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Review

Calcium signaling and endoplasmic reticulum dynamics during fertilization in marine protostome worms belonging to the phylum Nemertea

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ABSTRACT

Metaphase-I-arrested eggs of marine protostome worms in the phylum Nemertea generate a series of point-source calcium waves during fertilization. Such calcium oscillations depend on inositol-1,4,5-trisphosphate-mediated calcium release from endoplasmic reticulum (ER) stores that undergo structural reorganizations prior to and after fertilization. This article reviews fertilization-induced calcium transients and ER dynamics in nemertean eggs and compares these topics to what has been reported for other animals in order to identify unifying characteristics and distinguishing features of calcium responses during fertilization across the animal kingdom.

Contents

1. Introduction

In all animals examined, fertilization causes eggs to elevate their concentrations of intracellular free calcium ions [1–3], and such elevations help activate proper embryogenesis [4–6]. Because of the fundamental importance of fertilization-induced calcium signals for normal development, attempts have been made to delineate common themes of calcium responses during fertilization. For example, Jaffe [7,8] hypothesized that eggs of deuterostome animals (e.g. echinoderms and chordates) release calcium from internal stores to propagate point-source calcium waves during fertilization, whereas fertilized eggs of protostomes (e.g. arthropods, annelids, and mollusks) use external calcium influx to generate their calcium transients.

Since the advent of calcium-sensitive probes and confocal microscopy, most imaging studies of calcium signals during

Abbreviations: cafsw, calcium-free seawater; CG, calcium green; ER, endoplasmic reticulum; ERK, extracellular signal regulated kinase; GVBD, germinal vesicle breakdown; IP_3, inositol 1,4,5 trisphosphate; MAPK, mitogen-activated protein kinase; MPF, maturation-promoting factor; RB, rhodamine B.

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fertilization have focused on deuterostome eggs [9–14]. However, nemertean worms constitute a phylum of ~1300 species within the so-called lophotrochozoan clade of protostomes [15,16], and the gametes of many marine nemerteans [17–19] are well suited for in vivo confocal imaging analyses that employ ratioing methods to help minimize pathlength and dye loading artifacts [20,21].

This review summarizes features of calcium signals and endoplasmic reticulum (ER) reorganizations in nemertean eggs that are subjected to normal inseminations or injections with soluble sperm extracts. Such patterns are also compared with what has been reported for fertilizations in other animals. In doing so, the view that fertilization-induced calcium signals differ fundamentally in protostome vs. deuterostomes is re-evaluated, and alternative hypotheses regarding fertilization-induced calcium signals are examined.

2. Results and discussion

2.1. After oocyte maturation, nemertean eggs arrest at metaphase I in preparation for fertilization

Ripe female nemerteans possess multiple ovaries with prophase-I-arrested oocytes that contain a large nucleus, called the germinal vesicle (Fig. 1A). After removal from the ovary, immature oocytes undergo germinal vesicle breakdown (GVBD) in response to stimulation by seawater or alternative triggers [22–25]. Subsequently, the mature egg arrests at metaphase I, at which point successful fertilization can occur, as evidenced by a robust calcium response and subsequent embryogenesis [26]. Conversely, inseminations of immature nemertean oocytes fail to yield normal calcium signals or development [26].

2.2. The fertilization-induced calcium response begins with a cortical flash involving external calcium influx

At the onset of fertilization, mature nemertean eggs undergo a rapid increase in free calcium ions, or "cortical flash", that arises essentially synchronously around the egg periphery (Fig. 1B) [21,24,26–31]. The cortical flash apparently involves external calcium influx, given its lack of a distinct point-source origin and the fact that pre-treating eggs with cobalt chloride to block oolemmal calcium channels prevents the cortical flash [26] without inhibiting subsequent calcium wave production (see Section 2.3). Conversely, injecting eggs with heparin to impair IP_3 (inositol 1,4,5 trisphosphate) receptor function before insemination blocks calcium waves but not the cortical flash [26]. Moreover, in tests using KCl to depolarize the oolemma, KCl-stimulated unfertilized eggs exhibit a cortical flash [26].

Impairment of cortical flash production via either cobalt chloride treatment or incubation with inhibitors of Src family kinase signaling is correlated with multiple sperm incorporations [26,31]. Thus, the cortical flash appears to contribute to a block against polyspermy.

