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Calcium signals and oocyte maturation in marine invertebrates

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ABSTRACT In various oocytes and eggs of animals, transient elevations in cytoplasmic calcium ion concentrations are known to regulate key processes during fertilization and the completion of meiosis. However, whether or not calcium transients also help to reinitiate meiotic progression at the onset of oocyte maturation remains controversial. This article summarizes reports of calcium signals playing essential roles during maturation onset (=germinal vesicle breakdown, GVBD) in several kinds of marine invertebrate oocytes. Conversely, other data from the literature, as well as previously unpublished findings for jellyfish oocytes, fail to support the view that calcium signals are required for GVBD. In addition to assessing the effects of calcium transients on GVBD in marine invertebrate oocytes, the ability of maturing oocytes to enhance their calcium-releasing capabilities after GVBD is also reviewed. Furthermore, possible explanations are proposed for the contradictory results that have been obtained regarding calcium signals during oocyte maturation in marine invertebrates.

KEY WORDS: *GVBD, IP_3, meiotic reinitiation, metaphase arrest*

Introduction

During oogenesis in animals, immature oocytes initially stop cell cycle progression at prophase of the first meiotic division. Prophase-arrested oocytes are characterized by a large nucleus, called the germinal vesicle (GV), and in most animal groups, GV-containing oocytes cannot undergo normal fertilization without first completing complex nuclear and cytoplasmic reorganizations, collectively referred to as oocyte maturation (Voronina and Wessel, 2003).

The first readily recognizable indicator of maturation onset is a process called germinal vesicle breakdown (GVBD). During GVBD, prophase arrest is overridden, and the oocyte disassembles its GV in preparation for two unequal meiotic divisions that will ultimately yield a large haploid egg with small polar bodies located at the egg's animal pole. In many species, maturing oocytes complete their pre-fertilization phase of meiosis by entering into a secondary cell cycle arrest at metaphase I or metaphase II (Nishiyama *et al.*, 2010; Chiba, 2011), although various exceptions to this pattern occur throughout the animal kingdom (Fig. 1).

The complex signaling pathways regulating oocyte maturation have yet to be fully elucidated, but a well documented downstream effector of both meiotic reinitiation from prophase arrest and the establishment of a secondary metaphase arrest is the protein complex called maturation-promoting factor (=M-phase-promoting factor, MPF) (Kishimoto, 2003; Hara *et al.*, 2012). As the names suggest, MPF induces maturation in oocytes and drives interphase to M-phase transitions in general. Accordingly, in immature oocytes, MPF activity is relatively low, whereas in response to maturation-inducing stimuli, MPF is activated to trigger GVBD and meiotic progression. Subsequently, metaphase-arrested oocytes maintain high levels of MPF activity in association with a cytostatic arrest factor (CSF) that generally depends on mitogen-activated protein kinase (MAPK) signaling (Nishiyama *et al.*, 2010).

Recent reviews of upstream regulators of MPF and MAPK activities during the first cell cycle of development have summarized

Abbreviations used in this paper: 5HT, serotonin (5-hydroxytryptamine); ASW,artificial seawater; cADPR, cyclic ADP ribose; CaFSW, calcium-free seawater; CaM, calmodulin; CaMKII,calcium/calmodulin dependent kinase II; Epac, exchange protein directly activated by cAMP; DAG, diacylglycerol; ER, endoplasmic reticulum; GVBD, germinal vesicle breakdown; IP_3, inositol 1,4,5 trisphosphate; MAPK, mitogen-activated protein kinase; MPF, maturation-promoting factor; NSW, natural seawater; PKC, protein kinase C; RyR, ryanodine receptor; SW, seawater; TPA, tetradecanoylphorbol-13-acetate.

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the important roles played by such signaling molecules as cyclic nucleotides and divalent cations (e.g. Jaffe and Norris, 2010; Wakai and Fissore, 2013). In particular, oocytes or eggs are known to elevate their concentrations of intracellular calcium ions (Ca^{2+}) during fertilization, typically by releasing Ca^{2+} from stores within the endoplasmic reticulum (ER) via inositol-1,4,5 trisphosphate (IP_3)-dependent- or IP_3-independent pathways (Miyazaki, 2006). Accordingly, in order to generate effective Ca^{2+} signals during fertilization, oocytes of most animals must first enhance their calcium release capabilities during meiotic maturation (Stricker, 1999; Nader *et al.*, 2013), and in mature, metaphase-arrested oocytes undergoing fertilization, such enhanced capabilities enable the production of robust calcium responses that ultimately downregulate MPF and CSF activities to allow the completion of meiosis (Nishiyama *et al.*, 2010).

However, unlike the well established functions of intraoocytic calcium transients in triggering meiotic progression after fertilization, there is little consensus as to whether or not calcium signaling also mediates the pre-fertilization reinitiation of meiosis in prophase-arrested oocytes. For example, in two intensively studied groups of vertebrates, frogs and mammals, early studies suggest either a requirement (e.g. Moreau *et al.*, 1980; Goren *et al.*, 1990), or a non-essential role (e.g. Cork *et al.*, 1987; Tombes *et al.*, 1992), for calcium signals during GVBD. Based on more recent analyses showing intraoocytic Ca^{2+} elevations downregulate, rather than promote, progesterone-induced maturation in *Xenopus* (Sun and Machaca, 2004), it has been concluded that calcium transients are not required for GVBD in frog oocytes (Machaca, 2011). Alternatively, in spite of findings that follicle-enclosed mouse oocytes can undergo GVBD independently of calcium signals (Mehlmann *et al.*, 2006), calcium is still viewed as a key stimulator of GVBD in mammals (e.g. Silvestre *et al.*, 2011; Wang and Machaty, 2013), although whether or not mammalian GVBD routinely requires a Ca^{2+} elevation continues to be debated.

Given that data for and against calcium signals controlling GVBD in vertebrate oocytes have been thoroughly summarized, this article attempts to expand the scope of coverage by considering oocytes produced by marine invertebrates. Thus, studies related to calcium signals during the onset of oocyte maturation in several groups of marine invertebrates are reviewed, and some new data regarding calcium signaling in jellyfish oocytes are presented. In addition, since a robust calcium response during fertilization of post-GVBD oocytes is a well documented driver of meiotic completion, evidence showing an enhanced ability to generate calcium signals in mature vs. immature oocytes is also reviewed for marine invertebrates. Finally, potential explanations for apparent discrepancies in the literature are presented.

