Cell Calcium 53 (2013) 10–15

Contents lists available at SciVerse ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Calcium and egg activation in *Drosophila*

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ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form 2 November 2012
Accepted 3 November 2012
Available online 5 December 2012

Keywords:
Drosophila
Oocyte
Embryo
Egg activation
Calcium
Mechanosensitive
Calcineurin

ABSTRACT

In many animals, a rise in intracellular calcium levels is the trigger for egg activation, the process by which an arrested mature oocyte transitions to prepare for embryogenesis. In nearly all animals studied to date, this calcium rise, and thus egg activation, is triggered by the fertilizing sperm. However in the insects that have been examined, fertilization is not necessary to activate their oocytes. Rather, these insects' eggs activate as they transit through the female's reproductive tract, regardless of male contribution. Recent studies in *Drosophila* have shown that egg activation nevertheless requires calcium and that the downstream events and molecules of egg activation are also conserved, despite the difference in initial trigger. Genetic studies have uncovered essential roles for the calcium-dependent enzyme calcineurin and its regulator calcipressin, and have hinted at roles for calmodulin, in *Drosophila* egg activation. Physiological and *in vitro* studies have led to a model in which mechanical forces that impact the *Drosophila* oocyte as it moves through the reproductive tract triggers the influx of calcium from the external environment, thereby initiating egg activation. Future research will aim to test this model, as well as to determine the spatiotemporal dynamics of cytoplasmic calcium flux and mode of signal propagation in this unique system.

Through the process of egg activation, a single resting cell – the mature oocyte – suddenly is triggered to undergo several major changes that give it the capacity, once fertilized, to give rise to every type of tissue in the adult. Understanding the intricacies of egg activation is fruitful not only for understanding how cells can transition between differentiated and totipotent states, but also has relevance to the development of assisted reproductive technologies.

In many organisms, mature oocytes remain stalled in a species-specific stage of meiosis. Release from this arrest point occurs during egg activation. This process initiates a number of changes at the molecular and cellular levels in the oocyte that prepare it to undergo embryogenesis after fertilization. The molecular changes include modification of vitelline membrane structure, alterations in RNA and protein pools through translation or degradation of stored maternal RNAs, and post-translational modifications made to existing proteins. These molecular events drive major changes at the cell level, including release from meiotic arrest, pronuclear fusion, and finally progression to embryonic mitoses.

In the vertebrate and invertebrate organisms studied, egg activation requires an increase in intracellular calcium levels within the oocyte (reviewed in [1–4]). In most cases, the calcium increase is triggered through the fertilizing sperm, commonly through activation of an intracellular calcium signaling pathway. For example, in mice the sperm introduces a specific isoform of phospholipase C, which activates the inositol triphosphate pathway in the fertilized egg, ultimately causing release of calcium stores from the endoplasmic reticulum (ER) [5].

However, the specific trigger for egg activation is different in at least some insects, including fruit flies and hymenoptera such as ants and bees (whose unfertilized eggs develop into males) [6,7]. Egg activation in these insects does not require fertilization [8]. Instead, a mature oocyte begins to activate as it travels through the female reproductive tract. Activation is triggered by passage through the ovipositor in wasps [9], or upon the oocyte's release from the ovary into the oviduct in *Drosophila* [10]. Thus, insects afford a different view of egg activation: without the fertilizing sperm, their egg activation events are driven solely by maternal components within the oocyte. Experimental results derived from egg activation studies in *Drosophila* oocytes may therefore be of particular interest to researchers or physicians investigating the determinants of oocyte quality for application in assisted reproduction.

How does travel through the reproductive tract promote the egg activation program in *Drosophila*? The obvious candidate for an intermediary is calcium, but only very recently – six years ago – was evidence first obtained that indicated a requirement of calcium for *Drosophila* egg activation [11,12]. Since then, some important discoveries have been made concerning the source of the calcium, the relationship of calcium to the trigger of the release from the ovary into the oviduct,

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http://dx.doi.org/10.1016/j.ceca.2012.11.008

and the components of the subsequent signaling pathway, but these are still very early days for studies of *Drosophila* egg activation. Our goals in this article are to summarize what has been reported, highlight questions that are not yet answered, and to provide a framework for future investigation of this important area.

1. Genetic analysis indicates a requirement for calcium

Calcium signals in many cell types are commonly transmitted through the calcium binding protein calmodulin (CaM), which may then interact with other proteins to activate downstream events. Studies in vertebrates had previously pointed to roles for CaM-mediated pathways in egg activation. In mouse, for example, activity of the calmodulin-dependent kinase CaMKII increases with the multiple calcium spikes in the oocyte, and each wave of CaMKII activity progressively promotes further landmarks of egg activation [13]. CaMKII and the calcium-dependent phosphatase calcineurin also play a role in activation of *Xenopus* oocytes, where much of the signaling network downstream of the calcium rise has been identified. In resting *Xenopus* oocytes, meiosis is held in an arrested state by inhibition of the anaphase promoting complex, APC/C, whose action is required to initiate anaphase. The APC/C acts as an E3 ligase when bound to its coactivator CDC20 to signal the degradation of cell cycle regulators at the spindle assembly checkpoint, thus promoting progression from metaphase to anaphase (reviewed in [14]). In the resting cell, anaphase progression is blocked by inhibitory phosphorylation of CDC20 and by active Emi2, a spindle checkpoint regulator [15]. Upon fertilization (and thus egg activation), the resulting rise in cytosolic Ca^{2+} activates (1) CaMKII, which places an inhibitory phosphorylation on Emi2; and (2) the calcium-dependent phosphatase calcineurin, which removes CDC20 phosphorylation. These post-fertilization events release inhibition of APC/C and allow progression through anaphase [15–17].

