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Intersecting Roles of Protein Tyrosine Kinase and Calcium Signaling During Fertilization

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Abstract

The oocyte is a highly specialized cell that must respond to fertilization with a preprogrammed series of signal transduction events that establish a block to polyspermy, trigger resumption of the cell cycle and execution of a developmental program. The fertilization-induced calcium transient is a key signal that initiates the process of oocyte activation and studies over the last several years have examined the signaling pathways that act upstream and downstream of this calcium transient. Protein tyrosine kinase signaling was found to be an important component of the upstream pathways that stimulated calcium release at fertilization in oocytes from animals that fertilize externally, but a similar pathway has not been found in mammals which fertilize internally. The following review will examine the diversity of signaling in oocytes from marine invertebrates, amphibians, fish and mammals in an attempt to understand the basis for the observed differences. In addition to the pathways upstream of the fertilization-induced calcium transient, recent studies are beginning to unravel the role of protein tyrosine kinase signaling downstream of the calcium transient. The PYK2 kinase was found to respond to fertilization in the zebrafish system and seems to represent a novel component of the response of the oocyte to fertilization. The potential impact of impaired PTK signaling in oocyte quality will also be discussed.

Keywords

fertilization; oocyte; calcium; SRC; FYN; YES; FGR; FAK; PYK2; and protein kinase

Introduction

Calcium signaling, in the form of the inositol triphosphate-induced release from internal stores, is a central aspect of the response of oocytes to fertilization in species as evolutionarily diverse as marine sponges and humans [1–3]. The scope of this signaling event is massive when considered at the cellular level involving the entire oocyte cytoplasm which ranges in diameter from 50 μm in the mouse to over a millimeter in amphibians. Fertilization drives an increase in intracellular free calcium from the resting level in the range of 10^{-7} M to a maximal amplitude in the 10^{-4} M range in marine invertebrates [4] and 10^{-6} M in mammalian oocytes [5]. Oocytes from marine invertebrates and fish respond to fertilization with a rapid ‘cortical flash’ due to opening of L-type channels in the plasma

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membrane allowing entry of calcium, followed by a high amplitude calcium transient mediated by inositol triphosphate (IP₃) operated channels that begins in the immediate vicinity of the fertilizing sperm and spreads through the entire oocyte as a wave propagated across the entire oocyte [4;6]. In zebrafish, compartmentalization of the ooplasm into a central yolk mass and a thin layer of active cortical cytoplasm is reflected in the speed and amplitude of the high amplitude calcium transient. The calcium transient propagates rapidly through the cortical cytoplasm (9µm/sec) and more slowly with lower amplitude through the central yolk mass [7]. This pattern enables rapid induction of the cortical reaction and establishment of a block to polyspermy across these very large oocytes (700µm) and more leisurely activation of metabolism in the central yolk mass. The pattern characteristic of marine invertebrates, amphibians, and fish differs from that in mammals where the calcium transient occurs as a series of oscillations that occur throughout the ooplasm. The detailed study by the Miyazaki laboratory [8] demonstrated that a fast calcium transient was initiated in the mouse oocyte at the site of sperm interaction within 1 to 4 minutes after the flagellum of the attached sperm ceased beating. The transient traversed the oocyte at a rate of 20µm/second and accelerated as it progressed to the antipode. This first calcium transient exhibited a two step shape involving an initial 'shoulder' followed by a second, steeper rise that raised the possibility that different calcium channels or signaling mechanisms were involved at different stages of the transient as in marine invertebrates. The functional significance of this calcium transient is of tremendous importance to the individual since the very beginning of zygote development absolutely requires this signaling step [4]. The cytoplasmic machinery to support this signaling event is assembled during the process of oogenesis and arranged in final form during meiotic maturation [9;9;10]. In this way, the oocyte is highly specialized to respond to interaction with the fertilizing sperm with a rapid and decisive calcium signal that begins embryonic development.

The fertilization-induced calcium transient is interwoven with other signal transduction pathways which are arranged upstream and are thought to initiate, facilitate, and accelerate progression of calcium release in the oocyte. In turn, the calcium transient triggers a wave of downstream pathways critical to almost every aspect of egg activation [11]. Protein tyrosine kinases (PTKs), primarily from the SRC-family, have been recognized as important elements of the upstream pathways leading to the calcium transient in oocytes from species that fertilize externally [2]. However, these SRC-family protein tyrosine kinases (SFKs) play no essential upstream role in mammalian oocytes indicating that significant diversity exists in the pathways leading to the fertilization-induced calcium transient. The downstream pathways that respond to the fertilization-induced calcium transient appear to be essential for zygote development in all species studied to date and recent evidence has demonstrated that one or more PTKs may be an important component of these pathways as well [12;13].