Results and Discussion

Cnidarians (jellyfish)

The phylum Cnidaria contains approximately 10,000 marine species (Appletans *et al.*, 2012) and is viewed as a basal lineage that arose before the divergence of bilaterian animals. In gravid ovaries of jellyfish such as *Cytaeis*, oocytes normally begin meiotic maturation without surrounding follicle cells in response to light-dark cycles that can be accompanied by intraoocytic cAMP elevations (Deguchi *et al.*, 2011), and following spawning, mature haploid eggs undergo fertilization (Fig. 1). Similarly, in isolated oocytes of various hydrozoan jellyfish including *Cytaeis*, reinitiation from prophase I arrest is triggered by *in vitro* treatments that raise intraoocytic cAMP levels (Takeda *et al.*, 2006; Deguchi *et al.*, 2011), and in maturing *Cytaeis* oocytes that are stimulated by cAMP, a spike-like Ca^{2+} transient is generated after, rather than before, GVBD (Fig. 2A). Accordingly, preloading *Cytaeis* oocytes with the Ca^{2+} chelator BAPTA abolishes the cAMP-induced Ca^{2+} transient but does not prevent GVBD (Fig. 2B). In addition, artificial induction of a Ca^{2+} rise by the calcium ionophores A23187 and ionomycin fails to trigger GVBD in *Cytaeis* (Fig. 2 C,D) and other hydrozoan species (Freeman and Ridgway, 1993). Thus, based on the current evidence that has been gathered for jellyfish oocytes, Ca^{2+} does not appear to play an essential role in stimulating GVBD.

Nemerteans (ribbon worms)

Nemerteans constitute a phylum of about 1,300 marine species (Appeltans *et al.*, 2012) within a larger protostome clade that includes molluscs and annelids. Nemerteans are usually dioecious, and gravid females typically possess numerous ovaries with relatively small (~70-200 μm) and translucent oocytes that lack follicle cells (Stricker *et al.*, 2001). Whether nemertean oocytes in the field begin GVBD before or after being spawned has yet

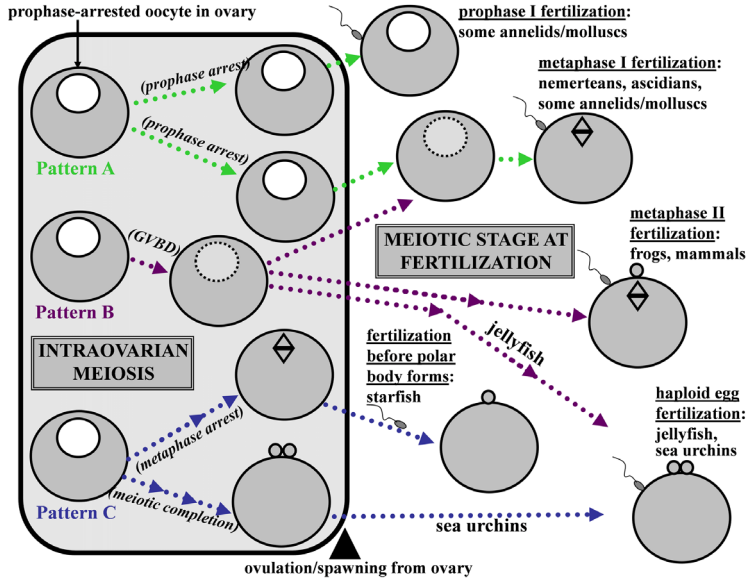

Fig. 1. Patterns of meiotic progression relative to fertilization for animal groups mentioned in this article. (Pattern A) *Prophase-arrested oocytes are released from the ovary.* **(Pattern B)** *Germinal vesicle breakdown (GVBD) is triggered within the ovary, and maturing oocytes are released before a secondary cell cycle arrest is attained either prior to or after meiotic divisions are completed.* **(Pattern C)** *Meiosis is reinitiated in the ovary, and maturing oocytes reach a secondary arrest before release from the ovary. Note: optimal rates of normal fertilization in starfish occur if post-GVBD oocytes are fertilized before polar body formation, and unlike intraovarian oocytes that arrest at metaphase I before spawning, prophase-arrested starfish oocytes that are removed from ovaries and treated with the maturation inducer 1-MA in the absence of sperm can complete meiotic divisions without establishing a metaphase arrest (not shown here). This figure is based mainly on data collated by Nishiyama* et al. *(2010) and Chiba (2011), which contain further details regarding various subtypes within these patterns.*

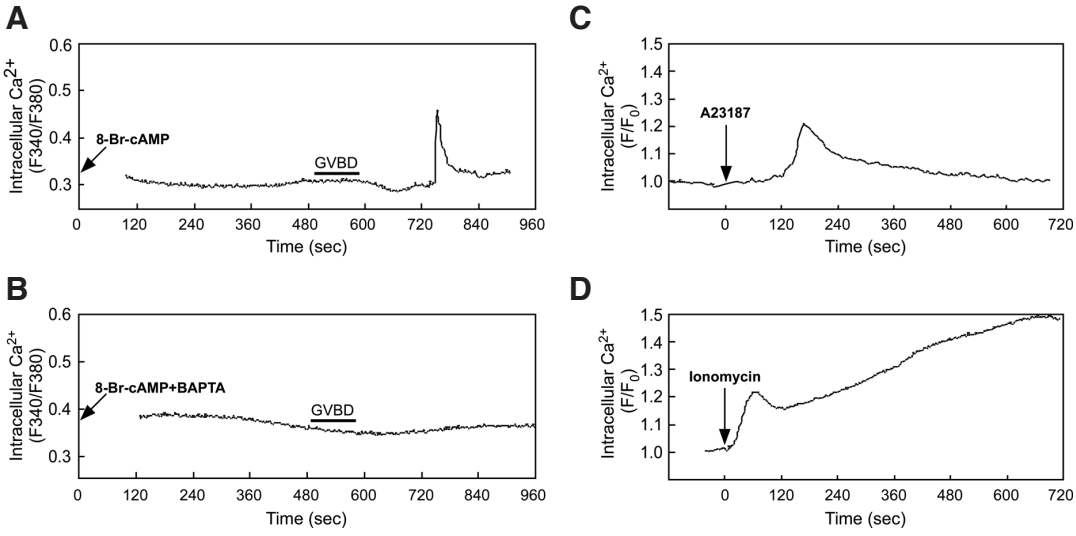

Fig. 2. Inability of a Ca^{2+} rise to trigger meiosis reinitiation in oocytes of the jellyfish cnidarian *Cytaeis uchidae*. (A) Cytaeis *oocytes isolated from the ovaries are arrested at prophase I and are induced to resume meiosis by injection of 8-Br-cAMP (final intracellular concentration: 25-50 μM). Based on fluorescence ratios of the Ca^{2+} indicator Fura-2 (F340/F380), 8-Br-cAMP triggers a spike-like Ca^{2+} transient, but this transient appears after, rather than before, GVBD.* **(B)** *If the 8-Br-cAMP-induced Ca^{2+} transient is blocked by co-injection of 8-Br-cAMP plus the Ca^{2+} chelator BAPTA (final intracellular concentration: 1-2 mM), the timing of GVBD is not altered.* **(C,D)** *Application of the Ca^{2+} ionophore A23187* **(C)** *or ionomycin* **(D)** *at 10 μM causes a substantial Ca^{2+} rise, which is measured using Calcium Green-1-dextran (10 kDa) and expressed as F/F_0, but both treatments fail to trigger GVBD.*

to be conclusively determined. However, fully-grown nemertean oocytes isolated from the ovary in the laboratory rapidly undergo GVBD in response to natural seawater (NSW) or cAMP-elevating stimuli before eventually arresting at metaphase I (Stricker *et al.*, 2013; Stricker, 2014). Such beneficial properties have facilitated analyses of intraoocytic calcium dynamics, and this section begins by summarizing findings related to calcium signals during meiotic reinitiation in the nemerteans *Cerebratulus lacteus* and *Micrura alaskensis* (Stricker and Smythe, 2000).

