^{2+}]_i$ responses induced either by fertilization or by agonists show slower frequency, smaller amplitude and shorter duration than in fresh eggs. Further, in these cells, injection of sperm extract/factor (SF) that recapitulates the oscillations induced by the sperm causes egg fragmentation rather than activation (Gordo et al. 2002; Gordo et al. 2000). Collectively, the data suggest that disturbed Ca^{2+} homeostasis is associated with reduced developmental competence and fragmentation/apoptosis in mouse eggs (Takahashi et al. 2009; Takahashi et al. 2000), although the precise mechanisms that underlie it are still poorly understood.

The inositol 1,4,5-trisphosphate receptor (IP_3R) is a large (~270KDa) tetrameric intracellular Ca^{2+} release channel primarily located in the ER. There are three isoforms of the receptor, 1, 2 and 3, which represent the main intracellular Ca^{2+} release channel in non-muscle cells (Berridge 1993); mouse oocytes/eggs express predominantly type-1 IP_3R isoform (IP_3R1) (Fissore et al. 1999; Parrington et al. 1998). IP_3R1 undergoes modifications during maturation that optimize Ca^{2+} release at the time of fertilization, including an increase in its concentration (Parrington et al. 1998; Xu et al. 2003), redistribution to the cortical area and formation of IP_3R1 clusters (Fissore et al. 1999; Mehlmann et al. 1996), and enhanced receptor phosphorylation (Deng and Shen 2000; Jellerette et al. 2004; Lee et al. 2006). The concentration of Ca^{2+} of the ER ($[Ca^{2+}]_{ER}$) also increases during maturation (Jones et al. 1995; Kline 2000; Kline et al. 1999) and may further potentiate Ca^{2+} release in MII eggs.

IP_3Rs can act as pro-apoptotic factors, as their misregulation alters Ca^{2+} homeostasis and promotes progression of the programmed cell-death program in somatic cells (Joseph and Hajnoczky 2007); further, elimination of IP_3R1 by genetic means prevents lymphocytes from undergoing apoptosis (Assefa et al. 2004; Jayaraman and Marks 1997). In addition, two signaling intermediaries of apoptosis, Cytochrome C (CytC) and caspase-3, modify the function of IP_3R1 . CytC, which is released from the mitochondria, directly increases IP_3R1

Ca²⁺ conductivity (Boehning et al. 2003), whereas caspase-3 cleaves IP₃R1 at a consensus site, D1888EVD, generating a 95-kDa C terminal fragment (C-IP₃R1) that exhibits unregulated Ca²⁺ release (Hirota et al. 1999). The presence of C-IP₃R1 has been detected in cells undergoing apoptosis such as Jurkat cells (Diaz and Bourguignon 2000), DT-40 (Assefa et al. 2004), SH-SY5Y cells (Haug et al. 2000) and PC-12 cells (Boehning et al. 2003).

In a previous study we detected C-IP₃R1 in aged mouse eggs (Zhang et al. 2011). We also found that overexpression of C-IP₃R1 blocked [Ca²⁺]_i oscillations and induced an apoptotic phenotype (Verbert et al. 2008). Nevertheless, important questions remain regarding the role of C-IP₃R1 in the aging of eggs, as C-IP₃R1 constitutes a very small proportion of the total IP₃R1 mass and expression of C-IP₃R1 cRNA induces apoptotic changes in only ~30% of injected eggs. Further, the effects of C-IP₃R1 during oocyte maturation are unknown. Therefore, to answer these questions, we injected oocytes and eggs with cRNA encoding for C-IP₃R1 and evaluated its effects on maturation and egg activation. We found that expression of C-IP₃R1 in MII eggs alters Ca²⁺ homeostasis parameters, although it has limited impact on egg fragmentation and developmental potential, whereas expression of C-IP₃R1 during maturation greatly compromises Ca²⁺ homeostasis and undermines activation competence.

MATERIAL AND METHODS

Animal care and welfare

Animals used for gamete collections herein were handled following the National Research Council's Animal Care and Welfare Guidelines. These procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts at Amherst, MA.

Egg collection and culture conditions

Superovulation was carried out as previously described (Gordo et al. 2002). CD-1 female mice, 6 to 8 weeks old, were injected with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma, St Louis, MO; all chemicals were purchased from Sigma unless otherwise specified). MII eggs were obtained from the oviducts 12-14 hr after injection of 5 IU hCG, which was administered 46-48 hr after PMSG injection. Eggs were released into HEPES-buffered tyrode-lactate solution (TL-HEPES) supplemented with 5% heat-treated fetal calf serum (FCS; Gibco BRL, Grand Island, NY) followed by treatment with 0.1% bovine testes hyaluronidase for 3-5 min to remove cumulus cells. MII eggs were thoroughly washed and transferred into 50 µl drops of KSOM (Potassium Simplex Optimized Medium; Specialty Media, Phillipsburg, NJ) containing 0.1% polyvinyl alcohol (PVA) under paraffin oil at 36.5°C and in a humidified atmosphere containing 5% CO₂. *In vitro* aging was accomplished by extending the culture time of eggs after collection in KSOM medium to 24-48 hr. Germinal vesicle (GV) oocytes were obtained from the ovaries 44 hours post PMSG in TL-HEPES supplemented with 5% heat-treated FCS and 100 µM 3-isobutyl-1-methylxanthine (IBMX). For maturation, GV oocytes were cultured for 12-14 hr in Chatot, Ziomek, and Bavister (CZB) medium (Chatot et al. 1989) containing 0.1% PVA at 36.5°C

and in a humidified atmosphere containing 5% CO₂. Fully matured eggs were evaluated by 1st polar body extrusion.

