

Research Article

Cite this article: Percivale G *et al.* (2022) Time-dependent expression of ryanodine receptors in sea urchin eggs, zygotes and early embryos. *Zygote*, **30**: 213–216. doi: [10.1017/S0967199421000514](https://doi.org/10.1017/S0967199421000514)

Received: 3 December 2020
Revised: 28 May 2021
Accepted: 11 June 2021
First published online: 28 July 2021

Keywords:

Ca²⁺ release; First cleavage; *Paracentrotus lividus*; Ryanodine receptor; Zygote

Author for correspondence:

Cristiana Picco. IBF CNR, Via de Marini 6, 16149 Genova, Italy. E-mail cristiana.picco@ibf.cnr.it

Time-dependent expression of ryanodine receptors in sea urchin eggs, zygotes and early embryos

G. Percivale¹, C. Angelini², C. Falugi², C. Picco¹  and G. Prestipino¹

¹CNR Institute of Biophysics, Via De Marini 6, 16149 Genoa, Italy and ²Laboratory of Developmental Neurobiology, Department of Earth, Environment and Life Sciences, University of Genoa, Viale Benedetto XV 5, 16132 Genoa, Italy

Summary

In this work, the presence of calcium-dependent calcium channels and their receptors (RyR) has been investigated in *Paracentrotus lividus* eggs and early embryos, from unfertilized egg to four-blastomere stages. Electrophysiological recordings of RyR single-channel current fluctuations showed that RyRs are functional during the first developmental events with a maximum at zygote stage, *c.* 40 min after fertilization, corresponding to the first cleavage. The nature of vertebrate-like RyRs active at this stage was established by specific activation/blockade experiments.

Introduction

The mechanisms modulating the electrical events of sperm–egg interaction (Whitaker, 2006), fertilization and cytoskeletal dynamics (Whitaker and Larman, 2001) have been intensively investigated by use of the sea urchin model. Harrison *et al.* (2002) found at about halfway through the first cell cycle (*c.* 40 min after fertilization) high spikes of calcium released from the inner stores due to excitation of muscarinic acetylcholine receptors (mAChRs) in two sea urchin species. A relationship between muscarinic receptors and the activation of ryanodine receptors (RyR) has been thoroughly demonstrated in different models and tissues (Galeotti *et al.*, 2008; Liu *et al.*, 2009). The presence of RyRs in sea urchin unfertilized eggs has been previously demonstrated (Galione *et al.*, 1993b), possibly localized in the vesicles of the smooth endoplasmic reticulum (SER) (McPherson *et al.*, 1992, Lokuta *et al.*, 1998). In fact, similar to excitable cells, the cytoplasm of sea urchin eggs, zygotes, and early embryos contains a reticulum of both rough and smooth endoplasmic organelles. The smooth organelles have the same functions as the vertebrate ones, including the presence of channels for intracellular [Ca²⁺] release. Here, the time-dependent presence and functionality of RyRs during the sea urchin first cell cycles was assessed by the method suggested by Lokuta *et al.* (1998) to understand the function of RyRs in sea urchin early developmental events.

Materials and methods

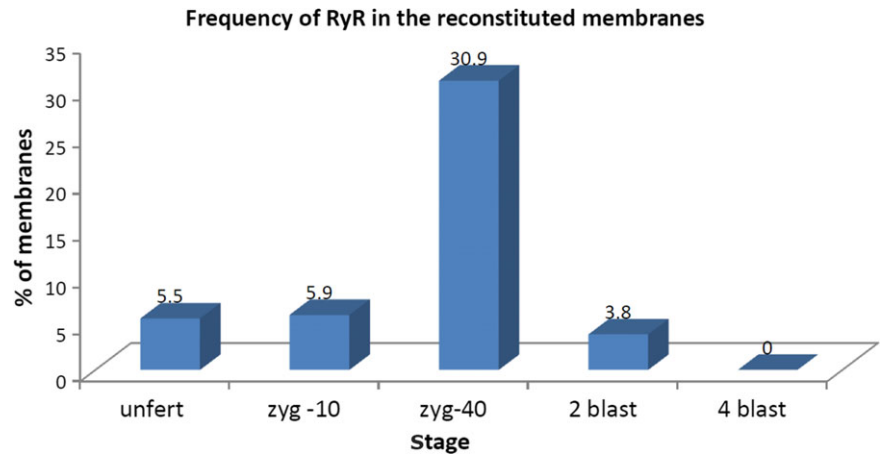
Adult specimens of the sea urchin, *Paracentrotus lividus* were taken from the Portofino marine park in different year periods, along all the fertile season from October to the beginning of June. Spawning and fertilization were performed in standard sea water (SSW) pasteurized, oxygenated and ultra-filtered through a 0.22- μ m Whatman filter. Samples were collected at different stages from unfertilized eggs to four-blastomere embryos.