Oocytes of *C. lacteus* and *M. alaskensis* typically remain arrested at prophase I after being transferred from the ovary to calcium-free solutions of artificial SW (CaFSW). Such inhibition is not simply due to morbidity, since CaFSW-treated oocytes undergo GVBD when subsequently placed in NSW. Moreover, nearly all GV-containing oocytes maintained in calcium-containing artificial seawater (ASW), but not in CaFSW, complete GVBD when treated with A23187, and A23187-induced maturation is prevented by the oolemmal calcium channel blocker cobalt chloride, suggesting that external calcium influx can promote GVBD in nemerteans. However, transferring intraovarian oocytes directly into calcium-containing ASW typically promotes lower levels of GVBD than are obtained with NSW, and although cobalt treatments block ASW-induced GVBD, adding cobalt to NSW fails to inhibit GVBD. Similarly, following incubation in CaFSW, calcium-containing ASW is not as effective as NSW in restoring GVBD, but conditioning ASW with marine sediments for several weeks allows ASW to become as potent a stimulator of GVBD as NSW. Collectively, such findings suggest that calcium-containing ASW does not fully mimic NSW and that external calcium influx is not the only mechanism mediating GVBD in nemerteans.

Accordingly, nemertean oocytes that are triggered to mature by 1 μM serotonin (5-hydroxytryptamine, 5HT) achieve ~90% GVBD rates in both calcium-containing and calcium-free ASW solutions. In addition, when monitored for intracellular calcium signals by time-lapse confocal microscopy, 5HT consistently induces GVBD without eliciting an obvious calcium transient, even though such maturing oocytes generate a series of marked calcium oscillations upon insemination and subsequently develop into normal blastulae. Taken together, these findings suggest that although external calcium influx can facilitate the reinitiation of meiosis under certain experimental conditions, neither the influx of calcium ions nor the generation of a distinct intraoocytic calcium transient is invariably required for normal GVBD in nemerteans.

Following GVBD, maturing oocytes of nemerteans enhance their ability to produce a robust calcium response, as evidenced by the fact that fertilization of metaphase-I-arrested oocytes routinely induces an oscillatory series of point-source calcium waves prior to normal embryogenesis, whereas inseminated prophase-arrested specimens typically generate only a single non-wavelike calcium transient before failing to develop (Stricker, 1996; Stricker and Smythe, 2003). The precise reasons for these differences remain unknown, but the homogeneous ER of prophase-arrested oocytes undergoes an MPF-dependent reorganization to form discrete clusters in metaphase-I-arrested specimens prior to fertilization. Such ER clusters then disassemble at the time when fertilization-induced calcium oscillations cease either in controls or in oocytes subjected to the MPF inhibitor roscovitine (Stricker *et al.*, 1998; Stricker and Smythe, 2003; Stricker, 2006). Collectively, such findings suggest maturation-associated reorganizations of the ER help ensure that proper calcium signals are generated during nemertean fertilizations.

Molluscs (bivalves, limpets)

Approximately 50,000 species of marine molluscs are grouped into eight classes of the phylum Mollusca (Appletans *et al.*, 2012). Among these taxa, intraoocytic Ca^{2+} changes during the reinitiation of meiosis have been examined in bivalves (class Bivalvia) (Fig. 3A) and limpets (class Gastropoda). In most bivalves and limpets, numerous fully-grown oocytes arrested at prophase I can be dissected from the ovaries. The diameters of oocytes are less than 100 μm in most bivalves (e.g. Deguchi and Osanai, 1993; 1994; Zhang *et al.*, 2009; Fig. 3B) and 120-200 μm in limpets (e.g. Guerrier *et al.*, 1986). Limpet oocytes remain enclosed in a thin layer of follicle cells after removal from the ovary (Guerrier *et al.*, 1986; Gould *et al.*, 2001), whereas bivalve oocytes lack surrounding cells (Degu-

chi and Osanai, 1994; Fig. 3B). Although some isolated oocytes of bivalves and limpets undergo spontaneous meiotic reinitiation in a species-, season-, or batch-dependent manner, most remain arrested at prophase I until further stimulation.

In a few bivalves such as *Spisula*, *Mactra*, and *Barnea*, isolated and naturally spawned oocytes normally do not resume meiosis and undergo GVBD until after being fertilized (Fig. 1) (Deguchi and Osanai, 1994 and references therein). During such fertilizations, prophase-arrested oocytes exhibit a Ca^{2+} rise that comprises an initial peak (cortical flash) and a subsequent elevated plateau lasting for several minutes (Deguchi and Osanai, 1994; Deguchi and Morisawa, 2003). Several lines of evidence suggest that the sperm-induced Ca^{2+} rise in these bivalves strictly depends on the influx of external Ca^{2+} (see Kashir *et al.*, 2013, for a recent review) and GVBD can be triggered only when Ca^{2+} influx and the resultant intraoocytic Ca^{2+} rise continue for at least a few minutes (Deguchi and Osanai, 1994).

On the other hand, most bivalves normally spawn oocytes that are already undergoing GVBD, and such oocytes subsequently arrest at metaphase I prior to fertilization (Fig. 1). In this type of bivalve (e.g. *Ruditapes*, *Hiatella*, *Patinopecten*, *Mercenaria*, and *Crassostrea*), as well as in *Spisula*, prophase-arrested oocytes that are removed from the ovary can be triggered to resume meiosis *in vitro* by micromolar concentrations of 5HT (Osanai and Kuraishi, 1988; Guerrier *et al.*, 1993; Deguchi and Osanai, 1995; Fong *et al.*, 1997; Colas and Dubé, 1998; Leclerc *et al.*, 2000; Zhang *et al.*, 2009; Yuan *et al.*, 2012). Accordingly, because of its bioactivity and localization within the gonad, 5HT is believed to function *in vivo* as a physiologically relevant neurohormone that generally triggers intraovarian meiotic reinitiation from prophase I, spawning of maturing oocytes, and a subsequent metaphase I arrest in bivalve oocytes.