DNA Constructs and cRNA preparation

Mouse C-IP₃R1 encoding the 95-kDa caspase-3-generated C-terminal region of the receptor and the pore-dead mutant C-IP₃R1 (D2550A) (a kind gift from Dr. Geert Bultynck, K.U. Leuven, Belgium) in pcDNA-3.1(+) vector were used as a template to generate Venus tagged version of these constructs. The Venus tagged C-IP₃R1 (V-CIP₃R1) was constructed by PCR amplification of the relevant region flanked by HindIII and XbaI. The PCR fragment was cloned into the pcDNA6/myc-his A plasmid (Invitrogen, Carlsbad, CA). The C-IP₃R1(D2550A) was amplified by PCR flanked by NotI and XbaI and the PCR fragment was ligated into the pcDNA6/myc-his B with Venus in between HindIII and BamHI. For Venus construct, which encodes Venus fluorescence protein, Venus in PCs2 vector was used as a template (Nagai et al. 2002) and amplified using PCR flanked by HindIII and BamHI with Kozak sequence at the beginning and ligated into pcDNA6/myc-his B. After the sequences were confirmed (Genewiz, Cambridge, MA), the constructs were used for *in vitro* cRNA synthesis. Plasmids were linearized with AgeI and then transcribed using the mMessage/mMachine T7 Kit (Ambion, Austin, TX). Poly A tail was added to the produced cRNA by Poly (A) Tailing kit (Ambion, Austin, TX) followed by purification using the MEGAclear Kit (Ambion, Austin, TX). cRNA was stored at -80°C in single-use aliquots.

[Ca²⁺]_i imaging

[Ca²⁺]_i measurements were carried out as previously described, a maximum of 20 eggs could be monitored each time (Kurokawa and Fissore 2003). Eggs were loaded with 1.25 μM fura-2 AM (Molecular Probes, Eugene, OR) supplemented with 0.02% pluronic acid (Molecular Probes, Eugene, OR) for 20 minutes (min) at room temperature (RT). Eggs were then thoroughly washed and attached to glass-bottom dishes (MatTek Corp, Ashland, MA) in drops of FCS-free TL-HEPES under mineral oil, because eggs are inclined to stick to glass or plastic surface in the absence of a protein source. [Ca²⁺]_i values were monitored using a Nikon Diaphot microscope fitted for fluorescence measurements. Eggs were simultaneously monitored using the software SimplePCI (C-Imaging System, Cranberry Township, PA), which controls a filter wheel rotating between excitation wavelengths of 340 and 380 nm illuminated by a 75 W Xenon arc lamp. Emitted light above 510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ) every 20 seconds (s) and used to calculate fluorescence ratios of 340/380 nm.

Total releasable Ca²⁺ pool in the ER was estimated by assessing the magnitude of the [Ca²⁺]_i responses induced by addition of 10μM thapsigargin (TG) (Thastrup et al. 1990b). Eggs were maintained in Ca²⁺-free conditions, which were created by using TL-HEPES without adding CaCl₂ and supplemented with 1mM EGTA; TG was added to this media during [Ca²⁺]_i monitoring. [Ca²⁺]_i responses were then assessed by comparing the area-under-the-curve, which was calculated using the Prism software (GraphPad Software, La Jolla, CA).

Venus fluorescence imaging

Eggs were thoroughly washed and attached to glass-bottom dishes (MatTek Corp, Ashland, MA) in drops of FCS-free TL-HEPES under mineral oil as described before (Zhang et al. 2011). To monitor the degradation of expressed protein, Venus fluorescence was captured using a Nikon Diaphot microscope and the software SimplePCI (C-Imaging System, Cranberry Township, PA), which controls the excitation wavelength of 513 nm illuminated by a 75 W Xenon arc lamp and the exposure time for taking pictures. Emitted light above 510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ). Fluorescence intensity was quantified using Image J software (NIH). To investigate the cellular distribution of V-C-IP₃R1 cells were examined at RT with a confocal laser-scanning microscope (510 META, Carl Zeiss Microimaging, Inc., Germany) using an Axiovert 2 microscope outfitted with a 63 × 1.4 NA oil immersion objective lens. Z-stack images were obtained from cortical to equatorial planes every 2 to 5 μm.

Immunofluorescence

Following removal of the zona pellucida with acid tyrode's solution (pH 2.7) and after washes in 0.1% BSA-supplemented Dulbecco's PBS (DPBS-BSA), eggs were first fixed in 3.7% paraformaldehyde supplemented with 0.02% Triton X-100 and subsequently permeabilized with 0.1% Triton X-100 supplemented DPBS-BSA. Eggs were transferred into DPBS+5% normal goat serum (NGS-DPBS) for 2 hr at 4°C followed by overnight incubation at 4°C with an anti-Venus primary antibody (MBL international corporation, Woburn, MA01801) (Nagai et al. 2002). Following washing of the primary antibody, eggs were incubated with Alexa fluor 555-conjugated goat anti-rabbit IgG (Molecular Probes) for 1hr at RT. Eggs were counterstained with 5 mg/ml Hoechst 33258 or with 1 mM TO-PRO-3 iodide (Invitrogen) and then mounted using Vectashield Mounting Media (Vector Laboratories, Burlingame, CA). Slides were examined at room temperature with a confocal laser-scanning microscope (510 META, Carl Zeiss Microimaging, Inc., Jena, Germany) using an Axiovert 2 microscope outfitted with a 63 1.4 NA oil immersion objective lens. Z-stack images were obtained from cortical to equatorial planes every 2–5 μm.

