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CHARACTERIZATION OF THE INVOLVEMENT OF INTEGRINS, FOCAL
ADHESION KINASE, AND PHOSPHOLIPASE C ENZYMES
ENDOGENOUS TO THE OOCYTE IN BOVINE
FERTILIZATION AND OOCYTE ACTIVATION

by

Benjamin Rand Sessions

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Animal Science

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2012

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ABSTRACT

Characterization of the Involvement of Integrins, Focal Adhesion Kinase, and
Phospholipase C Enzymes Endogenous to the Oocyte
in Bovine Fertilization and Oocyte Activation

by

Benjamin Rand Sessions, Doctor of Philosophy

Utah State University, 2012

Major Professor: Dr. Kenneth L. White
Department: Animal, Dairy, and Veterinary Sciences

The objectives of this research were to better characterize the protein signaling complexes that form in response to spermatozoa binding to the bovine oocyte vitelline membrane and to elucidate their potential involvement in oocyte activation.

Integrins located on the vitelline membrane of bovine oocytes have been implicated in mediating the sperm-oocyte interaction. Anti-integrin function blocking antibodies and immunofluorescence were utilized in order to reveal that the αV and $\beta 1$ integrin subunits are essential for fertilization in the bovine and could form the integrin heterodimer involved in the sperm-oocyte interaction.

Focal adhesion kinase is localized to focal adhesions and is a key component of signal transduction pathways mediated by integrins. The presence of focal adhesion kinase in bovine oocytes was verified by real-time polymerase chain reaction and immunoprecipitation and the localization of focal adhesion kinase at the site of sperm

binding to the oocyte plasma membrane was verified using immunohistochemistry. The inhibition of focal adhesion kinase resulted in fewer cleaved embryos in addition to a reduction in the number of oocytes responding with calcium transients.

Phospholipase C isoforms regulate the release of calcium from the endoplasmic reticulum and are known to interact with integrins and focal adhesion kinase. The experiments reported in this dissertation explored the involvement of phospholipase C isoforms endogenous to the oocyte in mediating the calcium release associated with fertilization. Reduction in phospholipase C messenger ribonucleic acid levels for the phospholipase C isoforms $\gamma 1$ and $\gamma 2$ resulted in significantly lower cleavage rates compared to the controls. Interestingly, the reduction in messenger ribonucleic acid levels for phospholipase ζ failed to impact cleavage. Maximizing protein levels for the phospholipase C isoforms ζ and $\gamma 2$ resulted in a significantly higher number of oocytes reaching the 2-cell stage compared to all other treatment groups and not significantly different than the activation control.

Together these data illustrate the involvement of the αV and $\beta 1$ integrin subunits, focal adhesion kinase, and the potential involvement of multiple endogenous phospholipase C isoforms ($\gamma 1$ and $\gamma 2$) in bovine oocyte activation. A more complete understanding of the molecular players involved in fertilization could have beneficial impacts for human fertility, assisted reproduction, and improved efficiency of animal somatic cell nuclear transfer.

PUBLIC ABSTRACT

Characterization Of The Involvement Of Integrins, Focal Adhesion Kinase, and

Phospholipase C Enzymes Endogenous to the Oocyte

in Bovine Fertilization and Oocyte Activation

Benjamin R Sessions

The Center for Integrated Biosystems (CIB) and Animal, Dairy, and Veterinary Sciences (ADVS) Department at Utah State University propose a multi-year molecular study of cattle gamete interactions to improve our basic understanding of fertilization. The CIB and ADVS will utilize existing collaborations in addition to recruiting state and federal funding sources to complete the extensive project.

Various laboratory techniques will be used to discover the molecular players of fertilization in hopes of shedding light on a variety of human infertility issues due to problems at the gamete level. A more complete understanding of fertilization could also lead to the development of improved contraceptives. Another potential outcome is improved efficiency associated with animal cloning. Currently cloning technology involves multiple non-natural techniques that could be contributing to the poor success rate of animal cloning. A better understanding of fertilization at the molecular level could lead to a more natural way of cloning animals. Cloning technology has a great potential of producing transgenic animals for biopharmaceutical production, creating animal models to study human diseases, rescuing endangered species, and producing stem cells. Improving the efficiency of animal cloning will allow for faster advancements and benefits of this technology.