Protein tyrosine kinases include multiple different families encompassing a wide variety of structural and functional variations that confer specific capabilities and signaling functions. PTKs share a tight specificity for tyrosine residues as the phosphate acceptor exhibiting a highly conserved catalytic domain [14]. PTKs are generally expressed in low quantities in cells relative to the more abundant serine-threonine protein kinases. However, within the PTK family, specific kinases are expressed at relatively high levels in certain cell types where they confer unique functional properties to that cell. Several PTKs that can function in calcium signaling cascades are expressed at relatively high levels in oocytes (Figure 1) consistent with the central roles that calcium plays in the function of male and female gametes. The goal of the present review is to describe the array of PTKs that function as part of the upstream triggers for the fertilization-induced calcium transient or act downstream of this event to execute specific functions in the fertilized egg.

PTKs expressed in oocytes

The functional diversity between PTK families is based on their unique domain structure which provides many opportunities to confer specialized characteristics to a cell. In addition to the highly conserved catalytic domain, PTKs characteristically include specialized protein-protein interaction domains such as the SRC Homology 2 (SH2) domain with strong binding specificity for phosphotyrosine (P-Tyr) and a limited set of flanking amino acids, and the SH3 domain which binds proline-rich regions. PH and transmembrane domains provide the capability of certain PTKs to become localized to specific membrane compartments of a cell. ERM, actin binding and microtubule interaction domains enable some PTKs to interact with cytoskeletal elements, and in some cases, nuclear localization signals target PTKs to the nucleus. A distinct subset of these structural features provide the opportunity for a limited group of PTKs to interact with components of calcium signaling pathways in a variety of cell types. Members of the SRC-family of PTKs and of the FAK family are frequently involved both upstream and downstream, of calcium signaling events in somatic cells and will be discussed at length below.

SRC- family PTKs

Description and relevance to calcium

The SRC-family of PTKs consists of nine genes (*Src*, *Yes*, *Fyn*, *Lyn*, *Lck*, *Hck*, *Blk*, *Fgr*, and *Yrk*) which encode 57–60kDa cytoplasmic kinases that are closely related structurally and functionally [15]. These kinases exhibit a common domain structure consisting of an N-terminal unique domain, the SH3 and SH2 protein interactions domains, a catalytic domain, and the C-terminal regulatory domain (Figure 2). The N-terminal unique domain contains myristylation and palmitoylation sites and represents the most structurally divergent domain among SRC-family PTKs. The SH3 domain binds proline rich sequences and often functions in interaction with other proteins while the SH2 domain binds P-Tyr containing sequences and is intimately involved in intra-molecular interaction that regulates catalytic function. The SH3 and SH2 domains are conserved in overall structure among SRC-family PTKs, yet retain features unique to each kinase allowing for preferential interaction with a set of signaling proteins characteristic for each kinase. Both SH3 and SH2 domains function in protein-protein interactions with upstream modulators and downstream effectors that are critical to the function of these kinases. As a result, even though they are often expressed simultaneously in the same cell, SRC-family PTKs are usually regulated differently and perform different functions in that cell. Still, they retain enough overlap in specificity among the SH2 and SH3 domains to enable them to compensate for each other if one gene is knocked out [16;17].

Within the SRC-family, SRC and FYN kinases have been shown to play important roles in calcium signaling in multiple cell types. Early work demonstrated activation of PLC γ by both direct and indirect mechanisms. For example, SRC, activated in response ligand binding to transmembrane receptor protein tyrosine kinases such as the epidermal growth factor receptor (EGFr), or the platelet derived growth factor receptor (PDGFr), acts during co-activation of these receptors and phosphorylates many of the signaling proteins, such as PI 3 kinase, that complex with the activated receptors [18;19]. The result is activation of PLC γ by direct phosphorylation (in the case of PDGFr [20] or indirect activation through activation of PI 3 kinase, etc. [21–23]. In many specialized cells, FYN kinase, acting through its N-terminal U domain, interacts directly with PLC γ and phosphorylates it [24]. However, SRC-family PTKs also impact calcium signaling through mechanisms other than stimulating IP₃ production. For example, FYN was shown to phosphorylate the IP₃ receptor in T-cells with the result that channel activity was increased [25]. In addition, FYN and/or SRC also interact with other calcium channels such as transient receptor potential cation

channels such as TRPC6 [26], or TRPV6 [27] which are phosphorylated and activated by SRC-family tyrosine kinases or by indirect means [28].