2.3. During fertilization, the cortical flash is followed by repetitive calcium oscillations that are mediated by IP_3-dependent calcium release

Within ~10 min after a cortical flash is initiated, each nemertean zygote begins to propagate a series of point-source calcium waves (Fig. 1C). Such waves, or "oscillations", travel at ~10–15 μm/s in eggs maintained at 10–14 °C and thus correspond to the "fast" type of calcium waves noted for eukaryotes in general [32]. Based on the transfer of dye from injected eggs into fertilizing sperm (Fig. 1D), fertilization-induced calcium oscillations begin after sperm-egg fusion, and successive calcium waves typically arise every 2–8 min for 20–60 min to generate ~5–15 transients over the entire oscillatory sequence. The oscillatory signal gradually dampens and ultimately ceases before completion of polar body formation and pronuclear development [26,29,33]. Thus, unlike in certain mammalian eggs [34], the presence of pronuclei is not necessary for terminating fertilization-induced calcium signals in nemertean eggs.

In the nemertean *Cerebratulus lacteus*, the initial one to three calcium waves of fertilization arise near the sperm entry site, which in turn occurs with similar frequency in the animal vs. vegetal hemisphere of the egg [26]. Such waves typically fail to spread across the entire ooplasm and thus resemble incompletely-propagated transients of ascidian zygotes [35] or what have been termed "tango waves" in computer simulations

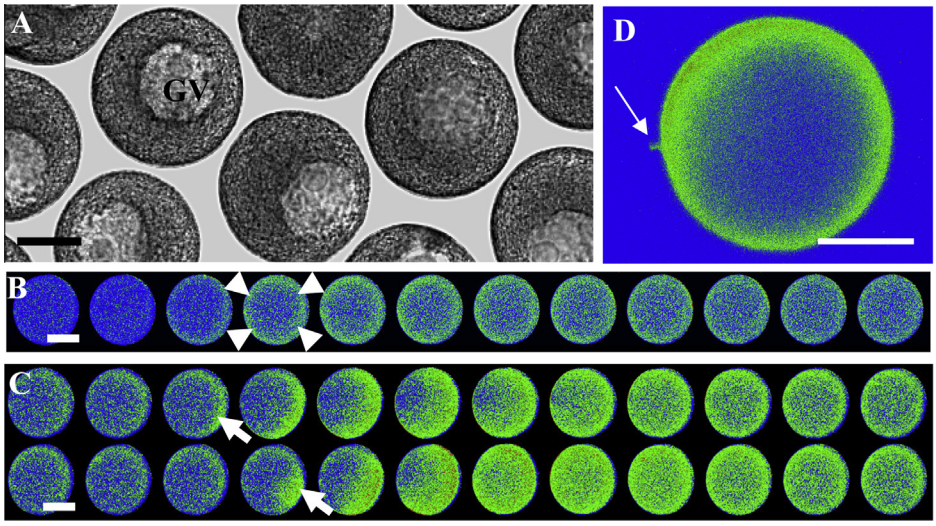

Fig. 1. (A) Immature oocytes of the nemertean *Cerebratulus* sp., showing a prophase-I-arrested germinal vesicle (GV). (B,C) Time-lapse confocal images of a *Cerebratulus* sp. egg that had been injected with the calcium indicator Calcium Green (CG) Dextran and imaged every 10 s during insemination to reveal a fertilization-induced cortical flash (arrowheads) and two subsequent calcium waves (arrows). Blues = relatively low calcium levels; yellows = elevated calcium. (D) Confocal image of a CG Dextran-injected *Cerebratulus* sp. egg, showing transfer of fluorescence to the fertilizing sperm (arrow), which occurs after sperm-egg fusion and before calcium oscillation production. Scale bars = 50 μm.

[36]. Thereafter, repetitive calcium transients in nemertean zygotes spread fully across the ooplasm as point-source waves that increase their fluorescence intensity an average of ~45% over pre-transient baselines. These values presumably correspond to substantially lower free calcium rises than the ~130% average fluorescence increases recorded for starfish zygotes, which in turn fall within an ~500–1000 nM range of peak free calcium amplitudes, based on correlative fura-2-based analyses [37].

In 19/24 *C. lacteus* zygotes, the later calcium waves eventually arise vegetally, even if the sperm enters the animal hemisphere, since a gradual animal-to-vegetal shifting of calcium wave onsets typically occurs in such cases [26]. Subsequently, the final waves tend to originate from a "vegetal pacemaker" [38], and these spatiotemporal patterns of calcium signals are typically accompanied by normal embryogenesis, at least as monitored up to blastular hatching.