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LIST OF ABBREVIATIONS

$\Delta\Delta Ct$	delta-delta Ct
ADAM	A Disintegrin And Metalloproteinase
AKAP	A-kinase anchor protein
AKT	protein kinase B
BSA	bovine serum albumin
Ca_2^+	calcium
cAMP	cyclic adenosine monophosphate
CAMs	cell adhesion molecules
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
COC	cumulus cell oocyte complex
CRISP1	cysteine-rich secretory protein 1
cRNA	complementary ribonucleic acid
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
FAK	focal adhesion kinase
FAT	focal-adhesion-targeting
FBS	fetal bovine serum
FSH	follicle stimulating hormone
GalT	β 1-4 Galactosyltransferase
GPI	glycosylphosphatidylinositol
HCO_3^-	sodium bicarbonate
HRP	horseradish peroxidase
IAM	inner acrosomal membrane
ICSI	intracytoplasmic sperm injection
ILK	integrin-linked kinase
IVM	<i>in vitro</i> maturation
kDA	kilodalton
LH	luteinizing hormone
MAbs	monoclonal antibodies
MAPK	mitogen activated protein kinase
OAM	outer acrosomal membrane
PAWP	postacrosomal sheath WW domain binding protein
PB1-	phosphate-buffered saline without calcium and magnesium
PB1+	phosphate-buffered saline with calcium and magnesium
PBST	phosphate-buffered saline with 0.05% Tween 20
PC	phosphatidylcholine
PDK1	3-Phosphoinositide-dependent kinase-1
PH	pleckstrin homology
PH-20	sperm adhesion molecule 1
PI-PLC	phosphatidylinositol-specific phospholipase C

PI3	1-phosphatidylinositol-3
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
qPCR	real time polymerase chain reaction
RGD	arginine-glycine-aspartic acid
SACY	soluble adenylyl-cyclase
scNT	somatic cell nuclear transfer
SFK	Src family kinase
SH	Src homology
siRNA	small interfering ribonucleic acid
SM	sphingomyelin
SOAF	sperm borne activating factor
Src	sarcoma
ZP	zona pellucida
ZP1	zona pellucida glycoprotein 1
ZP2	zona pellucida glycoprotein 2
ZP3	zona pellucida glycoprotein 3
ZP4	zona pellucida glycoprotein 4

CHAPTER 1

LITERATURE REVIEW

Mammalian fertilization is a complex multi-step sequential process involving the interaction and fusion of two distinct, highly specialized gametes. Mammalian spermatozoa must undergo a series of physiological and biochemical changes, known as capacitation, in the female genital tract before gaining the ability to fertilize an ovulated metaphase II arrested oocyte. Only after capacitation is a spermatozoa able to penetrate the cumulus oophorus, bind to the zona pellucida of the oocyte, undergo the acrosome reaction, interact with membrane receptors located on the oolemma, and successfully fertilize the oocyte. In response to sperm binding and entry into mammalian oocytes, calcium is released from intracellular stores in a series of spikes or oscillations considered a “hallmark of fertilization.” These oscillations release the oocyte from its metaphase II arrested state and are essential to activate the oocyte and initiate embryonic development.

Despite years of research and much inquiry, the molecular mechanisms of this complex multi-faceted process remain elusive. Perhaps the greatest debate has been centered on understanding the underlying mechanism of the initiation of the cytosolic calcium oscillations. Is it initiated by a sperm ligand binding to an oolemma receptor or by a soluble sperm factor introduced upon sperm fusion? Only after comprehensive reviews of the process by which spermatozoa gain developmental competence in addition to identifying potential molecular players in membrane binding and fusion and the subsequent signaling cascades can a cohesive theory of oocyte activation be proposed.