On the other hand, injecting ripe *Spisula* females with 5HT causes prophase-arrested oocytes to be spawned, and treatment of isolated GV-containing oocytes of *Spisula* with high concentrations of 5HT triggers meiotic completion without a metaphase I arrest (reviewed by Colas and Dubé, 1998). Thus, in *Spisula*, 5HT apparently regulates meiotic progression differently than in bivalve oocytes that arrest at metaphase I prior to fertilization. Nevertheless, nanomolar concentrations of 5HT are still believed to enhance the ability of *Spisula* oocytes to undergo successful fertilization (Masseau *et al.*, 2002).

Addition of exogenous 5HT to *Ruditapes*, *Hiatella*, and *Spisula* oocytes arrested at prophase I causes a transient Ca^{2+} rise followed by an elevated plateau (Guerrier *et al.*, 1993; Deguchi and Osanai, 1995; Fong *et al.*, 1997; Colas and Dubé, 1998). Both the amplitude and the duration of the Ca^{2+} rise depend on the concentration of 5HT (Deguchi and Osanai, 1995; Fong *et al.*, 1997), and the 5HT-induced Ca^{2+} rise is propagated as a point-source Ca^{2+} wave that rapidly spreads across the oocyte (Fig. 3C). Such a transmission pattern is in turn unlike the initial cortical flash of fertilization (Deguchi and Morisawa, 2003) or the non-wavelike calcium transient that is stimulated by excess K^+ (Fig. 3D). Ca^{2+} release from IP_3-sensitive stores may be the main mediator of the 5HT-induced Ca^{2+} wave, since: i) a Ca^{2+} rise persists in the absence of external Ca^{2+} (Guerrier *et al.*, 1993; Deguchi and Osanai, 1995; Leclerc *et al.*, 2000), but is suppressed by the IP_3 receptor antagonist heparin (Deguchi and Osanai, 1995), and ii) adding 5HT generates a transient increase in intraoocytic levels of IP_3 (Gobet *et al.*, 1994). However, in addition to internal calcium release, a Ca^{2+} influx pathway is also activated following application of 5HT, and such influx enhances the action of 5HT (Guerrier *et al.*, 1993; Deguchi and Osanai, 1995; Colas and Dubé, 1998). In any case, the amplitude and duration of 5HT-induced Ca^{2+} rises are tightly

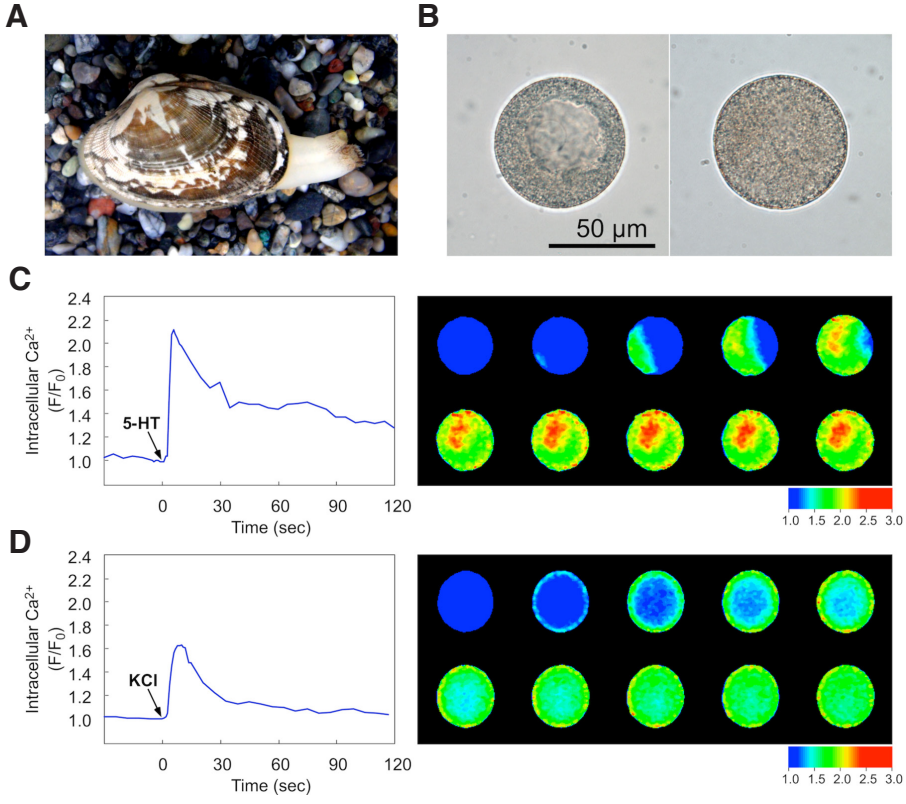

Fig. 3. Meiotic reinitiation by a 5HT-induced Ca^{2+} rise, but not by a K^+-induced Ca^{2+} rise, in oocytes of the Japanese littleneck bivalve mollusc *Ruditapes philippinarum*. (A) *Adult clams (shell length: 3-6 cm) can be collected in the field or purchased from Japanese fish shops and supermarkets.* **(B, left)** Ruditapes *oocytes isolated from the ovaries remain arrested at prophase I.* **(B, right)** *Alternatively, oocytes exposed to 5HT at 1 µM undergo GVBD and subsequently arrest at metaphase I.* **(C, left)** *Addition of 5HT immediately causes an intraoocytic Ca^{2+} rise comprised of an initial peak and a subsequent plateau, which are plotted in terms of the relative fluorescence intensity (F/F_0) of the Ca^{2+} indicator Calcium Green-1.* **(C, right)** *Pseudocolored images taken every 0.5 sec reveal the initial rising phase as a point-source Ca^{2+} wave starting from the cortex at the opening of a measurement chamber.* **(D)** *By contrast, in analyses using the same methods for monitoring and graphing calcium transients, a smaller Ca^{2+} rise in the form of a cortical flash is triggered by excess K^+ seawater (0.53 M KCl: normal seawater = 1: 4), and such a flash fails to induce GVBD.*

coupled with the presence or absence of GVBD, and 5HT cannot trigger GVBD without a significant intraoocytic Ca^{2+} rise (Guerrier *et al*., 1993; Gobet *et al*., 1994; Deguchi and Osanai, 1995; Fong *et al*., 1997; Leclerc *et al*., 2000; Deguchi and Morisawa, 2003). Similarly, the stimulatory or inhibitory effects of various agonists or antagonists of GVBD correlate well with the amplitudes and durations of the Ca^{2+} rises caused by these agents (Fong *et al*., 1997).