Part of the sample was fixed for immunostaining of molecules immunologically related to RyRs and to α -tubulin, while the major part was processed for microsome preparation and incorporated into artificial membranes for electrophysiological recording. All the reagents were obtained from Sigma Chemical Co. (Milan, Italy) except calmodulin (Boehringer-Mannheim GMBH) and anti-RyR antibody (ABCAM) and anti- α -tubulin (Proteintech).

Immunocytochemical staining

After fixation with cold paraformaldehyde (PFA) 4% in seawater, whole cells were incubated in the cold overnight in primary antibody 1/100 PBS. For molecules immunologically related to RyR, the antibody that gave the best result was the anti-ryanodine receptor antibody [34C] (ab2868) abcam, with a large spectrum of predicted activity (including amphibians and fish). For α -tubulins, the antibody used was polyclonal rabbit IgG, with a wide range of reactivity (mammalians, fish and beetles; Proteintech). After incubation, the materials were rinsed and incubated in the secondary antibody (Alexafluor).

Figure 1. Percentage of membranes presenting RyR responses obtained from eggs and embryos at different stages. unfert, unfertilized eggs; zyg-10, zygotes at 10 min after fertilization; zyg-40, zygotes at 40 min after fertilization; 2 blast, two-blastomere stage; 4 blast, four-blastomere stage.



Cortical microsomes preparation

Samples formed by 50 μ l of concentrated eggs/embryos (*c.* 95,000–100,000 eggs/embryos) were stored at -80°C . Cortical membranes were prepared according to the protocol suggested by McPherson *et al.* (1992), modified by Lokuta *et al.* (1998) to be adapted for sea urchin eggs. All the procedure was performed on ice. The eggs, zygotes and early morulae were homogenized and centrifuged for 2 min at 2000 rpm (refrigerated Beckman high speed centrifuge, 64xG). The pellet was resuspended in 1 ml of modified SW (MSW), in which NaOH is substituted by 30 mM TRIS pH 8.0 in 1 mM EGTA, and described by Mueller and colleagues (1962) and incorporated in the phospholipid bilayer, as described by Mueller and colleagues (1962), for single-channel recording. The signal from the single-channel current was recorded using a 200 A axopatch amplifier (Axon instruments) at 20 mV holding potential and is shown as upwards deflections.

Data were acquired at 200 μ s/point and filtered at 1 kHz. Single-channel analysis was carried out according to Colquhoun and Sakmann (1985). Channel open probability was determined from the areas of Gaussian distributions that best fitted the peaks of amplitude histograms from recordings of 30 s duration. Drugs activating/blocking single-channel currents were added to the *cis* compartment.

The planar bilayer measurements and data analyses were performed according to the experimental procedures described by Buratti *et al.* (1995).

Characterization of the receptors

Characterization of the receptor was carried out by analyzing the pharmacological properties of the RyR Ca^{2+} -releasing channel reconstituted into bilayers by adding in *cis* compartment compounds that specifically modulated the mammalian skeletal muscle RyR (Galione *et al.*, 1991). Specific blockers, such as 2 mM MgCl_2 and 25 nM ruthenium red (RuRed), and specific activators, such as 25 mM caffeine, were used to check the activation of cADPR-induced calcium channels, and inhibition of the IP_3 -induced calcium release (Lokuta *et al.*, 1998; Galione *et al.*, 1991). Here, 50 nM calmodulin (CaM) was used to activate the RyR channel at a calcium concentration corresponding to the SR one (from 390 μ M, reduced by RyR excitation to 8 μ M; according to Ziman *et al.*, 2010); 5 mM ATP, followed by 25 nM ryanodine and 10 μ M of ruthenium red was used to check the subsequent reversibility of activation.