Western blotting

Cell lysates from 20-40 cumulus-free eggs were prepared by adding 15μl of 2X sample buffer (SB) (Laemmli 1970), as described previously (Jellerette et al. 2004). Samples were boiled for 3 min, loaded onto NuPAGE Novex 3–8% Tris-Acetate gels (Invitrogen, Carlsbad, CA), proteins were separated using electrophoresis and transferred onto nitrocellulose membranes (Micron Separations, Westboro, MA). To detect IP₃R1 and its cleavage product, the Rbt03 antibody (1/1000; a generous gift of Dr J.B.Parys, Katholieke Universiteit, Leuven, Belgium) (Parys et al. 1995) was used to detect IP₃R1. The detection was accomplished by addition of a secondary HRP-conjugated goat anti-rabbit antibody and chemiluminescence technology (NEN Life Science Products, Boston, MA). Blots were digitally recorded using a Kodak 440 Image Station (Rochester, NY). The same membranes were stripped at 50°C for 30 min (62.5 mM Tris, 2% SDS and 100 mM 2-beta mercaptoethanol) and were then used for detecting the overexpressed Venus with Anti-Venus polyclonal antibody (1/1000, MBL International Corporation, Woburn, MA). The

detection was accomplished by a HRP-labeled secondary antibody and the blots were digitally recorded using a Kodak 440 Image Station (Rochester, NY).

Microinjection

Microinjection was performed as previously described (Gordo et al. 2000). In brief, eggs were microinjected under a Nikon Diaphot microscope (Nikon, Inc., Garden City, NY) using Narishige manipulators (Medical Systems Corp., Great Neck, NY). Reagents were loaded into glass micropipettes by aspiration and delivered by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus, Cambridge, MA). The injection volume was ~7-12 pL (1-3% of the total volume of the egg). For cRNA preparation, concentrated cRNA (2 µg/µL) was heated for 3 min at 85 °C and diluted as needed in nuclease free water before microinjection. PLC ζ cRNA was injected at 0.05 µg/µL; various C-IP $_3$ R1 cRNAs were injected at the original concentration; Venus cRNA was injected at 1 µg/µL.

IP $_3$ R1 knockdown

20 µM Adenophostin A diluted in microinjection buffer (MIB) containing 75 mM KCl and 20 mM Hepes, pH 7.0 were delivered into the ooplasm by microinjection technique described above. Eggs were kept in the presence of colcemid (Col, 100ng/ml) during the operation to prevent the cell activation.

Caspase-3 detection

Caspase-3 activity was detected by caspGLOW Fluorescein Active Caspase-3 Staining Kit (Biovision, Mountain View, California). The FIT-DEVD-FMK was diluted with KSOM (0.1% PVA) 1:300, and the cells were incubated in it for 2.5 hr. After washing with DPBS eggs were examined at RT with a confocal laser-scanning (510 META, Carl Zeiss Microimaging, Inc., Germany) using an Axiovert 2 microscope outfitted with a 63 × 1.4 NA oil immersion objective lens microscope as described before (Zhang et al. 2011).

Parthenogenetic egg activation

Eggs were incubated in Ca $^{2+}$ -free CZB medium dissolved with 10mM SrCl $_2$ for 2.5-3 hr at 36.5°C and in a humidified atmosphere containing 5% CO $_2$. After washing, eggs were transferred to KSOM medium for 6 hr and subsequently evaluated under a phase contrast microscopy for signs of activation.

Statistical analysis

Values from three or more experiments, performed on different batches of eggs, were used for evaluation of statistical significance. The Prism software (Graphpad Software) was used to draw graphs and perform the statistical comparisons using appropriate Student's t-test or one-way ANOVA. Values are shown as means±S.E.M, and significant differences were considered at *p* values <0.05.

RESULTS

Expression of C-IP₃R1 constructs in mouse oocytes and eggs

The basic structure of IP₃R1 comprises the N-terminal ligand binding domain, the regulatory domain and the channel domain (Fig. 1A) (Bosanac et al. 2002; Bosanac et al. 2004). To investigate the role of C-IP₃R1 on Ca²⁺ homeostasis in aging mouse eggs as well its effects on Ca²⁺ parameters during oocyte maturation, we generated a variety of constructs missing the N-term portion of the receptor, amino acids 1-1891, as Asp-1891 is the site of caspase-3 cleavage (Haug et al. 2000; Hirota et al. 1999); these constructs leave intact the channel portion of the receptor. We also produced a pore-dead version, CIP₃R1(D2550A), which abolishes the function of IP₃R1 and curtails its Ca²⁺ leaking properties (Khan et al. 2007). To track the cellular distribution of the exogenous receptors, C-IP₃R1 was conjugated with the sequence encoding for the fluorescent protein Venus (V-C-IP₃R1), a variant form of YFP (Nagai et al. 2002) (Fig. 1A). An added benefit of generating V-C-IP₃R1 was to enhance the analysis of IP₃R1 distribution in oocytes and eggs, which has been hindered by technical difficulties due in part to the size of mammalian eggs and difficulties in attaining high expression levels of IP₃R1 after cRNAs injections (Mehlmann et al. 1996; Shiraishi et al. 1995). Given that, V-C-IP₃R1 is significantly smaller than the wild type receptor, but still contains the transmembrane domain that localizes IP₃R1 to the ER (Galvan et al. 1999; Sayers et al. 1997; Takei et al. 1994), V-C-IP₃R1 could be a surrogate to investigate the distribution of IP₃R1 in mammalian eggs.

The cRNAs encoding for V-C-IP₃R1 and other versions of the receptor were injected into GV oocytes or eggs and protein expression was assessed by Western blotting (Fig. 1B) and by confocal laser scanning microscopy (Fig. 1C). Untagged and tagged versions of C-IP₃R1s with MWs of ~95 and 125 kDa, respectively, were detected in GV oocytes within 4 hr of injection (Fig. 1B, upper and lower panels, respectively) using an anti-IP₃R1 polyclonal antibody (Parys et al. 1995), which also detected the endogenous ~270-kDa IP₃R1 (Fig. 1B, upper and lower left panel). As expected, probing the blot with anti-Venus antibody only detected the V-C-IP₃R1 protein (Fig. 1B, lower right panel).