The first indication that calcium may play an important role in *Drosophila* egg activation came from genetic analysis: the gene for the calcineurin regulator *sarah* (Sra, calcipressin) was identified in a screen for mutations affecting early post-fertilization embryonic development. Embryos from *sra* mutant mothers arrest normally at metaphase I, but upon meiotic resumption at egg activation they are unable to proceed past anaphase I [11,12]. Calcipressins typically regulate the activity of the Ca^{2+}/CaM-dependent phosphatase calcineurin (CaN) through direct binding [18–20]. Recent evidence establishes that during egg activation in flies, the calcipressin Sra activates calcineurin. First, the phenotype of embryos derived from germline clones of a null allele of the CnB subunit gene of calcineurin is remarkably similar to that of *sra* mutants: meiosis arrests properly at metaphase I but is unable to continue past anaphase I upon release from meiotic arrest [21]. Second, a recent study confirms that Sra associates with calcineurin *in vivo* and further shows that phosphorylation of Sra at Ser215 is necessary for calcineurin activity [22]. This residue is phosphorylated only in activated eggs and not in mature oocytes, and this phosphorylation event is dependent upon activity of glycogen synthase kinase (GSK-3, *shaggy*) [22]; however, it is not known how GSK-3 activity is turned on specifically at egg activation. Taken together, these results indicate the following scenario: upon egg activation, Sra becomes phosphorylated at a specific residue, which allows Sra to activate calcineurin.

Interestingly, Sra is bound to calcineurin both in ovaries, where calciuneurin is not active, and in early embryos, where calcineurin is active [22]. In other organisms, calcipressin has been shown to bind groups of different targets for activating or repressive regulation depending on its phosphorylation status, and this association can be either repressive or activating [12,22]. The same may be true in *Drosophila*, where the association of Sra and calcineurin in the ovary may be inhibitory until Sra's phosphorylation at egg activation.

Roles for CaM and CaMKII in *Drosophila* egg activation have not yet been directly determined through genetics. We suspect that both proteins are important for signal transduction upon Ca^{2+} influx during egg activation. For example, the results just described indirectly point to at least one role for CaM through the calmodulin-dependent phosphatase calcineurin. In fact, CaM was identified in calcineurin-containing immunoprecipitates of Sra [22], further suggesting that CaM is required to convey the calcium signal to effector proteins. We discuss here the attempts to define genetically and directly the functions of CaM and CaMKII in *Drosophila* egg activation.

One challenge for such genetic analysis is that global knockout of either CaM or CaMKII is lethal to the animal, so, tissue-specific knockdowns must be tested. CaM-deficient germline clones were generated to study CaM's role in oogenesis and embryogenesis, but surprisingly, the germlines of these flies were not depleted of CaM protein. Though the germline cells were indeed *CaM*-null, it appeared that CaM protein was able to enter the germline cells from the surrounding somatic cells [23]. Thus, it was not possible to determine definitively whether CaM plays a role at egg activation. However, Andruss et al. observed that some mature oocytes absorbed less somatic CaM than others (anywhere between 28% and 76% that of heterozygous controls), and those that received relatively little somatic CaM show severe defects upon embryogenesis, though oogenesis occurs relatively normally [23]. These latter results support the idea that CaM plays a role in the egg-to-embryo transition, although further experiments are needed to fully prove this hypothesis.

It has not yet been possible to test for a role for CaMKII in *Drosophila* egg activation because the location of the CaMKII gene, on *Drosophila*'s fourth chromosome, has precluded the generation of germline clones. However, a method to generate mitotic clones for fourth chromosome genes in the soma has just been reported [24]. Future tweaks to this system may make it possible to induce loss of CaMKII expression in the germline while maintaining normal expression in the soma.

Because generating germline clones deficient for CaM or CaMKII has not yet been possible, attempts have been made to knock down the amount or activity of these proteins using other means. Interfering RNA (Harvard Medical School, TRiP) or inhibitory peptides targeted against CaM [25] or CaMKII [26] can be expressed in the germline. Preliminary experiments of this type have not produced an effect on egg activation or fertility (A. Krauchunas, K. Kumar, Y. Lai, K. Sackton, C. Sartain, and M. Wolfner, unpublished data), but it is possible that the abundant maternal stores of CaM or CaMKII in oocytes cannot be sufficiently inhibited by these methods. Thus, at present we cannot evaluate whether CaM and CaMKII are essential for egg activation. We expect that new genetic techniques for germline analysis will permit future studies of these proteins' roles.

2. Exogenous calcium is needed for *Drosophila* egg activation

Early observations of *Drosophila* oocytes noted that laid eggs were already activated, even if they had not been fertilized [6,8]. Oocytes in the ovaries were never activated, indicating that activation had to occur sometime between the release of eggs from the ovary and their being laid by the female. Subsequent detailed physiological studies indeed indicated that oocytes become activated as they pass into the oviduct [10]. Fig. 1 demonstrates that transport through the oviduct promotes activation: among oocytes that had