Spermatozoa Maturation

Sperm are highly differentiated cells with two main components, a head and a flagellum, joined together by a connecting piece. The head contains the nucleus, acrosome, cytoskeletal structures, and a small amount of cytoplasm while the tail consists of a motility apparatus, an axoneme, mitochondria, and cytoskeletal structures. The nucleus consists of highly condensed chromatin, the acrosomal cap is an enclosed cytoplasmic vesicle with hydrolytic enzymes and the flagellum is further divided into the midpiece, principal piece, and end piece. These specialized structures represent the unique functions of the spermatozoa and are critical for delivering the spermatozoa's nuclear material to the cytoplasm of the oocyte. Despite the critical role these structures play in transporting and fertilizing the oocyte, they are not functional until the modification of the spermatozoa plasma membrane during maturation in epididymal transport [1]. Some of these pivotal modifications to the spermatozoa plasma membrane during epididymal transport include modifications of surface proteins, changes in its lipid and protein composition, and an increased total negative charge of the extracellular surface [2].

The lumen of the epididymis contains epididymosomes, small membranous vesicles that are rich in sphingomyelin (SM) and arachidonic acids in addition to containing endoplasmic reticulum, a 70 kDA heat shock protein 5, chaperones, GPI –anchored proteins and other exosomes [3-9]. These epididymosomes aggregate to the sperm membrane during epididymal transport in order to modify and contribute to the formation of various membrane structures such as lipid rafts [2, 10].

Capacitation of Spermatozoa

After maturation in the epididymis and deposition in the female reproductive tract, mammalian spermatozoa are able swim, but are still incapable of fertilizing an oocyte. After remaining in the uterus for an appropriate period of time, the spermatozoa undergoes capacitation, a process resulting in removal of seminal plasma proteins adsorbed to the head of the spermatozoa and modification of sperm plasma membrane proteins/glycoproteins and sterols during passage through the female genital tract [11, 12]. Capacitation of spermatozoa *in vitro* can also be accomplished by exposing the spermatozoa to a defined media containing bovine serum albumin, energy substrates, and electrolytes [13]. The modification of membrane cholesterol levels results in a change in membrane permeability and fluidity, thus allowing entry of ions. These ions activate protein kinase(s), increase protein tyrosine phosphorylation, and result in hyperactive spermatozoa [13-18].

The efflux of cholesterol/sterols from the spermatozoa plasma membrane is believed to be the initial step in capacitation and thought to be mediated by albumin, the major protein in the female reproductive tract. This outward movement of sterols alters membrane potential, increases the membrane permeability and fluidity and restructures the phospholipid content of the asymmetrically distributed lipid leaflets of the plasma membrane [13]. This action allows movement of calcium (Ca^{2+}) and bicarbonate (HCO_3^-) ions across the spermatozoa plasma membrane in order to increase protein tyrosine phosphorylation and activation of secondary messengers including cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). An additional proposed function of the loss of cholesterol is increasing the fusogenicity of the

spermatozoa plasma membrane [13].

The flow of HCO_3^- across the spermatozoa plasma membrane results in the activation of a non-transmembrane soluble adenylyl-cyclase (SACY). Activated SACY increases the production of cAMP and ultimately results in the activation of a cAMP dependent protein kinase A (PKA) through the release of PKA's inhibitory subunits [19-23]. PKA is a ubiquitous serine-threonine kinase, consisting of four subunits, two of which are catalytic and the other two being regulatory. The two regulatory subunits form a dimer, which is disrupted upon cAMP binding and results in the activation of the catalytic subunits [24]. The regulatory subunits also function to anchor PKA to its respective cellular activation location where it can interact with A-kinase anchor proteins (AKAPs) [25, 26]. AKAPs are thought to serve as scaffolding proteins that mediate the activation of several signaling enzymes within a cellular region including PKA, protein phosphatase I, calcineurin, calmodulin and protein kinase C (PKC) [21, 27-31]. However, the intermediate kinase that is activated by PKA and initiates the process of protein tyrosine phosphorylation has yet to be identified. It has been proposed that a Src Family kinase [21, 23, 32, 33], the serine-threonine kinase MAPK [21, 34], or c-Abl, another non-receptor tyrosine kinase [35, 36] might be involved.