Expression & function in oocytes

Analysis of the diversity and extent to which SRC-family PTKs are expressed in oocytes has revealed that oocytes are highly specialized with regard to their PTK expression complement. The SRC-family PTKs expressed in marine invertebrates exhibit insufficient homology to vertebrate forms to enable classification via the classical naming system used in vertebrates, however a small number were found to be detectable at the protein level in oocytes. For example, the sea urchin SRC-family PTKs SpSFK1, 3, and 7 were expressed in oocytes at levels sufficient for detection by western blot. In starfish, the AmSFK1, 2, and 3 kinases were expressed at levels sufficient for detection in western blots of egg plasma membranes [29]. In frog oocytes, only the XYK kinase has proven amenable to study by proteomic methods [30]. Zebrafish oocytes were found to express YES and FYN kinases at levels sufficient to detect by enzymatic or western blot methods although highly purified plasma membrane fractions had to be prepared to overcome interference by the abundant yolk proteins [31–33]. In mice, FYN, YES, and FGR kinases were the only members of the SRC-family that were readily detected by Western blots of mature oocytes [34] or by immunofluorescence in mouse and rat oocytes [35], and most of the functional studies recently have focused on FYN and YES.

While the SRC-family PTKs are cytoplasmic enzymes, they typically are concentrated in the cortex of mature oocytes. High resolution confocal analysis was used to examine the relationship between SRC-family kinases and the cortical actin cytoskeleton in oocytes from sea urchins, zebrafish, and mice. In the sea urchin, the SpSFK1/7 kinase was found to be concentrated in close association with the cortical actin layer. SpSFK1/7 was also distributed within the sub-adjacent cortical cytoplasm which is typically enriched in endoplasmic reticulum [36]. A similar distribution of active SRC-family kinases was detected using a phosphorylation-site specific antibody in the zebrafish oocyte [37]. Interestingly SpSFK1/7 and activated SFKs were found to be highly concentrated in the fertilization cone of sea urchin and zebrafish oocytes, respectively [36;38]. In mammalian oocytes, FYN and YES were both localized to the cortex of the mature oocyte as demonstrated in the rat, and mouse systems [12;31;38;39]. FYN-GFP constructs have recently been used to localize Fyn kinase in live oocytes and confirmed that FYN is concentrated at the cortex and also near the meiotic spindle [40]. This localization pattern could be duplicated by injection of RNA encoding GFP constructs of mutant FYN modified to remain in the ‘open’ or active configuration [35].

A significant role of PTK activity in events leading up to the fertilization-induced calcium transient has been demonstrated in oocytes from a variety of externally fertilizing organisms including sea urchin, starfish and ascidians, as well as amphibian and fish species. While it had been known that fertilization induced a burst of PTK activity in sea urchin oocytes [41;42] the finding that catalytically active PTK constructs could activate starfish oocytes [43] stimulated an effort to establish whether these kinases could act as a trigger for the fertilization-induced calcium transient. Initially, PTK inhibitor studies suggested that these kinases had little effect on the early responses to fertilization such as the cortical reaction, but rather functioned primarily during pronuclear congression and mitosis [44]. However, following demonstration that PLC γ was phosphorylated on tyrosine following fertilization [45] a more detailed analysis of the fertilization-induced calcium transient revealed that PTK inhibitors partially suppressed the amplitude and timing of calcium release in the fertilized egg [46–50]. The inhibitor studies suggested that Src-family PTKs might play the most significant role in triggering the calcium transient and more detailed work using dominant-negative constructs encoded by SRC-family PTKs [47;51–53] demonstrated that dominant-

negative constructs containing the SH2 domain of SpFRK, SpSFK1, and SpSFK3 completely suppressed the fertilization-induced calcium transient in most eggs and delayed it in the few that did manage to elicit a transient [36;54]. In an effort to determine the mechanism by which these kinases promoted calcium release, co-immunoprecipitation or pull down studies performed in the sea urchin system demonstrated that SpSFK1 formed a strong interaction with PLC γ 1 [36]. An additional interaction between the tandem SH2 domains of PLC γ and SRC-family kinase activities was also detected in the fertilized starfish oocyte [52]. Interestingly, the results described above for sea urchins and starfish (deuterostomes) have not been duplicated in protostomes such as the nemertine worm *Cerebratulus* [55;56] which respond to fertilization with a series of calcium oscillations more similar in character to that in mammalian and tunicate oocytes than to oocytes from sea urchins and starfish.