Based on studies using pharmacological modulators of mammalian 5HT receptors (e.g. Gobet *et al*., 1994; Fong *et al*., 1997; Osada *et al*., 1998), 5HT receptors of bivalve oocytes appear to be distinct from any known mammalian 5HT receptor and instead tend to present a mixed pharmacological profile of $5HT_1$/$5HT_2$/$5HT_3$ receptors, with some subtypes of these three receptor classes having been shown to regulate calcium signals in somatic cells (Pytliak *et al*., 2011). Alternatively, a recently cloned 5HT receptor from the bivalve *Patinopecten* ($5HT_{py}$) exhibits specific sequence homology to mammalian $5HT_1$ receptors, which can also modulate intracellular calcium levels (Hill *et al*., 2000), and immunolocalization analyses reveal mRNA for such a receptor in intraovarian oocytes of *Patinopecten* (Tanabe *et al*., 2010).

Regardless of the type of 5HT receptor that may be expressed, intraovarian oocytes of bivalves are apparently protected from the premature action of 5HT by an "oocyte maturation arresting factor" (OMAF) that has been characterized in *Patinopecten* as a protein-containing substance with an estimated molecular mass of ~60 kDa (Tanabe *et al*., 2006; Yuan *et al*., 2012). Accordingly, OMAF inhibits a 5HT-induced Ca^{2+} rise and subsequent GVBD in *Ruditapes* oocytes and prevents *Patinopecten* ovaries from releasing maturing oocytes (Tanabe *et al*., 2006).

The effects of various chemical agents other than 5HT also suggest essential roles for Ca^{2+} rises during GVBD in some bivalve oocytes. For example, Ca^{2+} ionophores such as A23187 and ionomycin mobilize external and internal Ca^{2+} to produce an intraoocytic Ca^{2+} rise that is followed by GVBD (Osanai and Kuraishi, 1988; Guerrier *et al*., 1993; Deguchi and Osanai, 1994; 1995; Leclerc *et al*., 2000; Zhang *et al*., 2009). Similarly, excess K^+ seawater, which evokes Ca^{2+} influx as a result of membrane depolarization, is a reliable inducer of GVBD in bivalve oocytes that are normally fertilized at prophase I (Deguchi and Osanai, 1994 and references therein). In oocytes of *Ruditapes*, in contrast, excess K^+ causes a relatively small Ca^{2+} rise and fails to trigger GVBD (Guerrier *et al*., 1993; Leclerc *et al*., 2000; Fig. 3D). Moreover, although weak bases such as ammonia are frequently used to cause intracellular alkalization, such bases can also accelerate a pre-GVBD Ca^{2+} rise in some bivalve oocytes mainly via the release of Ca^{2+} from internal stores (e.g. Guerrier *et al*., 1993; Leclerc *et al*., 2000). Similarly, thapsigargin, which is known to induce an intracellular Ca^{2+} rise by inhibiting the Ca^{2+} pump of the ER, also triggers GVBD in *Ruditapes* oocytes (Guerrier *et al*., 1993). In addition, injecting IP_3 or photoreleasing caged IP_3 induces Ca^{2+} release and GVBD in *Spisula*, *Mactra*, and *Ruditapes* oocytes (Bloom *et al*., 1988; Guerrier *et al*., 1996; Deguchi and Morisawa, 2003). Accordingly, if the Ca^{2+} rises induced by the above mentioned chemicals are inhibited, GVBD is also specifically blocked (Guerrier *et al*., 1993; Deguchi and Osanai, 1994; Leclerc *et al*., 2000; Deguchi and Morisawa, 2003). For example, in *Mactra* oocytes, pre-treatments with heparin and D-600 inhibit the Ca^{2+} rises and subsequent GVBD that are normally induced by IP_3 and excess K^+, respectively, but neither heparin nor D-600 blocks these processes in oocytes that are stimulated by excess K^+ and IP_3, respectively (Deguchi and Osanai, 1994; Deguchi and Morisawa, 2003). Collectively, such findings indicate that an intraoocytic Ca^{2+} rise is a prerequisite for meiotic reinitiation from prophase I in the bivalves described above.

On the other hand, the role of calcium during GVBD either remains unclear or has been shown to be non-essential in bivalves such as *Crassostrea*, *Limaria*, and *Mytilus*, as well as in limpets such as *Patella* and *Lottia*, where naturally spawned oocytes arrest at metaphase I prior to fertilization. For example, in *Crassostrea* oocytes that undergo GVBD and arrest at metaphase I in response to 5HT (Osanai and Kuraishi, 1988), there are conflicting reports regarding the presence or absence of a Ca^{2+} rise during GVBD (Kyozuka *et al*., 1997; Leclerc *et al*., 2000). Similarly, it is unclear whether or not GVBD is indeed triggered by artificially elevated Ca^{2+} levels in *Crassostrea* (Kyozuka *et al*., 1997; Leclerc *et al*., 2000). In addition, the 5HT receptor profiles determined using specific agonists and antagonists are quite different in two published papers (Kyozuka *et al*., 1997; Osada *et al*., 1998), although such discrepancies may well be due to the remarkable variability in maturity and responsiveness to 5HT that *Crassostrea* oocytes can exhibit during the breeding season (Leclerc *et al*., 2000).

In the bivalves *Limaria* and *Mytilus*, oocytes cannot be stimulated to mature by 5HT (Osanai and Kuraishi, 1988; Deguchi and Osanai, 1993). Moreover, A23187 either only weakly induces or does not stimulate GVBD in *Limaria* (Deguchi and Osanai, 1993; 1994) and *Mytilus* (Osanai and Kuraishi, 1988), respectively. Furthermore, an intraoocytic Ca^{2+} rise is neither necessary nor sufficient for GVBD in the limpets *Patella* and *Lottia* (Guerrier *et al*., 1986; Gould *et al*., 2001). Collectively, such findings indicate a calcium-independent mechanism of GVBD, and indeed, meiosis reinitiation from prophase I arrest in these bivalves and limpets strictly depends on a pre-GVBD rise in intraoocytic pH (Guerrier *et al*., 1986; Deguchi and Osanai, 1994; Gould *et al*., 2001). Similarly, a pH-mediated mode of GVBD has also been reported for bivalve oocytes that require Ca^{2+} elevations for GVBD, as Ca^{2+} and pH rises may act synergistically to reinitiate meiosis in such species (see Deguchi and Osanai, 1994; 1995; Dubé and Eckberg, 1997; Colas and Dubé, 1998 and references therein).

The pathways that potentially link pre-GVBD elevations in intraoocytic Ca^{2+} and/or pH with the activation of cell cycle kinases such as MPF and MAPKs remain largely unknown in molluscs (reviewed by Colas and Dubé, 1998). However, since phospholipase C presumably produces diacylglycerol (DAG) along with IP_3 in oocytes stimulated by 5HT, it follows that DAG-sensitive isotypes of protein kinase C (PKC) would also be activated. Indeed, in *Spisula* oocytes, PKC agonists such as TPA (tetradecanoylphorbol-13-acetate) trigger GVBD, whereas antagonists of PKC can block GVBD that is normally induced by fertilization or TPA (Eckberg *et al*., 1987). Alternatively, TPA inhibits rather than accelerates GVBD in *Ruditapes* oocytes stimulated by 5HT, apparently by suppressing its action on a Ca^{2+} rise (Gobet *et al*., 1994).