During maturation, the IP₃R1 undergoes reorganization similar to that described for its host organelle, the ER (Fissore et al. 1999; Kline et al. 1999; Mehlmann et al. 1995; Shiraishi et al. 1995); we found that V-C-IP₃R1 and V-C-IP₃R1(D2550A) underwent similar reorganizations. For example, at the GV stage, V-C-IP₃R1 (Fig. 1C-a) and V-C-IP₃R1(D2550A) (Fig. 1C-c) were widely distributed in the ooplasm with slight accumulation in the area surrounding the GV, from which the signal was excluded. Following *in vitro* maturation (IVM), V-C-IP₃R1 (Fig. 1C-b) and V-C-IP₃R1(D2550A) (Fig. 1C-d) displayed a clear reticular organization and distinct clusters both in the cortex as well as in the ooplasm, which is reminiscent of the organization of the ER (Mehlmann et al. 1995; Nakayama et al. 2004). Expression of Venus alone caused a diffuse fluorescent signal in the ooplasm of both GVs and MII eggs, and such fluorescence was excluded only from the nucleolus of GV oocytes (Fig. 1C-e,f).

To confirm the distribution suggested by detection of live V-C-IP₃R1 fluorescence, we performed immune detection studies of both V-C-IP₃R1 and wild type IP₃R1 (V-IP₃R1) on

fixed eggs (IF; Fig. 1D). We found that both V-C-IP₃R1 and VIP₃R1 underwent redistribution during maturation and showed cortical enrichment in MII eggs (Fig. 1Da-d). In contrast, the fluorescence of oocytes and eggs injected with Venus cRNA was homogenous regardless of the stage of maturation and did not undergo cortical enrichment (Fig. 1De-f). Nevertheless, in these eggs, we observed enhanced staining of the spindle suggesting that Venus may have affinity for microtubules.

ER Ca²⁺ leak and spontaneous fragmentation rates in eggs expressing C-IP₃R1

In previous studies, a band corresponding to C-IP₃R1 was detected in aged mouse eggs undergoing fragmentation/apoptosis (Verbert et al. 2008; Zhang et al. 2011). By morphology, we found that apoptotic eggs usually showed an initial shrinkage of the cytoplasm, which was followed by protuberances of the plasma membrane and ultimately release of membrane-enclosed vesicles of unequal sizes, fragments. This is consistent with the previous reports from our lab and other groups (Perez et al. 1999; Zhang et al. 2011). Images representing the fragmented eggs at the final step are shown in Fig 2A (d and f). To more thoroughly ascertain the role of C-IP₃R1 on egg fragmentation and on Ca²⁺ homeostasis, we injected C-IP₃R1 or V-C-IP₃R1 cRNA into fresh MII eggs and evaluated their effects on both of these parameters. Approximately 30% of C-IP₃R1-expressing eggs became activated, as they formed PN by 8 hr after injection (Fig. 2Ac and e, B; P < 0.05), and by 24 hr these activated eggs became fragmented (Fig. 2Ad and f, B). Expression of C-IP₃R1(D2550A) and Venus induced lower rates of PN formation, ~10% (Figure. 2Aa and g; B), which were followed by lower rates of fragmentation (Fig. 2Ab and h; B).

To test whether expression of C-IP₃R1 altered Ca²⁺ homeostasis in MII eggs, we estimated its effects on the ER releasable Ca²⁺ pool. To accomplish this, eggs were exposed to thapsigargin (TG), a sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor (Thastrup et al. 1990), which causes a leak out of the ER, and the observed [Ca²⁺]_i rise was quantified by measuring the area under the curve. Expression of C-IP₃R1 and V-C-IP₃R1 reduced the [Ca²⁺]_i response to TG, while C-IP₃R1(D2550A) and Venus were without effect (Fig. 2C, D P < 0.05). We also examined the effects of C-IP₃R1 expression on Ca²⁺ homeostasis using ionomycin, an ionophore that promotes more generalized intracellular Ca²⁺ release; C-IP₃R1 expression did not affect ionomycin-induced Ca²⁺ release (Fig. 2E, F). These data are consistent with results showing that the TG-releasable and IP₃ releasable Ca²⁺ pools are significantly smaller than the ionomycin-releasable Ca²⁺ pool (Wakai and Fissore 2013). The present results suggest that C-IP₃R1 expression negatively impacts the TG-releasable Ca²⁺ pool in the ER and that addition of Venus does not interfere with the function of C-IP₃R1.

Role of IP₃R1 in fragmentation of *in vitro* aged eggs

To evaluate the effects of C-IP₃R1 on egg fragmentation without the confounding effects of endogenous IP₃R1, which is known to be a pro-apoptotic factor in somatic cells (Joseph and Hajnoczky 2007), endogenous IP₃R1 was downregulated by injection of 20 μM adenophostin A (AdA), a non-hydrolysable IP₃R agonist that causes robust receptor degradation in eggs (Brind et al. 2000; Jellerette et al. 2000). AdA injection also induces [Ca²⁺]_i oscillations and egg activation, and therefore to eliminate the confounding effects of

oscillations, control eggs were exposed to SrCl_2 . To prevent MII exit in stimulated eggs, both AdA- and SrCl_2 -treated eggs were incubated in colcemid (Col), a microtubule inhibitor that prevents MII exit (Fissore et al. 2002; Jellerette et al. 2004; Sablina et al. 2001; Sablina et al. 1998) for the duration of the oscillations. Consistent with previous results (Brind et al. 2000; Jellerette et al. 2000), AdA reduced $\text{IP}_3\text{R1}$ levels within 4 hr (Fig. 3A), although this was not the case for control eggs injected with buffer (MIB) or eggs treated with 10mM SrCl_2 +Col (Fig. 3A). Following their stimulation, these eggs were *in vitro* cultured for 24 or 48hr and evaluated for spontaneous fragmentation. Eggs with reduced levels of $\text{IP}_3\text{R1}$ showed lower rates of fragmentation (Fig. 3B, $P<0.05$), although Col-treatment alone also reduced fragmentation. Similar results were observed when aged eggs were injected with SF, which caused fragmentation in nearly all control eggs by 4 hr after injection, although it was nearly without effect in eggs devoid of $\text{IP}_3\text{R1}$ (Fig. 3C, $P<0.01$). Remarkably, expression of C- $\text{IP}_3\text{R1}$ partly rescued fragmentation in eggs with reduced levels of endogenous $\text{IP}_3\text{R1}$ (Fig. 3C, $P<0.05$). $\text{IP}_3\text{R1}$ downregulation also prevented activation of caspase-3 in SF-injected aged eggs, which was easily observable in both control groups (Fig. 3D). Taken together these data suggest that $\text{IP}_3\text{R1}$ has pro-apoptotic effects on aged eggs and C $\text{IP}_3\text{R1}$ is sufficient to induce fragmentation of aged eggs.