Working in the *xenopus* oocyte system, Sato and co-workers used PTK inhibitors initially to demonstrate a role for SRC-family PTKs in egg activation and later identified the XYK kinase and demonstrated its activation during fertilization [57–59]. As seen with starfish and sea urchins, SFK inhibitors could completely block the calcium transient in most oocytes. Furthermore, the XYK kinase was shown to associate with PLC γ and to be required for the fertilization-dependent increase in PLC γ phosphorylation and enzyme activity [57]. This group demonstrated that XYK kinase and other components present in lipid rafts from the *xenopus* oocyte plasma membrane could activate PLC γ and promote calcium release when injected into intact eggs which supported the results obtained from experiments using marine invertebrates [58]. More recent development of a cell-free extract model has significantly advanced the utility of the *xenopus* oocyte model for analysis of the role of intact protein signaling complexes associated within lipid rafts in PLC γ regulation [60].

The role of SRC-family PTKs upstream of the calcium transient in the zebrafish oocyte was found to be more complicated as a result of the fact that the cortical cytoplasm transmits a calcium wave more rapidly and at higher amplitude than the yolk rich central cytoplasm. In addition, once spawned into a hypotonic medium such as pond water, the zebrafish oocyte will undergo spontaneous activation which induces a calcium transient and polyspermy block that is similar to that triggered at fertilization. While this property may serve to maintain the competitive advantage of the male fish involved in the mating process since the oocyte only remains fertilizable for a short time (about 45seconds), it complicates resolution of signaling events that might result from sperm-oocyte interaction from those stimulated only by exposure to water. High resolution analysis of the calcium transient by confocal fluorescence with its narrow field of focus largely eliminated the complications resulting from the spherical geometry of the oocyte cortex and confirmed the earlier observation [7] that the calcium wave was propagated through the cortical cytoplasm significantly faster than through the central cytoplasm. Examination of oocytes injected with the dominant-negative FYN GST-SH2 fusion protein, demonstrated that propagation of the calcium transient through the cortical cytoplasm was suppressed by this fusion protein while that in the yolk-rich central cytoplasm was not [38]. This result supported the interpretation that FYN kinase might help ensure rapid propagation of the calcium transient in the oocyte cortex without degradation of the signal. However the observation that the cortical cytoplasm of the zebrafish oocyte was enriched in IP3 receptors as well as FYN kinase while the central cytoplasm had barely detectable levels of these enzymes made it impossible to differentiate the effect of SRC-family PTKs from that of the IP3r density. Together, the studies in frog and fish provided the first evidence that SRC-family PTK activity might also be important to fertilization among more advanced vertebrate species. The collective results of the studies performed in the invertebrates and lower vertebrates seemed consistent with a model in which fertilization triggered rapid activation of one or more SRC-family PTKs in the oocyte which in turn activated PLC γ and initiated the fertilization-induced calcium transient. The

fact that oocytes from marine invertebrates (except nemertine worms), amphibians, and fish share the properties of a high amplitude calcium transient that occurs rapidly (15–45 seconds) following sperm interaction and requires SRC-family PTK activity for optimal transmission led us to propose that involvement of these PTKs may represent an adaptation to ensure successful propagation of the calcium transient through these oocytes which are typically much larger than mammalian oocytes [61].

While the above model seemed well supported in studies performed on marine invertebrates and amphibians, subsequent attempts to duplicate the results in mammalian oocytes were not successful in mice. Pharmacological inhibition of SRC-family PTKs in mouse oocytes failed to suppress calcium oscillations induced by sperm extracts and injection of recombinant activated SRC constructs failed to induce any calcium response in oocytes [62]. Similarly, dominant-negative fusion proteins containing the SH2 domain of FYN and YES failed to suppress calcium transients in fertilized mouse oocytes even though they were effective in starfish oocytes [34] and were administered at concentrations sufficient to block pronuclear congression [12].