Another potential calcium-sensitive mediator of GVBD is calcium/calmodulin dependent kinase II (CaMKII), based on the inhibitory effects of calmodulin (CaM) antagonists on GVBD in some bivalve oocytes (Carroll and Eckberg, 1986; Zhang *et al*., 2009). However, as opposed to simply suppressing a pathway downstream to the maturation-associated Ca^{2+} elevation, such inhibitors can also prevent calcium influx itself (Carroll and Eckberg, 1986), thereby complicating interpretations of the drug-induced blockage of GVBD.

In any case, although the underlying regulatory mechanisms of GVBD have yet to be fully elucidated, many molluscan oocytes are known to enhance their abilities to generate proper fertilization-induced Ca^{2+} responses during maturation from prophase I to metaphase I, with optimal responses generated during fertilization typically taking the form of repetitive Ca^{2+} oscillations in bivalves versus a single but prolonged Ca^{2+} rise in limpets (Kashir *et al.*, 2013). Moreover, fertilization-induced Ca^{2+} signals are necessary and sufficient for these oocytes to be released from a metaphase I arrest (Kashir *et al.*, 2013), whereas an additional pH elevation is not required for such cell cycle progression (Deguchi and Osanai, 1995).

Annelids (echiuran and polychaete worms)

Recent molecular-based phylogenies have indicated that the phylum Annelida with its ~14,000 marine species (Appeltans *et al.*, 2012) should also include echiuran worms that were formerly viewed as constituting their own phylum (Struck *et al.*, 2011). Thus, the echiuran *Urechis* is treated here as an annelid.

Oocytes of annelids are fertilized at prophase I (e.g. *Urechis*) or at metaphase I (e.g. *Chaetopterus*, *Arenicola*, and *Pseudopotamilla*) (Meijer, 1979; Stephano and Gould, 1997; Kashir *et al.*, 2013), and in either case, fully-grown oocytes arrested at prophase I accumulate in the coelom after having already been freed from follicle cells. As in molluscs, the connection between a Ca^{2+} rise and subsequent meiotic progression is well documented for annelid oocytes that are normally fertilized at prophase I (Fig. 1). Accordingly, fertilized oocytes of *Urechis* exhibit a single Ca^{2+} rise initiated by a non-wave-like Ca^{2+} transient (Stephano and Gould, 1997), which is probably homologous to the cortical flash seen in mollusc oocytes. Based on several lines of evidence, Ca^{2+} influx is responsible for the Ca^{2+} rise at fertilization, although the supplemental contribution of IP_3-dependent Ca^{2+} release remains possible (Stephano and Gould, 1997). Moreover, a Ca^{2+} rise in *Urechis* oocytes is necessary but not sufficient for meiosis reinitiation from prophase I, as GVBD proceeds only when an intraoocytic Ca^{2+} and pH rise occur simultaneously (Stephano and Gould, 1997 and references therein).

In oocytes of polychaete annelids that arrest at metaphase I before fertilization (Fig. 1), induction of GVBD in isolated oocytes is triggered in various ways, including: i) transfer into natural, but not artificial, seawater in *Chaetopterus* (Ikegami *et al.*, 1976); ii) exposure to a protease purified from digestive tract fluids in *Sabellaria* (Peaucellier, 1978), and iii) application of coelomic fluid supplemented with prostomial homogenates in *Arenicola* (Watson *et al.*, 1998). *Chaetopterus* oocytes incubated in artificial seawater can be stimulated to undergo GVBD by excess K^+ in the presence of external Ca^{2+} (Ikegami *et al.*, 1976) and by A23187 even in Ca^{2+}-free seawater (Eckberg and Carroll, 1982). A23187 also triggers GVBD in *Sabellaria* oocytes (Peaucellier, 1978) but not in *Arenicola* oocytes (Meijer, 1979; Watson *et al.*, 1998). Neither A23187 nor ionomycin induces GVBD in *Pseudopotamilla* oocytes (Nakano and Deguchi, unpublished observation), and instead an intraoocytic cAMP elevation seems to be essential for this process (Deguchi *et al.*, 2011). It should be noted that release from the secondary arrest at metaphase I absolutely depends on an intraoocytic Ca^{2+} rise in all of the polychaetes listed above (Meijer, 1979; Kashir *et al.*, 2013), regardless of the importance of a Ca^{2+} rise at prophase I.

In *Chaetopterus*, it is thought that activations of PKC and CaM are essential steps in the activation of MPF, and that PKC directly phosphorylates CDK1 (cdc2) to yield active MPF (Eckberg *et al.*, 1996). However, the actual interactions between a Ca^{2+} rise and PKC or CaMKII activities have not been fully documented.

Echinodermata (starfish, sea urchins)

Approximately 2,000 starfish species constitute the class Asteroidea within the deuterostome phylum Echinodermata (Appeltans *et al.*, 2012). Most starfish have separate sexes, and ripe females store numerous prophase-arrested oocytes in their ovaries. When triggered by environmental cues, a relaxin-like peptide is released from the nervous system of females to cause follicle cells around fully grown oocytes to produce a maturation-inducing substance (=1-methyladenine, 1-MA) that initiates the signaling cascade leading to GVBD (Mita, 2013). In prophase-arrested oocytes that are removed from the ovary and treated with 1-MA *in vitro*, meiosis is completed without a secondary arrest, whereas injecting 1-MA into females to replicate a more natural mode of maturation induction causes oocytes to arrest at metaphase I, owing to a reduced intraoocytic pH that is established in the ovary, but not in seawater (Harada *et al.*, 2003). Following the spawning of metaphase-arrested oocytes from 1-MA-injected females, seawater triggers an intraoocytic pH rise that eventually allows meiotic progression, and given that the lowest levels of polyspermy occur when inseminations are carried out between GVBD and first polar body formation, the metaphase-I arrest is viewed as an adaptation for ensuring that oocytes released during protracted spawnings in the field have not already undergone polar body formation before encountering sperm (Chiba, 2011).

Although the role of calcium during GVBD in starfish oocytes that undergo a metaphase I arrest has not been elucidated, numerous investigations have assessed calcium dynamics in isolated starfish oocytes that mature *in vitro* and complete their meiotic divisions without arresting at metaphase. For example, pioneering analyses of the starfish *Marthasterias glacialis* have shown that external applications of 1-MA in CaFSW trigger a pre-GVBD Ca^{2+} transient and that blocking the 1-MA-induced Ca^{2+} transient via EGTA injection prevents GVBD (Moreau *et al.*, 1978). These and various other findings support the view that a Ca^{2+} rise normally promotes GVBD in starfish (Moreau *et al.*, 1985). However, in additional studies of *M. glacialis* and other starfish, GVBD can proceed in control 1-MA treatments without a marked Ca^{2+} transient being generated, even though subsequent inseminations trigger a recognizable Ca^{2+} response, and, contrary to previous reports, 1-MA-induced GVBD is not blocked by calcium chelators in such studies (Picard and Doree, 1983, Eisen and Reynolds, 1984; Witchel and Steinhardt, 1990; Kikuyama and Hiramoto, 1991; Stricker, 1995).

