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Mechanical stimulation by osmotic and hydrostatic pressure activates *Drosophila* oocytes *in vitro* in a calcium-dependent manner

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Abstract

Embryogenesis in vertebrates and marine invertebrates begins when a mature oocyte is fertilized, resulting in a rise in intracellular calcium (Ca^{2+}) that activates development. Insect eggs activate without fertilization via an unknown signal imparted to the egg during ovulation or egg laying. One hypothesis for the activating signal is that deformation of eggs as they pass through a tight orifice provides a mechanical stimulus to trigger activation. Ovulation could produce two forms of mechanical stimulus: external pressure resulting from the passage of oocytes from the ovary into the narrow oviducts, and osmotic pressure caused by hydration-induced swelling of the oocyte within the oviducts. Ovulation could also trigger activation by placing the oocyte in a new environment that contains an activating substance, such as a particular ion. Here, we provide the first evidence that *Drosophila* oocytes require Ca^{2+} for activation, and that activation can be triggered *in vitro* by mechanical stimuli, specifically osmotic and hydrostatic pressure. Our results suggest that activation in *Drosophila* is triggered by a mechanosensitive process that allows external Ca^{2+} to enter the oocyte and drive the events of activation. This will allow exploitation of *Drosophila* genetics to dissect molecular pathways involving Ca^{2+} and the activation of development.

Keywords: Egg activation; Calcium; Mechanosensation; Pressure; French press; Gadolinium

Introduction

Mature oocytes require an external signal to begin development. This signal, which differs among animals, 'activates' the oocyte to resume and complete meiosis, modify its outer coverings, reorganize its cytoskeleton, and translate or degrade certain maternal mRNAs. In most animals, activation is triggered by fertilization, but changes in the ionic environment, changes in pH, or mechanical deformation can initiate egg activation in some species (Harada et al., 2003; Horner and Wolfner, 2008; Lindsay et al., 1992; Runft et al., 2002; Went and Krause, 1974). A frequent response to the activating trigger in vertebrates and marine invertebrates is a rise in free calcium within the egg (reviewed in Lee et al., 2006; Parrington et al., 2007; Sato et al., 2006; Townley et al., 2006). In these organisms calcium acts as a second messenger to drive the downstream processes of egg activation. In insects, the requirement for calcium during egg activation has never been directly tested; however, recent reports show that a calcium-responsive regulator is essential for egg activation in *Drosophila melanogaster* (Horner et al., 2006; Takeo et al., 2006).

Drosophila egg activation, as in other insects that have been examined, is independent of fertilization. Unfertilized laid eggs can complete meiosis (Doane, 1960), modify their vitelline membranes (Heifetz et al., 2001; LeMosy and Hashimoto, 2000), and translate some maternal RNAs (Macdonald and Struhl, 1986) while degrading others (Tadros et al., 2003). Thus, *Drosophila* sperm trigger none of the traditional metrics of egg activation. Instead, activation initiates during ovulation (Heifetz et al., 2001) but the activating signal itself remains unknown.

One hypothesis for the activating signal in *Drosophila* derives from studies of Hymenoptera in which embryo development is triggered by oviposition. It has been proposed that mechanical stress imparted upon the egg during passage through the ovipositor is the signal that starts development in Hymenoptera. For instance, in the haplodiploid wasp, *Pimpla turionellae*, the diameter of the ovipositor is about one-third of the width of the egg, suggesting that physical deformation during egg laying initiates development. Consistent with this

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hypothesis, when *P. turionellae* eggs are dissected from the ovary and squeezed through a narrow capillary tube, over 70% of eggs activate, as measured by their ability to develop into haploid male larvae (Went and Krause, 1974). Pressure exerted on the egg can also activate eggs of another wasp, *Nasonia vitripennis*, as 23% of eggs dissected from the ovary and pressed with a needle were able to develop to larvae (King and Rafai, 1970).

The hypothesis that mechanical stimulation could also trigger *Drosophila* egg activation was initially suggested by two observations. First, Mahowald et al. (1983) briefly commented that application of hydrostatic pressure of an unspecified level or duration to *Drosophila* oocytes resulted in an increase in nuclear number in those oocytes. Whether such oocytes had properly completed meiosis and were undergoing haploid mitotic divisions was not reported. Second, Endow and Komma (1997) reported that pulling manually on the dorsal chorionic appendages of *Drosophila* oocytes triggered the resumption of meiosis in 3/3 cases. These intriguing observations suggested that mechanical stimulation might trigger the resumption and completion of meiosis, one aspect of egg activation. No other aspects of egg activation in response to mechanical stimulation were examined in those studies.