The results of these studies by different laboratories seem to convincingly rule out the role of SRC-family PTK/PLC γ activation as having a significant role in the fertilization-induced calcium transient in mammalian oocytes. However, examination of the calcium oscillation pattern of a substantial number of FYN-null oocytes demonstrated that lack of FYN kinase expression was correlated with calcium oscillations that occurred with a shorter interval and lower amplitude than control oocytes. Interestingly, in FYN-null oocytes, the total integrated calcium input was not different from controls because FYN-null oocytes had more oscillations during the measurement period [13]. In any case, the fertilization rate of FYN-null oocytes was not different from controls although developmental potential was affected. The fact that FYN-null oocytes also exhibited reduced organization and polarity of components of the oocyte cortex raises the possibility that FYN may indirectly affect the organization of the cortical endoplasmic reticulum that plays an important role in calcium oscillations at fertilization [63–66].

During this period, another series of studies described a novel, sperm-specific phospholipase in mammalian systems, PLC zeta (PLC ζ) that provided an alternative mechanism by which the fertilization-induced calcium transient could be triggered [67; 68; 69]. The model which developed to explain the dominant role of PLC ζ in mammalian fertilization simply required sperm-oocyte fusion as a means to deliver PLC ζ to the ooplasm. Since PLC ζ remains fully active at very low calcium concentrations typical of an unfertilized oocyte [70] it would immediately begin the process of PtdIns 4,5,P2 hydrolysis and IP3 production and would not require activation by protein kinases as is the case for PLC γ . This feature is significant in that it could enable oocytes to do without a sophisticated PTK activation pathway for initiation of the fertilization-dependent calcium transient. Despite some conflicting reports [71–73] an increasing body of evidence supports the model in which PLC ζ rather than a SRC-family PTK provides the trigger for the fertilization-induced calcium transient in mammals, a model clearly different than that proposed for invertebrate and amphibian oocytes.

The contrasting models described above to explain the upstream regulation of the fertilization-induced calcium transient in marine invertebrates, amphibians, fish, and mammals undoubtedly reflect the outcome of continued gamete evolution in response to changing conditions. While mammals make use of a calcium signal to trigger a membrane block to polyspermy as well as cortical vesicle exocytosis and a more permanent block polyspermy via modification of the egg plasma membrane and zona pellucida, the speed with which this happens is much slower than in species that fertilize externally [74–76]. The

oocytes of invertebrates and vertebrates that fertilize externally typically face a high concentration of sperm that present a severe risk of polyspermy. In addition, sperm from multiple males have access to the oocyte which may reduce the advantage of male fitness. These oocytes respond to fertilization with rapid, high amplitude calcium transient that achieves maximal levels of $[Ca^{2+}]_i$ between 0.5 – 2.5 minutes post fertilization. The available data suggests that externally fertilizing species take advantage of the fast SFK activated PLC γ signaling pathway that is used by cell types such as platelets [77], T-cells [78], and many growth factor receptor-mediated processes [79] that require a rapid calcium response. Since most oocytes from externally fertilizing species are large in size, they face the additional challenge of rapidly transmitting a calcium transient across a large amount of cytoplasm without attenuation of the signal. The combined SRC-family kinase-PLC γ activation pathway may facilitate rapid propagation of IP₃ production through the egg cortex and enable the calcium transient to reach the vegetal pole of the oocyte before polyspermy occurs. Mammalian oocytes are fertilized internally and have a reduced requirement for a permanent block to polyspermy within the first minute or two after penetration of the first sperm. It would seem likely, therefore, that mammalian oocytes have less need for the rapid, SFK-mediated calcium release pathway described above and can rely instead on the sperm-derived PLC ζ which triggers calcium release [72] following delivery of the phospholipase by the fertilizing sperm [80;81]. The apparent lack of a highly excitable calcium signaling pathway in the mammalian oocyte cortex may have advantages in that parthenogenetic activation would be less likely during ovulation and transport of the oocyte to the oviductal ampulla.

The differing requirement for SFK signaling in initiation of the fertilization-induced calcium transient between mammals and species that fertilize externally also has implications regarding the significance of cell-cell contact-mediated signaling between sperm and egg. The theory that sperm-egg contact would lead to SFK activation either through a specific sperm receptor on the oocyte [29], or through rearrangement of plasma membrane lipid domains [58] which would act as the trigger for the calcium transient is supported by the fact that SFK inhibition could completely block a calcium response to fertilization in most starfish, sea urchin, *xenopus* and (probably) zebrafish oocytes. While one might consider that small, localized instances of calcium release might precede SFK activation yet be below the limit of detection, the simplest interpretation of available data is that SFK activation precedes and is required for the calcium transient in species that fertilize externally. However, as seen below, the finding that PYK2, a calcium-sensitive PTK is activated during fertilization opens up additional possibilities.