In attempting to reconcile these contradictory results, it should be noted that 1-MA causes oocytes of the starfish *Asterina pectinifera* and *Astropecten aranciacus* to generate both cytoplasmic and nuclear Ca^{2+} elevations before GVBD (Santella and Kyozuka, 1994; Santella *et al.*, 1998; Lim *et al.*, 2003), and that injections of BAPTA into the nucleus, but not into the cytoplasm, can inhibit 1-MA-induced GVBD (Santella and Kyozuka, 1994). Similarly, in 1-MA-treated *A. pectinifera* oocytes, all elevations in Ca^{2+} and subsequent GVBD are eliminated by the membrane-permeable calcium release blocker TMB-8 (Tosuji *et al.*, 2007), presumably because, unlike BAPTA, TMB-8 can cross the nuclear envelope to affect nuclear Ca^{2+} signals. Moreover, injecting IP_3 or cyclic ADP

ribose (cADPR) into GVs of immature oocytes can trigger nuclear Ca^{2+} transients, and following such elevations, some of the injected oocytes complete GVBD in the absence of 1-MA (Santella and Kyozuka, 1997a). Accordingly, some 1-MA-treated oocytes do not undergo GVBD, if IP_3 and cADPR blockers are injected into the GV (Santella and Kyozuka, 1997a; Stanella *et al*., 1998), whereas cytoplasmic delivery of the inhibitors routinely fails to prevent GVBD in 1-MA-stimulated oocytes (Santella and Kyozuka, 1997a; Iwasaki *et al*., 2002). In addition, nuclear injections of antibodies to CaM, a likely downstream target of Ca^{2+} signals, block GVBD in 1-MA-treated oocytes, whereas co-injecting the antibodies with excess CaM allows GVBD (Santella and Kyozuka, 1997b). Collectively, such findings suggest that in these two starfish nuclear Ca^{2+} transients promote GVBD via a CaM-dependent mode of MPF activation.

In terms of upstream processes that might regulate such Ca^{2+} signals in starfish oocytes, reorganizations of the cortical actin network may play a key role, based on: i) the rapid restructuring of actin filaments that routinely occurs before GVBD in 1-MA-treated controls; ii) the fact that the 1-MA-induced calcium wave propagates exclusively in the cortical region where actin filaments are concentrated, and iii) the findings that experimental manipulations to hypo- or hyperpolymerize cortical actin serve to disrupt normal Ca^{2+} dynamics and maturation (Kyozuka *et al*., 2008; 2009; Chun and Santella, 2009; Chun *et al*., 2013). The precise mechanisms by which a remodeling of the actin network might affect Ca^{2+} release capabilities in starfish oocytes are not known. However, based on results from analyses of somatic cells, changes in the organization of actin filaments could both directly and indirectly modulate IP_3 receptor function (Chun and Santella, 2009). Alternatively, the actin network itself might serve as an intracellular calcium pool that releases Ca^{2+} during 1-MA-induced restructuring (Chun and Santella, 2009).

As opposed to the more variable results that have been reported regarding the role of calcium signals in triggering GVBD, starfish oocytes have consistently been demonstrated as developing an enhanced capacity for IP_3-mediated calcium release following GVBD, owing to a heightened sensitivity to IP_3 rather than to any marked increase in IP_3 receptor mass (Chiba *et al*., 1990; Iwasaki *et al*., 2002; Lim *et al*., 2003). The enhanced capacity for Ca^{2+} release in turn allows a more robust and effective fertilization-induced calcium response to be generated in post-GVBD vs. prophase-arrested specimens (Stricker *et al*., 1994), and although ER reorganizations have also been documented in maturing starfish oocytes (Jaffe and Terasaki, 1994), such changes occur well after actin remodeling is induced by 1-MA, suggesting that at least the early enhancements in calcium signaling capabilities rely more on actin reorganizations (Chun and Santella, 2009). Accordingly, the increased sensitivity to IP_3 during starfish oocyte maturation depends on MPF activation, which apparently modifies IP_3-mediated signaling not by directly phosphorylating IP_3 receptors but instead by reorganizing the cortical actin network (Lim *et al*., 2003).

Approximately 1,000 species of sea urchins and their allies constitute the class Echinoidea of the phylum Echinodermata (Appeltans *et al*., 2012). Sea urchins normally complete meiotic divisions inside the ovary without a metaphase arrest prior to spawning, but fully-grown prophase-arrested oocytes of sea urchins can be removed from the ovary and allowed to undergo GVBD *in vitro* (Wessel *et al*., 2002). Although several types of inhibitors have been shown to block such *in vitro* GVBD (Wessel *et al*., 2002), the roles played by calcium signals during meiotic reinitiation from prophase arrest have not been fully elucidated. However, calcium release capabilities mediated by both IP_3- and non- IP_3-dependent pathways are enhanced during *in vitro* maturation of sea urchin oocytes, and sperm generate more robust calcium responses along with lower levels of polyspermy in post-GVBD vs. prophase-arrested specimens (Miyata *et al*., 2006).

Ascidians (sea squirts)

As deuterostome invertebrates closely related to vertebrates, ~3,000 species of ascidians belong to the subphylum Tunicata within the phylum Chordata (Appeltans *et al*., 2012). All ascidians are hermaphrodites that produce follicle-enclosed oocytes, and depending on the species examined, fully grown oocytes in the ovary can either remain arrested at prophase I or undergo GVBD (Lambert, 2005). After spawning, ascidian oocytes mature to a metaphase I arrest before being fertilized (Costache *et al*., 2014), although *in vitro* fertilization of certain GV-containing ascidian oocytes also yields some normal development (Gallo *et al*., 2013).

Immature oocytes of several ascidian species can be triggered to undergo GVBD by treatment with calcium-containing seawaters, whereas spontaneous maturation is reversibly inhibited if oocytes are incubated in CaFSW (Sakairi and Shirai, 1991; Cuomo *et al*., 2006; Lambert, 2011). Similarly, compared to the levels of forskolin-induced GVBD in low pH SW, oocytes of *Boltenia villosa* undergo less maturation following pre-treatment with either the calcium chelator BAPTA or with inhibitors of ryanodine receptor (RyR) functioning that normally allows internal calcium release via non-IP_3-dependent mechanisms (Lambert, 2011). Conversely, GVBD in *Boltenia* can be induced by an agonist of the exchange protein directly activated by cAMP (Epac) (Lambert, 2011), which has been shown to trigger calcium release from RyRs in other cells, and although actual evidence has yet to be published, preliminary observations of calcium elevations occurring during GVBD in *Boltenia* oocytes have been reported (Lambert, 2011).