To evaluate whether the protective role of $\text{IP}_3\text{R1}$ downregulation on egg fragmentation was associated with the inability of these eggs to exit MII, we examined if it still was effective when the block to the MII exit was bypassed by a pharmacological approach. We used roscovitine (Ros), a cdc2 kinase inhibitor that induces egg activation without promoting $[\text{Ca}^{2+}]_i$ release (Deng and Shen 2000; Phillips et al. 2002). We found that regardless of $\text{IP}_3\text{R1}$ mass, all aged eggs that exited the MII stage underwent fragmentation within 8 hr after the treatment of Ros (Fig. 3E). Collectively, these results suggest that the Ca^{2+} release through $\text{IP}_3\text{R1}$ or C- $\text{IP}_3\text{R1}$ facilitates the unfolding of the apoptotic program in aged eggs by promoting MII exit.

C- $\text{IP}_3\text{R1}$ and mouse embryo development

To examine if expression of C- $\text{IP}_3\text{R1}$ could affect egg activation and embryo development, MII eggs were injected with the aforementioned cRNAs and 4 hr later activated by exposure to SrCl_2 for 2.5 hr. We found that expression of V-C- $\text{IP}_3\text{R1}$ or V-C $\text{IP}_3\text{R1}$ (D2550A) did not affect activation rates, as evaluated by PN formation or cleavage to the 2-cell stage (Fig. 4A, $P>0.05$). The effects of C- $\text{IP}_3\text{R1}$ on embryo development were evaluated on *in vivo*-fertilized zygotes collected ~16 hr post-hCG and injected with cRNAs encoding for Venus, V-C- $\text{IP}_3\text{R1}$ and V-C- $\text{IP}_3\text{R1}$ (D2550A) and cultured for 72 hr. Expression of V-C- $\text{IP}_3\text{R1}$ did not affect embryo development (Fig. 4B; $P>0.05$).

The ineffectiveness of C- $\text{IP}_3\text{R1}$ to undermine embryo development was unexpected. We noticed nevertheless that the levels of fluorescence in eggs expressing VC- $\text{IP}_3\text{R1}$ or V-C- $\text{IP}_3\text{R1}$ (D2550A), which was grossly used to estimate protein expression, declined rapidly after injection; this was not the case for eggs injected with Venus cRNA. To determine whether this decline in fluorescence was due to decreasing protein concentrations, Western blots were performed. We detected V-C- $\text{IP}_3\text{R1}$ 4 hr post injection, but not by 24 hr (Fig. 4C), which suggests that the V-C- $\text{IP}_3\text{R1}$ is unstable in MII eggs. Therefore, the lack of

observable effects of C-IP₃R1 expression on long-term embryo development might be due at least in part to the transient presence of the protein in MII eggs.

C-IP₃R1 and proteolytic degradation

V-C-IP₃R1 cRNA injected GV oocytes displayed persistent fluorescence, which was confirmed by western blotting 24 hr after the injection of the cRNA (Fig. 5A). These results raised the question of whether V-C-IP₃R1 cRNA in GV oocytes is more efficiently translated or its product is less actively degraded or a combination of both. To gain insight into this, the fluorescence intensity of V-C-IP₃R1 cRNA injected GV oocytes and MII eggs was monitored and quantified every 5 hr for 22 hr. In addition, a cohort of oocytes and eggs were treated with MG132, a proteasome inhibitor (Gao et al. 2005). Time-lapse images (Fig. 5B) and line graphs (Fig. 5C) show that V-C-IP₃R1 fluorescence decreases in GV oocytes and that MG132 slows down/prevents degradation in both stages (Fig 5C). These results suggest that the proteasome plays a role in the degradation of V-C-IP₃R1.

To investigate whether all proteins resulting from cRNA injection are degraded/undergo turnover during maturation, we monitored the fluorescence of another Venus-tagged protein, IRBIT (V-IRBIT), a cytosolic IP₃R1 binding protein (Ando et al. 2003). Accordingly, V-C-IP₃R1 and V-IRBIT cRNAs were injected into GV-arrested oocytes, allowed to undergo protein translation for 11 hr, after which they resumed maturation. While V-C-IP₃R1 fluorescence decreased steadily during maturation, VIRBIT fluorescence remained stable (Fig. 5D), suggesting that C-IP₃R1 degradation is a selective process.

The selective loss of C-IP₃R1 in oocytes/eggs could be due to the presence of a signal peptide that targets the protein for degradation. We found a sequence reminiscent of a proteolytic signal known as PEST, which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (Rechsteiner and Rogers 1996; Rogers et al. 1986), between amino acids 1891 to 1911 of C-IP₃R1. To ascertain the impact of this sequence, we expressed a cRNA that lacked it [V-C-IP₃R1-(pest)] and evaluated the impact on the stability of the fluorescence. Expression of V-C-IP₃R1-(pest) produced a more sustained fluorescence (Fig. 5E), suggesting that the PEST sequence plays a role in the degradation of C-IP₃R1 during maturation.