FAK-family PTKs and calcium signaling in oocytes

The FAK-family includes two protein kinases FAK and PYK2 which are cytoplasmic protein tyrosine kinases that transduce signals from cell surface receptors to cytoplasmic pathways through their unique capability as scaffolding proteins as well as through their kinase activity. FAK functions in response to integrin clustering in response to external stimuli and plays a key role in transducing these stimuli into cytoplasmic responses. It also controls assembly/disassembly of adherens junctions at regions of cell-cell or cell-substrate contact and may also regulate plasma membrane ion channels such as the voltage-sensitive calcium channel [82;83]. PYK2 also responds to integrin signaling, as well as environmental stress and growth factor stimuli. Its function has less to do with cell junctions and instead integrates a wide variety of extracellular and intracellular signals to regulate Rho activity [84], implement actin cytoskeleton dynamics relating to junction turnover [85], cell process formation [86] and phagocytosis [87;88]. PYK2 is also active in cell motility, regulation of ion channels and mitogenic and hypertrophic responses [89–91]. These large (119 – 125KDa) protein kinases share a domain structure shown in Figure 2. An N-Terminal

FERM domain homologous to band Four point one, Ezrin, Radixin, and Moesin family proteins binds integrins via an adapter protein and participates in an intramolecular interaction with the catalytic domain that is autoinhibitory to catalytic function [92]. Two proline-rich domains bind SRC, FYN, YES and other SH3 containing proteins such as PI 1-3kinase [93–95]. The C-Terminal: Focal Adhesion Targeting (FAT domain) interacts with cytoskeletal elements such as paxillin & talin and with Rho-specific GDP/GTP exchange proteins leading to enhanced Rho signaling [96]. The combined PR1, PR2, and FAT domain FAK Related Non-Kinase (FRNK domain) or PRNK in the case of PYK2 acts as a negative regulator of FAK or PYK2 [97]. Both FAK and PYK2 contain several tyrosine phosphorylation sites that are involved in interactions with SRC-family PTKs and other signaling proteins. PYK2 differs from FAK in that it contains a calmodulin binding site within the ERM domain and requires calcium for activation of kinase activity [98]. During PYK2 activation, the initial autophosphorylation of Tyr⁴⁰² in the catalytic domain occurs in response to cell surface stimuli, but dimerization and transphosphorylation of Tyr⁵⁷⁹ & 580 to achieve full kinase activity is calcium-dependent [99]. The concentration of $[Ca^{2+}]_i$ required in vivo is not known, but in vitro experiments indicate that initial dimerization required micromolar (8–12 μ M) calcium, while maintenance of the dimerized state requires mid-nanomolar concentrations. The source of calcium for PYK2 activation is usually considered to be plasma membrane cation channels. For example, PLC γ /IP3-mediated calcium responses to EGF triggers PYK2 activation in fibroblasts, while the L-type voltage gated calcium channel activates PYK2 in gonadotropes [100] and the TRPM2 channel activates PYK2 in monocytes [101]. As seen in Table 1, both FAK and to some extent PYK2 are expressed in oocytes at levels higher than in most other cells. FAK expression has been reported in oocytes of the sea urchin [102] as well as zebrafish [31], pig [103;104], rat, and mouse [61;105] where it was localized to the oocyte cortex and the perinuclear region of the immature oocyte. Whether FAK responds to fertilization and plays a role in zygote development is an open question at present. Since the activation state of PYK2 can be detected by phosphorylation of key tyrosine residues that play a role in the activation process, phosphorylation-site-specific antibodies were used to monitor kinase activation in the oocyte by immunofluorescence. This technique was able to demonstrate activation of PYK2 during meiotic maturation of the rat oocyte [105] and raised the question of whether PYK2 also played a role in the response to fertilization. We have recently studied the activation and function of PYK2 during fertilization of the zebrafish oocyte [106]. By examining the appearance of phosphorylated PYK2 in the oocyte, we were able to determine that PYK2 is activated in the oocyte cortex within the first few minutes of fertilization (Figure 3). The effect of fertilization on PYK2 phosphorylation could be duplicated by injecting unfertilized oocytes with IP3 (Figure 4) suggesting that PYK2 can respond to calcium transients in the oocyte. Taken together, these results demonstrate that PYK2 is one of the signaling enzymes activated downstream of the fertilization-induced calcium transient in zebrafish oocytes and opens the possibility that it plays a similar role in mammals.