Following transfer of nemetean zygotes from seawater to calcium-free seawater containing the calcium chelator EGTA, calcium waves continue to be generated [26], indicating that external calcium ions are not required for maintaining fertilization-induced calcium oscillations. Accordingly, UV-mediated photorelease of caged IP_3 invariably causes a calcium transient in unfertilized specimens, and pre-loading eggs with heparin consistently prevents normal calcium oscillations during fertilization [26]. Conversely, treatment with ryanodine to mobilize non-IP_3-dependent calcium stores only occasionally causes a calcium increase, collectively suggesting that fertilization-induced calcium oscillations in nemerteans are mainly dependent on IP_3-mediated calcium release [26].

Given that immature nemertean oocytes fail to generate the oscillatory calcium response displayed by mature eggs [26,29], potential roles played by maturation-associated kinases such as MPF (maturation-promoting factor) and MAPK (mitogen-activated protein kinase) have been examined. In such tests, various inhibitors prevent the activation of ERK1/2 (extracellular signal regulated kinases 1/2) types of MAPK but fail to block fertilization-induced calcium oscillations [29]. Conversely, the MPF inhibitor roscovitine either blocks calcium oscillations when used on mature eggs prior to fertilization, or dampens the frequency of ongoing oscillations if added after a fertilization-induced calcium response has begun [29]. Accordingly, treatments with colchicine to maintain MPF activity prolongs post-fertilization calcium oscillations, collectively indicating that the oscillatory calcium response depends on MPF, but not on ERK1/2 MAPK activity [29].

2.4. Fertilization-induced calcium oscillations are accompanied by reorganization of the ER

Given that the ER of eggs is a major IP_3-regulated calcium store [4,5,39] that reorganizes its structure during oocyte maturation [40–45], maturing nemertean oocytes were injected with the vital ER probe DiI [46] and monitored by confocal microscopy. Based on such studies, immature oocytes display a homogeneous ER (Fig. 2A), but within about 1 h after GVBD, the ER of mature eggs forms numerous ~1–5-μm diameter microdomains, or "clusters" [18,29,30,33], within the animal and vegetal ooplasm (Fig. 2B).

Depending on the species of nemertean examined, ER clusters normally disassemble ~20–60 min post-fertilization, and such disassembly is tightly correlated with the termination of fertilization-induced calcium oscillations [18,29]. Inhibition of ERK-type MAPK activation blocks neither cluster formation during maturation nor post-fertilization disassembly [29]. Alternatively, the MPF inhibitor roscovitine triggers ER loss in unfertilized, mature specimens, and, when applied post-fertilization, such treatments initiate precocious breakdown of ER clusters while also disrupting calcium oscillations [29]. Conversely, pre-treating mature eggs with colchicine to delay MPF degradation triggers persistent ER clustering and prolonged calcium oscillations post-fertilization [29]. Such findings indicate that an oscillatory calcium response requires ER clusters that depend upon MPF, but not ERK1/2 MAPK activity.

2.5. Injections of soluble sperm extracts mimic the fertilization-induced calcium response

In various animals, including all mammals examined, fertilization-induced calcium signals appear to be driven by a soluble factor that the sperm delivers into the ooplasm following gamete fusion [3,47–50]. Consistent with this view are the findings that fertilization-induced calcium oscillations in nemerteans normally occur after sperm-egg fusion and that injecting metaphase-I-arrested nemertean eggs with either whole sperm or extracts of sperm obtained from nemerteans or pigs consistently generates calcium oscillations (Fig. 3A) [26,27,51]. Compared to fertilizations, sperm extract-induced oscillations in nemertean eggs display similar peak amplitudes, overall durations, and tendencies toward vegetal onsets [26,27]. Moreover, as in fertilized eggs, such oscillations continue to be produced after transfer to calcium-free seawater (Fig. 3B). In addition, nearly all eggs that are injected with nemertean sperm extract become activated and undergo polar body formation [27]. Conversely, numerous control injections