C-IP₃R1 reduces the TG releasable Ca²⁺ pool in the ER and the Ca²⁺ mounting ability of IVM eggs

Given the importance of maturation in preparing oocytes for the optimum [Ca²⁺]_i response and the extended presence of V-C-IP₃R1 at the GV stage, we sought to determine if its expression throughout maturation might alter Ca²⁺ homeostasis and undermine the ability of eggs to mount oscillations. Accordingly, GV oocytes were injected with the selected cRNAs, maintained at this stage for 5 hr, after which resumption of meiosis was allowed and maturation occurred for 14 hr; only those eggs that have extruded the 1st polar body by this time were examined. We first evaluated the releasable Ca²⁺ pool in the ER using the TG method previously described. Our results show that expression of V-C-IP₃R1 greatly reduced Ca²⁺ levels in the ER compared to controls, although V-IP₃R1(D2550A) expression also reduced Ca²⁺ levels in the ER (Fig. 6 A,B). Consistent with the previous findings,

exposure to SrCl_2 , which induces $\text{Sr}^{2+}/[\text{Ca}^{2+}]_i$ oscillations and causes egg activation (Kline and Kline 1992; Sato et al. 1998; Swann 1992), induced low rates of egg activation in eggs expressing V-C-IP₃R1, whereas higher rates of activation were observed in eggs expressing Venus or V-CIP₃R1(D2550A)(Fig. 6C; $P < 0.05$). Further, nearly all eggs expressing V-C-IP₃R1 failed to mount $\text{Sr}^{2+}/[\text{Ca}^{2+}]_i$ oscillations, whereas the majority of control or V-IP₃R1-(D2550A) expressing eggs displayed $\text{Sr}^{2+}/[\text{Ca}^{2+}]_i$ oscillations (Fig. 6D). Last, we examined whether V-C-IP₃R1 expressing eggs were resistant to the initiation of oscillations induced by injection of PLC ζ cRNA (Fig. 7A, B), the male specific phospholipase C (PLC) isoform thought to initiate oscillations at fertilization (Saunders et al. 2002). V-C-IP₃R1 expressing eggs displayed delayed time to the 1st spike, reduced number of rises and abnormal shape of the 1st spike compared to uninjected controls or V-C-IP₃R1(D2550A) expressing eggs (Fig. 7A, B). Together, our results show that expression of C-IP₃R1 during maturation compromises Ca^{2+} homeostasis and undermines the activation competence of IVM eggs.

DISCUSSION

In this study we characterized the functional properties and developmental implications of C-IP₃R1 expression in mouse oocytes and eggs. We conclude that expression of C-IP₃R1 altered Ca^{2+} homeostasis in oocytes and eggs, possibly by creating an unregulated channel that leaks Ca^{2+} from the ER. Nevertheless, the impact of C-IP₃R1 expression in MII eggs and *in vitro* egg aging was modest, as it was quickly degraded at this stage. In contrast, expression of C-IP₃R1 at the GV stage, which resulted in C-IP₃R1 accumulation during maturation, prevented initiation of robust $[\text{Ca}^{2+}]_i$ oscillations in MII eggs and egg activation. Therefore, alteration of Ca^{2+} homeostasis during maturation compromises the activation competence of eggs.

Expression of C-IP₃R1 in our studies caused only modest effects on Ca^{2+} homeostasis and fragmentation in MII eggs. This is in contrast to results in Cos and HeLa cells where expression of EGFP-C-IP₃R1 depleted $[\text{Ca}^{2+}]_{\text{ER}}$ and caused high rate of apoptosis (Nakayama et al. 2004). In those cells, expression levels of C-IP₃R1 might have been higher possibly due to persistent production of C-IP₃R1, given the use of stably transfected cells; we also cannot discount the possibility that somatic cells experience less degradation of C-IP₃R1. Therefore, the inability of C-IP₃R1 expression to replicate apoptotic results in eggs might be due to several different possibilities. One explanation is that the heterologous protein is not efficient at disrupting Ca^{2+} homeostasis in fresh eggs, as endogenous IP₃R1 is very abundant and stable in these cells. It is also conceivable that heterotetramers containing truncated and intact IP₃R1 monomers form (Monkawa et al. 1995), which might moderate the conductivity of C-IP₃R1 (Boehning and Joseph 2000; Devogelaere et al. 2008; Galvan et al. 1999). In addition, our studies were performed on healthy MII eggs, which have robust Ca^{2+} homeostatic mechanisms and express the anti-apoptotic Bcl-2 protein (Gordo et al. 2002; Igarashi et al. 2005; Takahashi et al. 2000), all of which might attenuate the pro-apoptotic effects of C-IP₃R1. Finally, the low translational capacity of MII eggs coupled to the rapid turnover of C-IP₃R1 at this stage might limit the accumulation of C-IP₃R1 in MII eggs. Therefore, despite its potential for a primary role in MII egg aging and fragmentation, the effects of C-IP₃R1 are limited by its transient presence at this stage.

Our study reveals for the first time that IP₃R1 is required for mouse eggs to undergo fragmentation, as downregulation of endogenous IP₃R1 prevented spontaneous or [Ca²⁺]_i oscillations-stimulated fragmentation of *in vitro* aged eggs. Moreover, expression of C-IP₃R1 partly restored the susceptibility to fragmentation. These data suggest that loss-of-regulation of endogenous IP₃R1 and emergence of C-IP₃R1 may underlie a “Ca²⁺ leak” from the ER, which can act as a pro-apoptotic stimulus in aged eggs. The impact of this leak may be enhanced by the lower activity of SERCA in these aged eggs (Gordo et al. 2002; Igarashi et al. 2005; Takahashi et al. 2000). The “Ca²⁺ leak” may undermine the function of the molecular components responsible for the MII arrest and promote resumption of meiosis, which is consistent with data from *in vivo* and *in vitro* aged eggs showing lower M-phase kinase activities than fresh eggs (Abbott et al. 1998; Xu et al. 1997; Zhang et al. 2011). Further, the resistance of IP₃R1-downregulated eggs to undergo fragmentation/apoptosis was overcome by exposure to Roscovitine, which promotes cell cycle progression. This resistance of MII eggs to undergo fragmentation was also reported in *Xenopus* eggs (Faure et al. 1997) and in somatic cells, which also failed to undergo apoptosis during mitosis (Andersen et al. 2009). Thus, future studies should elucidate the molecular mechanism(s) that render aged eggs susceptible to apoptosis and how MII arrest confers resistance to fragmentation.