Given the fact that FAK-family PTKs can modulate the activity of ion channels in other systems, the possibility remains that FAK and/or PYK2 respond to sperm-oocyte contact and play some role upstream of the fertilization-induced calcium transient. Both FAK and PYK2 interact with SFKs in a reciprocal co-activation process where complex formation between the two kinases stabilizes the active ‘open’ conformation in the other. This might provide a mechanism by which SFKs could be initially activated in response to sperm-oocyte contact. For example, in species that fertilize externally, it is possible that PYK2 might be activated directly by sperm-oocyte contact via specific sperm receptors or as a result of cadherin interactions and then trigger activation of SFKs in the oocyte cortex which would then initiate and amplify calcium release through a PLC γ -mediated mechanism. Another possibility is that sperm-oocyte contact or fusion could lead to highly localized calcium influx via plasma membrane channels or via PLC ζ activity which would be

sufficient to activate PYK2 locally. Then a reciprocal PYK2/SFK activation wave might propagate across the oocyte and stimulate PLC γ activity leading to the high amplitude calcium transient. Future analysis will be required to test these possibilities and to determine whether similar events occur in mammalian oocytes.

Summary

The fertilization-induced calcium response in oocytes is a universal aspect of oocyte activation but the signaling pathways acting upstream to initiate, accelerate, or amplify calcium transients has diversified during evolution of the mechanism through which fertilization occurs. Animals that fertilize externally face the need for a very rapid permanent block to polyspermy that is usually met by modification of the oocyte plasma membrane and extracellular matrix to inactivate cell surface proteins involved in sperm binding and to establish a mechanical barrier to additional sperm. The need to accomplish this polyspermy block is frequently complicated by the requirement in these oocytes to maintain a large reserve of yolk which results in very large oocytes through which a calcium transient must travel in order to trigger a cortical reaction. It is probably no accident that many of the animals that fertilize externally make use of a pathway in which SRC-family PTKs activate PLC γ to trigger or possibly amplify and accelerate calcium release in response to fertilization. In contrast, mammalian oocytes fertilize internally and are not normally challenged by contact with high concentrations of sperm that could cause polyspermy. Mammalian oocytes are also small in diameter so calcium transients have less geography to cover following fertilization and may face lower risk of signal attenuation. Mammalian oocytes seem to express the same SRC-family PTKs in their cortex that are used to stimulate calcium transients in externally fertilizing species, but these kinases are not required for the fertilization-induced calcium transients or for effective fertilization. In fact, SRC-family PTK activity in mammalian oocytes is not intensely activated in the cortical cytoplasm as it is in species that fertilize externally. These correlations suggest that the use of the SRC-family PTK/PLC γ pathway to stimulate calcium release has been implemented in oocytes from species that fertilize externally in order to ensure a rapid calcium response to fertilization that can establish a permanent block to polyspermy very quickly. Thus, it is likely that mammalian oocytes have not retained this pathway as part of oocyte biology because it was not required to prevent polyspermy. It remains possible, that SRC-family PTKs as well as FAK family PTKs do contribute to the organization of the oocyte cortex and thus may impact calcium signaling indirectly as shown in the starfish system [107]. Also, the realization that FAK and PYK2 kinases are expressed in the oocyte cortex raises the possibility that these PTKs may have an impact on plasma membrane calcium channels and play some role in fertilization. PTK signaling and other pathways important for maintenance of optimal cortical cytoskeleton and endoplasmic reticulum structure remains an important area for future research that could provide new insight into the factors needed for good oocyte quality. In addition, the observation that PYK2 is activated in the fertilized zebrafish oocyte opens up the possibility of a novel aspect of the downstream responses to the fertilization-induced calcium transient that may be essential for optimal zygote development.

The diverse actions that PTK signaling may regulate in oocytes raises the question of what impact suppression or unintentional activation of these pathways may have for oocyte quality. For example, the fact that SRC-family PTK signaling is not required for initiation of a calcium transient in mammalian oocytes does not imply that inappropriate activation or suppression of SRC-family PTKs prior to fertilization by endocrine, cell culture, or environmental factors would not impair oocyte quality. In some tissues, even minor changes in the oscillatory pattern of IP3-induced calcium transients can have extensive consequences to organ physiology and lead to pathological conditions (reviewed in [108]). Perhaps even

subtle alterations in the fertilization-induced calcium transient(s) may have an impact on oocyte quality that results in changes (perhaps subclinical) in embryonic development. The PYK2 and FAK kinases also have the potential to impact calcium physiology in the oocyte as well as pathways downstream of the fertilization-induced calcium transient. Other kinases not directly linked to calcium signaling in oocytes (such as the epidermal growth factor receptor) could, if inappropriately activated during culture, indirectly impact SRC-family or FAK-family kinases and disrupt the ability of an oocyte to initiate and maintain normal zygote development. Future study of the factors and procedures needed to preserve normal calcium signaling in the oocyte will undoubtedly benefit assisted reproductive techniques important for animal and human reproduction.