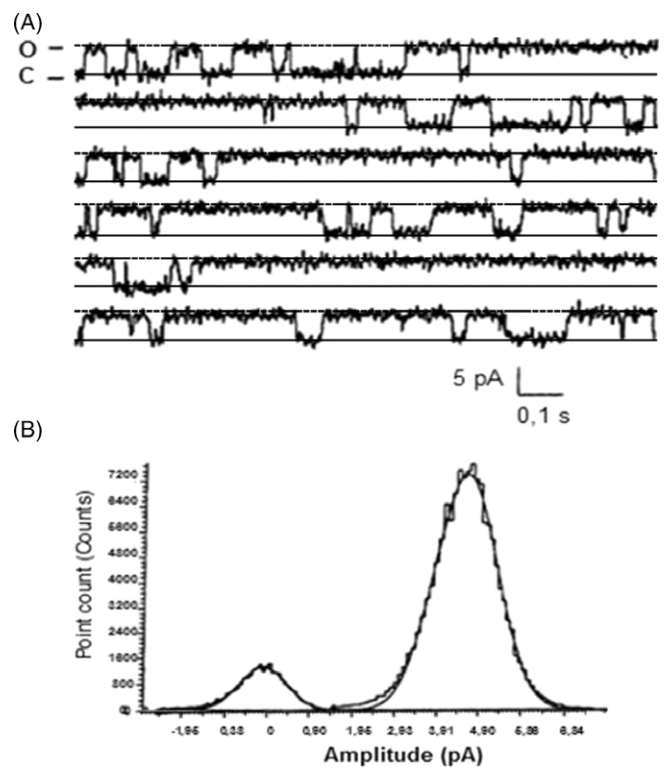


Figure 2. Single-channel recordings after incorporation of microsomes obtained from membranes of *Paracentrotus lividus* (Pl) zygotes at 40 min after fertilization. (A) Current recordings at +20 mV in the presence of 1:6 *cis/trans* concentration gradient. Here, 5 μ l of microsomes was previously added in *cis*. (B) Amplitude histograms, showing two spikes at 0 and 4.75 pA corresponding to the closed and open states of the channel, respectively.

Results

Frequency of incorporated RyR channels

The presence of RyR channels was identified at the unfertilized egg and 10 min zygote stage with almost the same frequency (frequency = percentage of responding membranes/number of tested membranes). The maximum frequency of reactive receptors was found at *c.* 40 min after fertilization, this decreased at the two-blastomere stage and disappeared at the four-blastomere stage (Figure 1).

Positive membranes showed traces of a Ca^{2+} -conducting channel elicited by a pulse of 20 mV, with a rather intense activity with opening times longer than closures (Fig. 2A). The related current amplitudes showed a peak at 4.75 pA corresponding with the open state (Fig. 2B), with a conductance level of 240 pS. The open probability was 0.84. These recordings were obtained from microsomes of sea urchin zygotes at 40 min after fertilization, synchronous with the calcium-induced retraction of the spindle fibres (shown in Fig. 3B) by an α -tubulin immunoreaction (Fig. 3).

Characterization of the receptor

Evidence for these results is supplied in Supporting Information Figures S1–S3:

- **2 mM MgCl_2** in the *cis* compartment caused channel closure at 0 pA (Fig. S1A).
- **Ruthenium red** (RuRed) blocked all the current fluctuations and maintained the channel in a long-lasting close state (Fig S1B).
- **25 mM caffeine** caused a strong increase in current fluctuations increasing the probability of channel opening to 0.73 (Fig S2A).
- **50 nM calmodulin** in the presence of Ca^{2+} -EGTA in the *cis* compartment activated the RyR channel at a Ca^{2+} concentration corresponding to the SR one, increasing the probability of the channel opening to 0.81.
- **ATP and ryanodine** (Fig. S3B) caused a marked increase in channel activity, rapidly fluctuating between the open and closed states; the subsequent addition of ryanodine blocked the channel in the open state (Fig. S3C), causing almost complete inhibition of the fluctuation between the two channel states.

This experiment shows an apparent long-lasting state of sub-conductance, similar to the effect shown by Rousseau *et al.* (1987) and Lindsay *et al.* (1994) for skeletal and cardiac muscle. Further addition of 10 μM RuRed also caused strong inhibition of channel activity (Fig. S3D), suggesting that RuRed may antagonize the RyR effect by inducing a long-lasting closed state. These results showed the similarity between the RyRs present in the sea urchin zygote and in vertebrate skeletal and cardiac muscle membranes (Table 1).

Whole mount immunostaining of the zygote at 40 min

RyR-like molecules were faintly stained in the membrane of vesicles in the cortical region of the cell, just beneath the plasmalemma (Fig. 3A)

Molecules immunologically related to α -tubulin showed the different aspects of microtubules in normal (Fig. 3B) and inhibited (Fig. 3C) zygotes.

Additional experiments were performed to check the presence of RyR expression along the development from unfertilized egg to pluteus stage. RT-PCR showed the presence of the signal at all the stages, with a peak at the zygote stage (Fig. S4).