Our findings that MG132 reduced degradation of V-C-IP₃R1 suggest that the proteasome is involved in processing C-IP₃R1. Consistent with this, deletion of the PEST sequence also decreased degradation of V-C-IP₃R1. The PEST motif has been found in many cellular proteins that exhibit short half lives such as metabolic enzymes, protein phosphatases, cyclins and other proteins that give rise to immunogenic peptides (Rechsteiner and Rogers 1996). Remarkably, IP₃R1 is a very stable protein in mouse eggs (Jellerette et al. 2000), which suggests that the PEST motif is not exposed in the wild-type protein. Nevertheless, upon processed by caspases and generation of C-IP₃R1, the PEST sequence is exposed and C-IP₃R1 is targeted for degradation, as its presence would undermine maturation and activation competence.

Unlike results in MII eggs, expression of C-IP₃R1 at the GV stage results in accumulation of C-IP₃R1. This higher expression of C-IP₃R1 affected Ca²⁺ homeostasis during maturation and undermined the activation potential of MII eggs. These eggs showed reduced TG releasable Ca²⁺ pool from the ER and were unable to mount normal [Ca²⁺]_i responses after PLC ζ cRNA injection or be activated after treatment with SrCl₂. Lower Ca²⁺ levels in the ER, which may alter IP₃R1 sensitivity, may explain the abnormal shape of the 1st [Ca²⁺]_i rise, the premature termination of oscillations after injection of PLC ζ cRNA, or the complete absence of response to SrCl₂. In addition, low Ca²⁺ levels in the ER may compromise protein synthesis during maturation, which may further disturb Ca²⁺ influx and Ca²⁺ homeostasis. These findings suggest that alteration of Ca²⁺ homeostasis during maturation impacts the ability of eggs to initiate oscillations and undergo activation. Nevertheless, additional studies are needed to precisely determine what aspect of the eggs' Ca²⁺ signaling machinery is affected by expression of C-IP₃R1.

In summary, we found that although IP₃R1 is cleaved by caspase-3 in eggs, its impact on MII aging and apoptosis is limited by its short half-life at this stage. Importantly, expression

of C-IP₃R1 during maturation markedly affected Ca²⁺ homeostasis and activation competence, suggesting that Ca²⁺ homeostasis and ER function are important factors in determining developmental competence. Future studies should elucidate in oocytes and eggs the molecular target(s) affected by alteration of Ca²⁺/ER homeostasis during maturation, as its understanding may assist in preventing changes associated with *in vitro* culture and may improve *in vitro* maturation conditions.

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Figure 1. Expression of C-IP₃R1 constructs in mouse oocytes and eggs

(A) Molecular structure of IP₃R1 comprising three functionally distinct domains, specific cleavage site for caspase-3 (DEVD 1891) and pore forming site (D2550). Different CIP₃R1 constructs are depicted. LBD, ligand-binding domain. (B) Top panel: Immunoblotting of cell lysates from GV oocytes injected with MIB or C-IP₃R1 cRNA probed with Rbt03 antibody. Bottom panel: Immunoblotting of cell lysates from GV oocytes 4 hr after being injected with MIB or V-C-IP₃R1 cRNA probed with Rbt03 antibody. The same blot was probed with anti-Venus antibody and displayed to the right. (C) Confocal images of

distribution of V-C-IP₃R1 (a,b), V-C-IP₃R1 (D2550A) (c,d) and Venus (e,f) in live mouse GV oocytes (a,c,e) and IVM eggs (b,d,f). Control cells are oocytes injected with MIB (g,h). The area denoted by rectangles in red color are magnified and shown below. (D)

Immunostaining images of distribution of V-C-IP₃R1 (a,b), V-IP₃R1 (c,d) and Venus (e,f) in fixed mouse GV oocytes (a,c,e) and IVM eggs (b,d,f). Control cells are oocytes injected with MIB (g,h). The area denoted by rectangles in red color are magnified and shown below.

Figure 2. Effects of C-IP₃R1 in mouse MII eggs

(A) Representative pictures of MII eggs 8 hr (a,c,e,g) or 24 hr (b,d,f,h) after being injected with cRNAs encoding for Venus (a,b), C-IP₃R1(c,d), V-C-IP₃R1(e,f) and V-C-IP₃R1(D2550A)(g,h). (B) Comparisons of the activation and apoptosis rates obtained from Fig. A. (C) Representative [Ca²⁺]_i profiles of Ca²⁺ release from the ER by addition of thapsigargin (TG) to Venus, C-IP₃R1, V-C-IP₃R1 and V-C-IP₃R1(D2550A) expressing MII eggs. (D) Area under the curve for TG-induced Ca²⁺ release was calculated from each group in C; MII eggs expressing Venus only was chosen as 100% and values for the other

overexpression groups are presented relative to 100%. (E) Representative $[Ca^{2+}]_i$ profiles of Ionomycin-induced Ca^{2+} release in MIB, Venus, V-C-IP3R1 and V-CIP3R1(D2550A) expressing MII eggs in the absence of extracellular Ca^{2+} . (F) Area under the curve for Ionomycin-induced Ca^{2+} release was calculated from each group in E; MII eggs injected with MIB was chosen as 100% and values for the other overexpression groups are presented relative to 100%. Bars with different superscripts represent treatments that are significantly different ($P < 0.05$).