In addition, the activity of T-type calcium channels increases as immature oocytes of *Styela plicata* grow larger during prophase I arrest, and in this species, such increased activity is correlated with the ability of some fully-grown prophase-arrested oocytes to undergo normal post-fertilization GVBD and subsequent embryogenesis (Gallo *et al*., 2013). Furthermore, compared to metaphase-arrested oocytes of *Ciona intestinalis*, prophase-arrested specimens generate lower amplitude calcium transients in response to either IP_3 injections or ionophore treatments (Cuomo *et al*., 2006). Collectively, such findings support the views that in ascidians Ca^{2+} elevations mediated by external calcium influx and/or internal calcium release help to promote GVBD and that calcium-mobilizing mechanisms are enhanced during oocyte growth and maturation. Exactly how Ca^{2+} elevations might elicit GVBD in ascidian oocytes remains unknown, but based on tests using pharmacological reagents, it has been proposed that a calcium-dependent activation of CaMKII may facilitate activation of the Cdc25 phosphatase, which in turn could help convert inactive pre-MPF into active MPF (Lambert, 2011).

Conclusions

A well unified literature has shown that compared to prophase-arrested specimens, mature oocytes of marine invertebrates develop an enhanced capacity to mobilize their calcium stores.

Moreover, in many cases, such enhanced capabilities depend on structural and functional reorganizations that the ER undergoes during oocyte maturation (Stricker, 2006). These findings in turn help to explain why prophase-arrested oocytes of most animals are incapable of generating an effective fertilization-induced calcium response that promotes normal meiotic completion and subsequent development (Stricker, 1999).

Alternatively, although the key roles of calcium transients during fertilization and the later phases of meiotic maturation are well established, there is little consensus regarding the contributions of calcium signals to the initial resumption of meiosis from prophase I arrest. The strongest data for an essential role being played by intraoocytic Ca^{2+} signals during GVBD come from analyses of nuclear Ca^{2+} transients in starfish oocytes, as well as from investigations of oocyte maturation in molluscs, annelids, and ascidians. However, other studies of cnidarian, nemertean, mollusc, annelid, and starfish oocytes provide evidence that tend to refute the view that calcium signals are routinely needed for GVBD.

In attempting to reconcile these apparently conflicting results, experimental protocols need to be carefully assessed. For example, along with exhibiting different patterns of meiotic arrest (Nishiyama *et al.*, 2010), marine invertebrate oocytes can also vary in the timing of GVBD onset relative to the initial stimulation of maturation. Thus, as proposed for mammals (Homa *et al.*, 1993), variable results might be obtained, depending on exactly when an experimental manipulation such as calcium chelation is carried out with respect to the delivery of a maturation-inducing stimulus. Similarly, although the notion of nuclear envelopes being impermeable to calcium ions remains controversial (Bootman *et al.*, 2009), variable experimental conditions have been postulated as causing starfish GVs to differ from being freely permeable to relatively impermeable to Ca^{2+} flow (Santella and Kyozuka, 1997a). Hence, depending on the physiological state of the nucleus at the time that maturation is stimulated, differing conclusions regarding the roles of Ca^{2+} during GVBD could be reached. Similarly, the precise mode of stimulus delivery could differ and thereby affect the presence or absence of calcium signals, as has been demonstrated for oocytes of the starfish *Asterina miniata*, where the relatively slow diffusion of subthreshold levels of maturation-inducing hormone fails to elicit a calcium response prior to GVBD, whereas a more rapid perfusion consistently generates a Ca^{2+} transient that nevertheless has been deemed neither necessary nor sufficient for meiotic reinitiation (Witchel and Steinhardt, 1990).

In addition to such considerations, the potential contributions of false-positive and false-negative results should also be evaluated. For example, the ability of certain EGTA injections to block 1-MA-induced oocyte maturation has been attributed to possible toxic effects of the particular experimental protocol that was used (Picard and Doree, 1983). Similarly, the fact that BAPTA-loaded *Xenopus* oocytes fail to mature may not be due to calcium chelation, but rather to the sequestration of such transition metals as zinc (Machaca, 2011). Conversely, in cases where calcium chelators fail to block GVBD, the chelator treatment should also be demonstrated as actually preventing a pre-GVBD calcium rise. Moreover, the fact that GVBD might not be triggered by exogenous calcium modulators such as ionomycin does not rule out an essential role for endogenous calcium transients, as not all calcium-mobilizing agents may be capable of triggering signals that fully mimic the complex temporal and spatial features that are normally required for maturation onset (Vasilev *et al.*, 2012).

Aside from assessing experimental protocols, the possibility that variability in the reported results arises from inherent species-specific differences needs to be considered. In fact, given the wide diversity in body plans and modes of reproduction exhibited by extant marine invertebrates, variable patterns and mechanisms of meiotic progression could certainly have evolved from one taxon to another, or even among closely related species within the same group. For example, unfertilized oocytes of some starfish species are known to arrest at G1 after the second meiotic division, whereas others terminate at G2 (Nishiyama *et al.*, 2010). Similarly, oocytes of one starfish (*Asterina pectinifera*), but not of another (*Astropecten aranciacus*), respond to cADPR (Chun and Santella, 2009). Collectively, such findings indicate that not all starfish oocytes regulate meiotic progression and calcium signaling in precisely the same way.

Perhaps by examining additional species and taxa, a clearer set of patterns will become evident across the animal kingdom, thereby helping to clarify the roles played by calcium signals during the onset of oocyte maturation. Similarly, although attempts have been made to identify specific downstream targets of Ca^{2+} elevations during cases of calcium-mediated GVBD, further analyses are needed to link Ca^{2+} signals with MPF activation and GVBD. In any case, various gaps in the existing literature as well as substantial variability in the reported results indicate that our understanding of calcium signals during oocyte maturation in marine invertebrates remains a work in progress.

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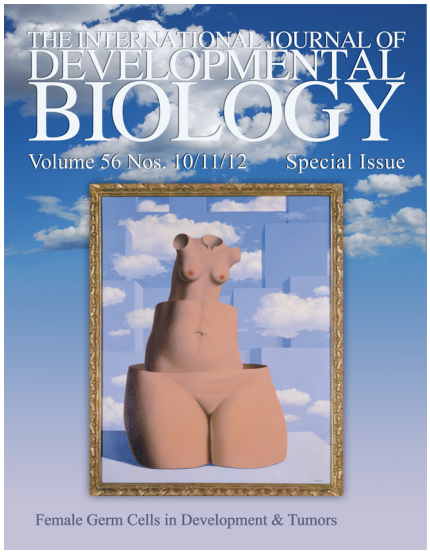

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