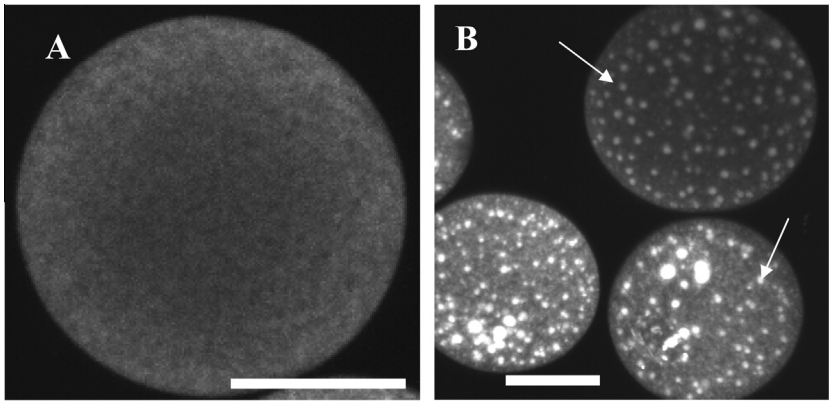

Fig. 2. (A) Projected z-series of confocal images taken of an immature oocyte of the nemertean *Cerebratulus lacteus* that had been injected with the vital endoplasmic reticulum (ER) dye DiI, showing a relatively homogeneous ER. (B) Confocal image of a mature *C. lacteus* egg (larger egg, upper right corner) and three mature *Cerebratulus* sp. eggs, showing distinct ER clusters (arrows). Scale bars = 50 μm.