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Figure 1.

Relative expression of PTKs in mouse tissues

Analysis of relative transcript abundance of selected SRC-family and FAK-family PTKs was retrieved from the mouse BioGPS expression array database (Novartis BioGPS, <http://biogps.gnf.org>; [109]). Data for the *src*, *fyn*, *yes*, *fgr*, *fak*, and *PYK2* genes obtained from arrays representing oocytes, zygotes, ovary, and several additional tissues is presented along the horizontal axis (bottom).

Src-family PTK Structure

FAK-family PTK structure

Figure 2.

Domain Structure of SRC-family and FAK-family PTKs

SRC-family PTKs are characterized by an N-terminal (U) domain responsible for plasma membrane localization, a SRC homology 3 domain (SH3) which binds proline-rich sequences, a SRC homology 2 domain (SH2) which binds P-Tyr containing sequences, the catalytic domain and a C-terminal regulatory domain (Reg) which inactivates kinase activity when phosphorylated. *Fak*-family PTKs contain an N-terminal band four point one homology domain that is important for integrin binding, a single catalytic domain, two proline-rich (PR) domains for interaction with the SH3 domain of signaling proteins, and a focal adhesion targeting (FAT) domain that interacts with components of focal adhesion complexes.

Figure 3.

Phosphorylation and activation of PYK2 in response to fertilization.

Zebrafish oocytes were fixed and prepared for confocal immunofluorescence microscopy [37] prior to fertilization (Unfertilized) and at 2.5 minutes post-insemination. The fixed and permeabilized oocytes were probed with an antibody to the phosphorylated tyrosine 881 (anti-PYK2 PY⁸⁸¹, BioSource, Grand Island, NY) a target of SRC-family PTK activity that participates in full activation of PYK2 [91]. Bound antibody was detected with alexa-488 conjugated anti-rabbit IgG (InVitrogen, Carlsbad, CA) and samples were imaged by confocal microscopy. Magnification is indicated by the bar which represents 100 μm.

Figure 4.

Activation of PYK2 by injection of unfertilized oocytes with IP3.

In order to establish whether PYK2 could be activated by artificially increasing intracellular calcium levels in zebrafish oocytes, unfertilized oocytes were injected with injection buffer only as a control (A) or with injection buffer containing 25μM IP3 (B) to artificially drive an increase in intracellular free calcium levels as described elsewhere [38;110]. The control and IP3-injected oocytes were maintained in Hanks-BSA [32] for 4.5 minutes in the complete absence of sperm, then fixed and labeled with anti-PYK2-PY⁵⁷⁹ (Biosource, Grand Island, NY) which detects phosphorylation of a tyrosine in the catalytic site of activated PYK2. Bound antibody was detected with alexa-488 conjugated anti-rabbit IgG (InVitrogen, Carlsbad, CA) as in Figure 3.

Table 1

Expression of PTKs that function in calcium signaling in oocytes of different species

Kinase	Kinase	Organism	Function	Reference
SRC-family PTKs				
	SpFRK	Sea urchin	Necessary for Ca ²⁺ release at fertilization	Townley et al., 2009
	SpSFK1,3	Sea urchin	Necessary for Ca ²⁺ release at fertilization	Townley et al., 2009
	SpSFK7	Sea urchin	Inhibitory effect on Ca ²⁺ release	Townley et al., 2009
	AmSFK1,3	Starfish	Necessary for Ca ²⁺ release at fertilization	O'Neill et al., 2004
	XYK	Frog	Necessary for Ca ²⁺ release at fertilization	Sato et al., 1996
	Fyn	Zebrafish	Necessary for rapid cortical Ca ²⁺ release at fertilization	Sharma & Kinsey, 2008
		Mouse	Not required for Ca ²⁺ release at fertilization	Kurokawa et al., 2004; Mehlmann & Jaffe, 2005
FAK-family	FAK	Zebrafish, mouse	Not likely as oocyte knockout appears to be fertilized normally.	Kinsey, unpublished
	PYK2	Zebrafish, mouse	unknown	