Figure 3. Role of C-IP₃R1 in the stimulated apoptotic cell death of *in vitro* aged eggs

(A) Immunoblotting of 20 cell lysates from MIB injected MII eggs (1st lane, MIB), IP₃R1 knockdown cells (2nd lane, AdA) and eggs treated with 10mM SrCl₂ + 100ng/ml Col for 4 hr (3rd lane, SrCl₂+Col), probed with Rbt03 antibody and anti-α-tubulin antibody. (B) Comparison of the spontaneous fragmentation rate for the 48 hr aged eggs with or without endogenous IP₃R1. MII eggs were injected with 20μM AdA in the presence of 100ng/ml Col (AdA) or injected with MIB in the absence of Col (MIB) followed by 48 hr aging. Control eggs were treated with 100 ng/ml Col (Col) or 10mM SrCl₂ + 100ng/ml Col

($\text{SrCl}_2 + \text{Col}$) for 4 hr before aging to mimic the Ca^{2+} signal in AdA eggs while arresting the eggs at MII stage. (C) Comparison of SF induced apoptosis rates of the 24 hr aged eggs with or without endogenous $\text{IP}_3\text{R1}$. AdA, Col and $\text{SrCl}_2 + \text{Col}$ eggs were treated similarly as in Fig B. A cohort of these $\text{IP}_3\text{R1}$ knockdown eggs (AdA) were injected with V-C- $\text{IP}_3\text{R1}$ cRNA 4 hr after AdA injection (AdA+V-C- $\text{IP}_3\text{R1}$). Eggs from all groups were injected with SF ($0.5 \mu\text{g}/\mu\text{l}$) after 24 hr aging. (D) Representative images of caspase-3 activity from the cells that were treated similarly to the cells in Fig B. The FIT-DEVD-FMK was used to detect caspase-3 activity in cells. (E) Comparison of the Ros induced apoptosis for the 24 hr aged $\text{IP}_3\text{R1}$ knockdown eggs. AdA, Col and $\text{SrCl}_2 + \text{Col}$ eggs were treated similarly as in Fig B before aging for 24 hr. All the eggs from MIB and $\text{SrCl}_2 + \text{Col}$ groups and a cohort of AdA eggs were treated with $50 \mu\text{M}$ Ros after 24 hr aging. Bars with one asterisk are different from those without them with $P < 0.05$. Bars with two asterisks are different from those without them with $P < 0.01$.

Figure 4. C-IP₃R1 exerts minor effects on mouse embryo development

(A) 4 hr after injection of Venus, V-C-IP₃R1 and V-C-IP₃R1 (D2550A) cRNAs, eggs were activated by SrCl₂ treatment. Representative pictures were taken 8 hr and 24 hr post activation. Bar graphs on the right are comparisons of the PN formation and 2-cell cleavage rates obtained from the left figure. (B) Representative blastocyst formation outcomes of *in vivo* fertilized eggs expressing Venus, V-C-IP₃R1 and V-C-IP₃R1 (D2550A) respectively. Bar graphs on the right are comparisons of the blastocyst formation rates obtained from the

left figure. (C) Immunoblotting of MII egg lysates from control and V-C-IP₃R1 expressing eggs 4 and 24hr after cRNA injection.

Figure 5. PEST sequence targets C-IP₃R1 for rapid proteolytic destruction

(A) Immunoblotting of GV oocyte lysates 24 hr after being injected with MIB or cRNAs encoding for V-C-IP₃R1 and V-C-IP₃R1 (D2550A). (B) Representative fluorescence of V-C-IP₃R1 in GV oocytes and MII eggs 5hr, 10hr and 22hr after expression in the absence (dotted circle) or presence (solid circle) of MG132. The exposure time were 0.3s and 1.25s for GV oocytes and MII eggs respectively. (C) Line graphs represent the degradation rate of the fluorescence in MII and GV oocytes from Fig. B. treated with (line a) or without MG132 (line b). Values are expressed as absolute values of mean pixel intensity. (D) Line graph

represents the intensity of fluorescence of mouse oocytes expressing MIB, V-C-IP₃R1 and V-IRBIT during the maturation process. (E) The fluorescence intensity of mouse oocytes expressing the MIB, V-C-IP₃R1 and V-CIP₃R1(pest) during the maturation process is represented as line graph. Lines with one asterisk are different from those without them with $P < 0.05$. Lines with two asterisks are different from those without them with $P < 0.01$. Lines with different superscripts represent treatments that are significantly different ($P < 0.05$)

Figure 6. C-IP₃R1 reduces the total releasable Ca²⁺ pool in the ER and the SrCl₂ induced Ca²⁺ release in IVM eggs

(A) Release of Ca²⁺ from the ER by addition of TG in MIB, Venus, V-C-IP₃R1 and V-C-IP₃R1(D2550A) cRNAs injected IVM mouse eggs. (B) Area under the curve for TG-induced Ca²⁺ release was calculated from each group in Fig A; MIB group was chosen as 100% and values for the other overexpression groups are presented relative to it. (C) Comparison of the parthenogenetic activation rates (6 hr) in the IVM eggs injected with MIB, Venus, V-C-IP₃R1 and V-C-IP₃R1(D2550A) cRNAs. (D) Changes in [Ca²⁺]_i induced by 10mM SrCl₂ in IVM eggs injected with MIB, V-C-IP₃R1 and V-C-IP₃R1(D2550A)

cRNAs. Bars with one asterisk are different from those without them with $P < 0.05$. Bars with two asterisks are different from those without them with $P < 0.01$.

Figure 7. C-IP₃R1 reduces Ca²⁺ oscillatory responses

(A) Changes in [Ca²⁺]_i induced by injection of PLCζ cRNA (0.05μg/μl) in IVM eggs expressing MIB, V-C-IP₃R1 and V-C-IP₃R1(D2550A) cRNAs. Dotted lines to the right are magnified 1st spikes of [Ca²⁺]_i traces of each group. (B) Comparison of different parameters of Ca²⁺ oscillatory responses in the eggs injected with MIB, V-C-IP₃R1 and V-C-IP₃R1(D2550A) cRNAs, such as initiation time, number of spikes, rate of responsive eggs and rate of eggs with normal shape of 1st spike. Bars with one asterisk are different from those without them with $P < 0.05$. Bars with two asterisks are different from those without them with $P < 0.01$.