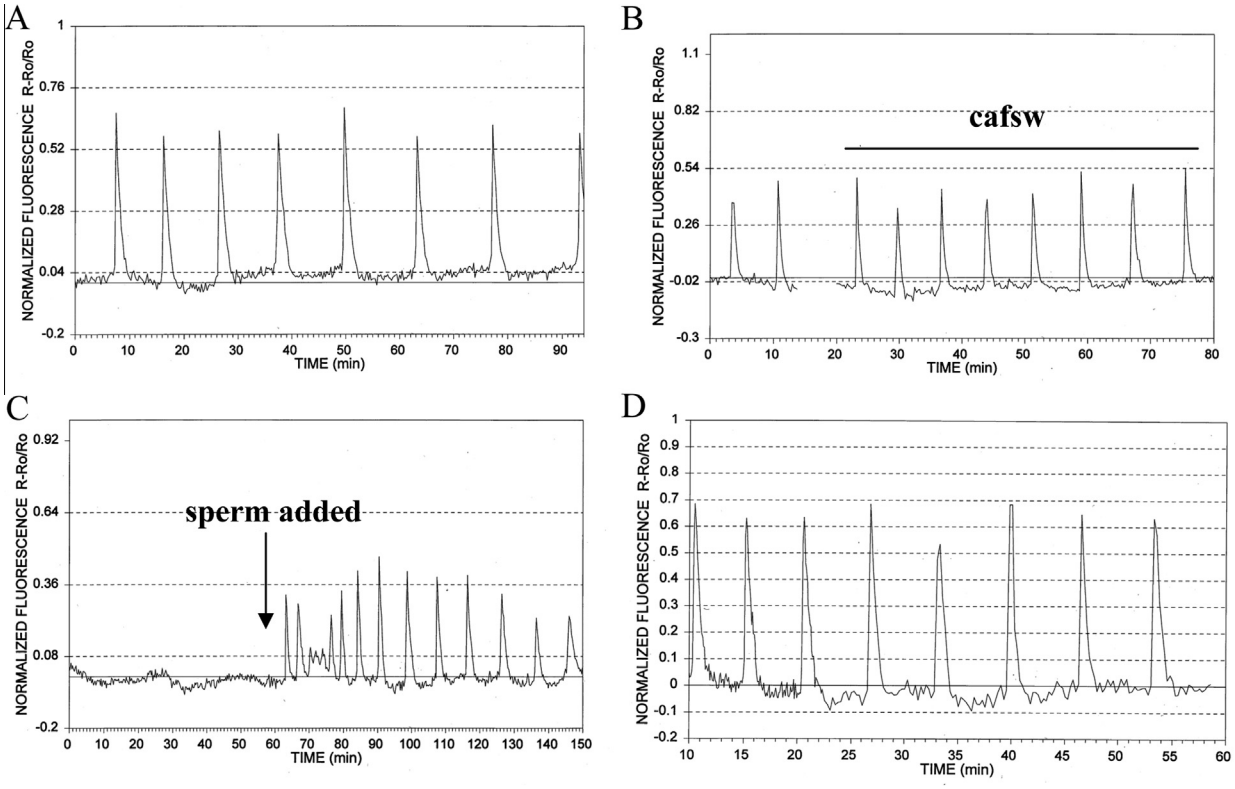

Fig. 3. Calcium ion concentrations expressed as normalized changes in the ratio (R) of Calcium Green (CG) Dextran/Rhodamine B (RB) Dextran fluorescence intensities relative to the initial CG/RB ratio (Ro) at the onset of a confocal run. (A) Fertilization-like calcium oscillations induced by injection of soluble sperm extract from the nemertean *Cerebratulus lacteus* into an unfertilized, mature egg of *C. lacteus*. (B) Persistence of sperm-extract-induced calcium oscillations in an unfertilized egg of *C. lacteus* after transfer to calcium-free seawater (cafsw). (C) Lack of calcium oscillations for 1 h after control injection of body wall extract into a mature *C. lacteus* egg and the subsequent triggering of oscillations after addition of sperm to specimen dish, indicating that the initial body wall extract injection did not simply cause morbidity. (D) Fertilization-like calcium oscillations in a mature *C. lacteus* egg injected with a 10–100 kD fraction of *C. lacteus* sperm extract.

(e.g. buffer alone, extracts from somatic tissues, protease-treated sperm extracts, or low-MW fractions of sperm extracts) as well as external applications of sperm extracts fail to trigger calcium oscillations or polar body formation [27], even though control eggs routinely produce repetitive calcium waves when subsequently inseminated (Fig. 3C). Collectively, such findings indicate that the calcium response of nemertean eggs is not dependent upon an external oolemmal receptor but rather on a soluble component of sperm, and although the precise nature of the putative sperm factor of nemerteans has yet to be elucidated, oscillogenic activity is heat labile [27] and routinely retained in 10–100 kD fractions of sperm extracts (Fig. 3D).

2.6. Fertilization-induced calcium dynamics in nemerteans and other protostomes do not support Jaffe's hypothesis and instead resemble those reported for some deuterostomes

Previous analyses of the prophase-I-arrested oocytes of such lophotrochozoan protostomes as the echiuran annelid *Urechis* [52] and the bivalve mollusc *Mactra* [53] have documented a single centripetally propagating cortical flash of elevated calcium occurring during fertilization. Similarly, in metaphase-I-arrested eggs of the polychaete annelid *Pseudopotamilla* [54] and several species of limpet molluscs [55,56], fertilization triggers a cortical-flash-like calcium elevation. Collectively, such examples of lophotrochozoans support the hypothesis proposed by Jaffe [7,8] that protostome eggs utilize calcium influx to generate their calcium signals during fertilization.

However, contrary to a global influx mechanism, observations of non-lophotrochozoan (i.e. ecdysozoan) protostomes suggest that fertilization promotes a point-source calcium wave from the putative site of sperm entry in the shrimp arthropod *Sicyonia* [57] and the nematode *Caenorhabditis* [58,59], and the overall propagation patterns of these waves are consistent with a mobilization of internal calcium pools, as has been shown for other eggs [1]. Moreover, Jaffe's hypothesis is not supported by data either reviewed here for nemerteans or reported elsewhere for several other protostome eggs [3,60–63], since internal calcium release, which was originally proposed as a hallmark of deuterostomes [7,8], has also been demonstrated for such protostomes.

Accordingly, although nemertean calcium oscillations are unlike the solitary calcium wave generated by starfish [12], sea urchin [64], amphibian [13], or fish [14,65] deuterostomes, fertilization-induced calcium responses in nemertean eggs resemble those of ascidian [9,66] and mammalian [10,48] deuterostomes. In fact, except for differences in overall amplitude and duration [48], or minor discrepancies such as the lack of a cortical flash in mammalian eggs [67] and the presence of a distinct break in oscillations between meiosis I and meiosis II in ascidians [9,68], fertilization-induced calcium oscillations in nemertean and bivalve protostomes are otherwise difficult to discriminate from those produced by deuterostomes [24].