Analogous to oviposition in wasps, a mechanical trigger might occur as *Drosophila* eggs move from the ovary into the narrow lateral oviduct during ovulation. Mechanical stimulation could rearrange egg contents, leading to new structural or molecular combinations. Alternatively, mechanical stimulation could stimulate a mechanically-gated (MG) process, such as the opening or closing of stretch-activated (SA) ion channels. Such alterations to ion channels could lead to ionic changes analogous to those that trigger egg activation in other metazoans.

Another potential activation trigger in *Drosophila* is hydration. Mature oocytes in the ovary appear desiccated, whereas laid eggs are taut and expanded. Some evidence suggests that the hydrated contents of the oviduct lumen are transferred to eggs during ovulation (Mahowald et al., 1983). Support for a hypothesis that hydration could lead to egg activation is that incubation in a hypotonic buffer *in vitro* causes oocytes to swell and activate (Mahowald et al., 1983; Page and Orr-Weaver, 1997). Such hypo-osmotic swelling *in vivo* or *in vitro* could serve as another form of mechanical stimulation, by altering membrane tension to trigger a MG process (Martinac, 2004; Sachs, 1988). Additionally, specific ion(s) in the hydrating medium could provide the activation signal.

To better understand the activating signal in *Drosophila*, we wished to determine if pressure exerted on the egg effects activation. We found that external hydrostatic pressure accelerates activation, as assessed by vitelline membrane permeability changes and protein translation. In addition, an inhibitor of MG processes was able to inhibit hypo-osmotically induced activation, suggesting for the first time that the mechanism by which hydration leads to activation is through a MG response triggered by osmotic pressure. We also found that external calcium is necessary for both hypo-osmotic and pressure-accelerated activation. Therefore we demonstrate that the phenomenon of calcium-dependent egg activation extends to a new and important class of metazoans: insects. Taken together, these results suggest that mechanical stimulation from hydration and/or physical deformation during ovulation triggers activation in *Drosophila* by causing an influx of calcium into the egg. *Drosophila* is now poised to join organisms traditionally used to study activation, with the advantage of valuable genetic resources to discover the likely conserved pathways that mediate egg activation.

Materials and methods

Drosophila strains

D. melanogaster stocks were raised on yeast–glucose–agar medium at 23±2 °C in a 12-h L:12-h D photoperiod. The P2 strain of Oregon R was our wild-type (Allis et al., 1977).

In vitro egg activation

Eggs were activated *in vitro* by a procedure modified from Page and Orr-Weaver (1997). Virgin females were aged on yeasted food for 3–5 days. Mature stage 14 oocytes were dissected from ovaries in hypertonic Isolation Buffer (IB), which does not activate eggs. Oocytes were then incubated in 1–5 changes of hypotonic Activation Buffer (AB) for 5 min each. Activated oocytes were selected in 50% bleach (see below), or allowed to age for an additional period in Zalokar's Buffer (ZB), a physiological buffer that can support development (Page and Orr-Weaver, 1997). In experiments involving inhibitors we substituted an equimolar amount of PIPES for NaH_2PO_4 and KH_2PO_4 in AB and ZB because certain physiological anions such as phosphate can bind free gadolinium (see below), effectively removing it from solution (Caldwell et al., 1998). In addition, we found that calcium phosphate did not precipitate from AB made with PIPES, adding to the stability of AB. The PIPES-modified buffers performed identically to the original buffers from Page and Orr-Weaver (1997) (data not shown).

Vitelline membrane hardening

Assays were performed exactly as described in Horner et al. (2006). Briefly, activated eggs reorganize and cross-link proteins within their vitelline membranes, becoming impermeable to small molecules such as bleach (Heifetz et al., 2001; LeMosy and Hashimoto, 2000). Unactivated oocytes are permeable to bleach and lyse within 2 min of exposure to 50% bleach. Eggs activated *in vitro* were placed in 50% bleach for 2 min, and the number of resistant and lysed eggs was recorded.

Hydrostatic pressure

50–100 mature oocytes were dissected from females in IB. IB was replaced with AB, and approximately 10 mL of oocytes in AB were transferred to a standard 35 mL 20,000 psi FRENCH® Pressure Cell Body (Thermo-Spectronic, Rochester, NY), which was sealed shut by inserting the closure plug to force air from the cylinder, and closing the flow valve. The Pressure Cell was placed on the FRENCH® Press (Thermo-Spectronic), which is a hydraulic press that uses a motor-driven pump to vary hydrostatic pressure within the Pressure Cell. The pressure level was adjusted using a set of control valves, and pressure was applied for 7 min. Oocytes were removed from the Pressure Cell by pipetting through the re-opened closure plug.

RT-PCR

To obtain 0–2 h embryos, virgin females were aged on yeasted vials for 3–5 days and then mated to males the night before embryo collection. Mated females were allowed to deposit eggs on Petri plates containing grape juice agar for 2-h periods. The first collection was discarded, to eliminate