Discussion

The reports available in the literature about the presence of RyRs in echinoderms refer to unfertilized eggs (Galione *et al.*, 1993a; Lokuta *et al.*, 1998) evoking calcium waves at fertilization (Galione *et al.*, 1993b). The presented outcomes suggest that the RyR channels are mainly involved in Ca^{2+} fluctuations occurring

Table 1. Comparison between the responses of RyR of sea urchin cortical SER and mammalian skeletal muscle (SR)

Exposure	MgCl_2	RuRed	Caffeine	CaM	Rya
Mammalian ^a	-	-	+	+	+
Sea urchin	-	-	+	+	+

CaM = Calmodulin; RuRed = ruthenium red; Rya = Ryanodine.

^aRousseau *et al.*, 1987; McPherson *et al.*, 1992; Lindsay *et al.*, 1995; Lokuta *et al.*, 1998; Pérez *et al.*, 1998.

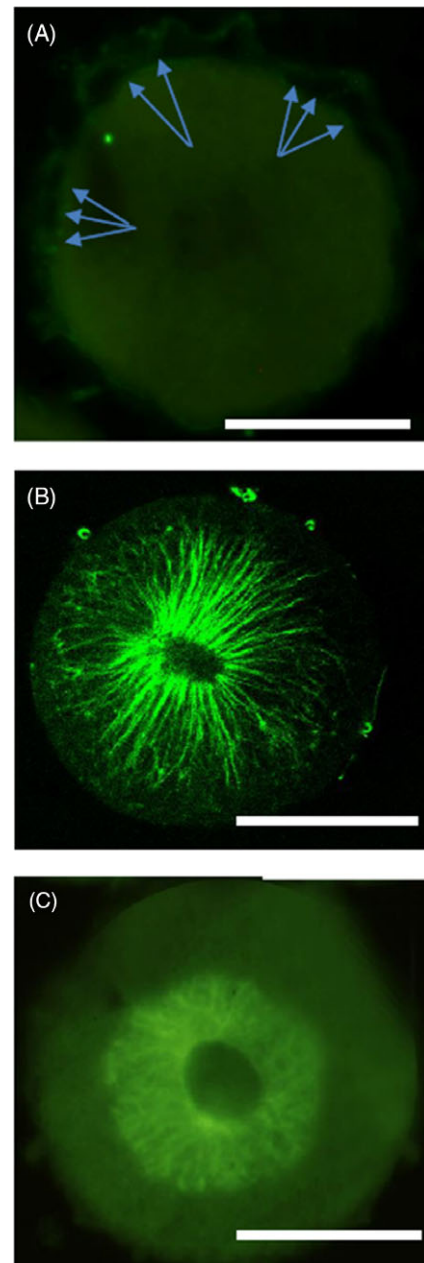


Figure 3. Whole mount immunoreactivity of 40 min zygote. (A) RyR-like staining of cortical vesicles. Arrows point to some vesicles, just beneath the plasmalemma. (B) α -Tubulin immunostaining showing the spindle fibres 40 min after fertilization. (C) Disorganization of microtubules after calcium release inhibition. Nucleus breakdown also seems slower. Scale bars, 50 μm .

at the first cleavage. In fact, high intracellular $[Ca^{2+}]$ is known to activate nucleus breakdown (Whitaker and Larman, 2001) and microtubule depolymerization involved in chromosome transport (Wolniak et al., 1983). The absence of responsiveness in the membranes obtained from the four-blastomere stages agrees with the finding that, at these stages, the cleavages presented Ca^{2+} waves of lower amplitude and were due to L-type channels (Dale et al., 1997), while the high frequency found at the 40 min zygote stage seemed to correspond rather well with the Ca^{2+} spikes evoked by muscarinic drugs at the beginning of the first prophase stage (Harrison et al., 2002).

The RyRs present in sea urchin zygote and in skeletal and cardiac muscle seem to be similar, with small differences. (Pérez et al., 1998) (Table 1). These results suggested the pre-eminent role of RyR function during the cleavage of the first blastomeres (Dale et al., 1997; Galione and Churchill, 2002; Harrison et al., 2002; Whitaker, 2006), while other calcium-dependent events, such as egg activation or cleavage at the four-blastomere stage may depend on alternative or parallel pathways for intracellular calcium dynamics that could alternate or cooperate in the different phases of sea urchin development.

Supplementary material. To view supplementary material for this article, please visit <https://doi.org/10.1017/S0967199421000514>

Acknowledgements. We thank Marco Millio (IBF) for skilled help in figure acquisition.