2.7. What accounts for the observed variations in calcium signals across the animal kingdom?

Given that fundamentally different patterns of fertilization-induced calcium responses do not neatly partition according to a protostome vs. deuterostome dichotomy, are there other explanations for the variations in calcium signals that are exhibited by fer-

tilized eggs across the animal kingdom? Based on an increased capacity for mobilizing internal calcium stores as maturing oocytes elevate their MPF levels and progress beyond GVBD [37,69–73], it is possible that oocytes fertilized at the prophase-I stage, such as those of *Urechis* [52] and *Mactra* [53], simply rely on external calcium influx in the absence of a pre-fertilization MPF elevation and the concomitant opportunity to mature their calcium release mechanisms sufficiently. Accordingly, in mammalian eggs, which undergo both an extended MPF/MAPK-mediated metaphase II arrest before being fertilized and a several-hour-long exit from meiosis after fertilization, a robust oscillatory response driven by internal calcium release may be needed to deal with such a relatively complex and protracted cell-cycle progression [74]. Conversely, in metaphase-II-arrested eggs of frogs [75] and fish [76] that can fertilize soon after spawning and complete meiosis within only ~30 min post-insemination, calcium release produces a single calcium transient that is presumably sufficient for transitioning through a rapid first cell cycle [74]. Similarly, a solitary calcium wave mediated by internal calcium release has evolved both in post-GVBD starfish oocytes that do not arrest at metaphase prior to fertilization and in sea urchin eggs that complete meiosis before being fertilized [74].

However, such strictly cell-cycle-based proposals regarding influx vs. release and oscillations vs. solitary signals do not seem to be supported by data obtained for some invertebrate eggs. For example, metaphase-I-arrested eggs of limpets [55,56] and *Pseudopotamilla* [54] rely on external calcium influx to generate their calcium response during fertilization, whereas other metaphase-I-arrested eggs, including those of nemerteans, release internal stores of calcium during fertilization [3,9,60,62,63]. Moreover, after correcting for relatively low culture temperatures, fertilized metaphase-I eggs of ascidians, bivalves, the polychaete annelid *Chaetopterus*, and the nemertean *Cerebratulus* sp. actually complete meiosis in comparable, or even shorter, times than those reported for metaphase-II-arrested frog and fish eggs, and yet unlike the single calcium wave generated by those rapidly transitioning vertebrate eggs, such marine invertebrates produce distinct calcium oscillations [9,29,60,61]. Thus, the speed with which meiosis is exited following fertilization [74] is not necessarily a good indicator of whether a solitary or oscillatory calcium response will be generated.

As an alternative explanation, it has been postulated that the ER clusters generated during oocyte maturation can optimally aggregate IP_3 receptors and thereby facilitate the production of calcium oscillations during fertilization of metaphase-arrested eggs [33,41]. Such a hypothesis was originally based on data linking ER clusters in mammalian, ascidian, and nemertean eggs to an enhanced capacity for generating calcium oscillations, as well as the finding that ER clusters normally disassemble following nemertean fertilizations at the time when oscillations cease [29]. More recently, metaphase-I-arrested limpet oocytes that produce only a single calcium transient via external calcium influx have been shown to lack both a network of ER clusters and a well developed capacity for IP_3-mediated calcium release [56]. Conversely, metaphase-I-arrested bivalve eggs with ER clusters respond to IP_3 and generate calcium oscillations upon fertilization before disassembling their clusters as oscillations stop [56].

However, mature frog eggs have ER microdomains that are somewhat similar to those of mammals, ascidians, and nemerteans, and nevertheless such substructures disassemble relatively quickly after fertilization and allow only a single calcium wave to be generated [40]. Moreover, fertilized mouse eggs continue to generate calcium oscillations for hours after their ER clusters have disassembled [42], indicating that the mere presence or absence of ER clusters may not explain oscillation production. Thus, along with an overtly visible restructuring of the ER, other changes in the sensitivity of IP_3 receptors, influx capabilities, and/or interactions with additional cellular organelles may be required for proper calcium responses [77–80].

Similarly, calcium signals during fertilization can also be shaped by sperm-dependent parameters, such as whether or not a sperm factor is involved, and in the case of sperm-factor-mediated fertilization, the precise type of sperm factor may markedly affect calcium dynamics [3]. In any case, although proposals based on simple dichotomies related to phylogenetic affinities, egg attributes, or sperm-derived stimuli may have some explanatory value, it seems likely that a more multifaceted suite of parameters needs to be considered when attempting to elucidate the various mechanisms that regulate fertilization-induced calcium signals in animals.

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