Conflicts of interest. All the authors declare no competing financial interests.

Financial support. This work was supported by endowment funds of CNR Biophysics Institute and Laboratory of Developmental Neurobiology of University of Genoa.

Ethical standards. Not applicable

Supporting information. Additional Supporting information is available with the online version of the article.

References

- Buratti R, Prestipino G, Menegazzi P, Treves S and Zorzato F (1995). Calcium dependent activation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by calmodulin. *Biochem Biophys Res Commun* **213**, 1082–90.
- Colquhoun D and Sakmann B (1985). Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle endplate. *J Physiol* **369**, 501–57.
- Dale B, Yazaki I and Tosti E (1997). Polarized distribution of L-type calcium channels in early sea urchin embryos. *Am J Physiol* **273**(3 Pt 1), C822–5.
- Galeotti N, Quattrone A, Vivoli E, Bartolini A and Ghelardini C (2008). Type 1 and type 3 ryanodine receptors are selectively involved in muscarinic antinociception in mice: An antisense study. *Neuroscience* **153**, 814–22.
- Galione A and Churchill GC (2002). Interaction between calcium release pathways: Multiple messengers and multiple stores. *Cell Calcium* **32**(5–6), 343–54.
- Galione A, Lee HC and Busa WB (1991). Ca^{2+} -induced Ca^{2+} release in sea urchin egg homogenates: Modulation by cyclic ADP-ribose. *Science* **261**, 348–52.
- Galione A, White A, Willmott N, Turner M, Potter BVL and Watson SP (1993a). cGMP mobilizes intracellular Ca^{2+} in sea urchin eggs by stimulating cyclic ADP-ribose synthesis. *Nature* **365**(6445), 456–9.
- Galione A, Galione A, McDougall A, Busa WB, Willmott N, Gillet I and Whitaker M (1993b). Redundant mechanisms of calcium-induced calcium release underlying calcium waves during fertilization of sea urchin eggs. *Science* **261**(5119), 348–52.
- Harrison PK, Falugi C, Angelini C and Whitaker MJ (2002). Muscarinic signalling affects intracellular calcium concentration during the first cell cycle of sea urchin embryos. *Cell Calcium* **31**, 289–97.
- Lindsay AR, Tinker A and Williams AJ (1994). How does ryanodine modify ion handling in the sheep cardiac sarcoplasmic reticulum Ca^{2+} -release channel? *J Gen Physiol* **104**, 425–47.
- Liu QH, Zheng YM, Korde AS, Yadav VR, Rathore R, Wess J and Wang YX (2009). Membrane depolarization causes a direct activation of G protein-coupled receptors leading to local Ca^{2+} release in smooth muscle. *Proc Natl Acad Sci USA* **106**, 11418–23.
- Lokuta AJ, Darszon A, Beltrán C and Valdivia HH (1998). Detection and functional characterization of ryanodine receptors from sea urchin eggs. *J Physiol* **510**, 155–64.
- McPherson SM, McPherson PS, Mathews L, Campbell KP and Longo FJ (1992). Cortical localization of a calcium release channel in sea urchin eggs. *J Cell Biol* **116**, 1111–21.
- Mueller P, Rudin DO, Tien HT and Wescott WC (1962). Reconstitution of excitable cell membrane structure *in vitro*. *Circulation* **26**, 1167–71.
- Pérez CF, Marengo JJ, Bull R and Hidalgo C (1998). Cyclic ADP-ribose activates caffeine-sensitive calcium channels from sea urchin egg microsomes. *Am J Physiol* **274**, C430–9.
- Rousseau E, Smith JS and Meissner G (1987). Ryanodine modifies conductance and gating behaviour of single Ca^{2+} release channel. *Am J Physiol* **253**, 364–8.
- Whitaker M (2006). Calcium at fertilization and in early development. *Physiol Rev* **86**, 25–88.
- Whitaker M and Larman MG (2001). Calcium and mitosis. *Semin Cell Dev Biol* **12**, 53–8.
- Wolniak SM, Hepler PK and Jackson WT (1983). Ionic changes in the mitotic apparatus at the metaphase/anaphase transition. *J Cell Biol* **96**, 598–605.
- Ziman AP, Ward CW, Rodney GG, Lederer WJ and Bloch RJ (2010). Quantitative measurement of Ca^{2+} in the sarcoplasmic reticulum lumen of mammalian skeletal muscle. *Biophys J* **99**, 2705–14.