The protein tyrosine phosphorylation of the flagellum is believed to allow the spermatozoa to acquire capacitation-associated hyperactive motility [13, 37-39]. The epidermal growth factor receptor (EGFR) has also been shown to be partially activated by PKA resulting in increased phospholipase D (PLD) dependent actin polymerization during acquisition of hyperactive motility [24, 40].

In addition to affecting the increase in intracellular cAMP levels via SACY, Ca^{2+}

is believed to influence capacitation through other Ca^{2+} binding proteins, namely Calcium/Calmodulin (CaM)-dependent kinase IV and Calmodulin, which undergo conformational changes in response to Ca^{2+} binding. CaM kinase IV is located in the flagellum of human spermatozoa, increases in abundance during capacitation, and is proposed to regulate the motility of human spermatozoa during this process [13, 41]. In the head and flagellum regions, spermatozoa also contain high amounts of calmodulin, a 17-kDa Ca^{2+} -binding acidic protein [13, 42-45]. Experimental evidence indicates that calmodulin accelerates the early stages of spermatozoa priming in preparation for capacitation in addition to influencing the protein tyrosine phosphorylation events in response to capacitation by increasing cAMP levels via stimulation of SACY [15, 18, 46].

After capacitation *in vivo*, sperm move into the ampulla region of the oviduct in an attempt to locate the ovulated cumulus cell oocyte complex (COC). Chemotaxis, in response to progesterone release by the cumulus layer, has been shown to be the method in which spermatozoa are able to locate the COC [47-51]. Progesterone is able to influence sperm through a nongenomic receptor that activates phospholipase C, elevates intracellular calcium levels, and regulate cellular behavior [52-56]. The cumulus oophorus is composed of several thousand ovarian granulosa cells that serve to nurture the oocyte and assist in the process of ovulation [57, 58]. The cumulus layer is not required for fertilization; however, an intact cumulus layer results in higher fertilization rates and may play a critical role in reducing polyspermy by trapping compromised sperm [59, 60]. Recently the cumulus layer has been suggested to promote the spermatozoa acrosome reaction and this “premature” activation may serve to limit the

number of sperm capable of binding to the zona pellucida [57, 61-64].

Penetration of Cumulus Oophorus

The major component of the cumulus layer is hyaluronan, an anionic, nonsulfated glycosaminoglycan; consequently, the current proposed theory for sperm penetration through the cumulus layer involves a sperm surface glycosylphosphatidylinositol (GPI) anchored protein, PH-20 [65-68]. PH-20 is a GPI-anchored membrane protein initially found on guinea pig sperm [69]. PH-20 is located in the postacrosomal region of the sperm head as well as within the acrosome. In accordance with having two areas of localization, PH-20 appears to have two distinct functions and two independent roles in the sperm-egg interaction. Despite being originally identified by a monoclonal antibody that blocks zona pellucida binding of acrosome reacted guinea pig sperm [70, 71], PH-20 has been found to also demonstrate hyaluronidase activity [72]. PH-20 is believed to be important in degrading hyaluronic acid during transit through the cumulus layer [73]. Thus the PH-20 molecules located on the postacrosomal surface are used during cumulus penetration, while those found within the acrosome participate in secondary binding to the zona pellucida [74]. However, spermatozoa from PH-20 knockout mice are still able to fertilize oocytes despite a delayed penetration of the cumulus layer indicating the potential involvement of another spermatozoa surface protein [75].

Penetration of Zona Pellucida

After penetrating the cumulus layer the sperm comes in contact with another vestment of the oocyte, the zona pellucida. The composition of the mammalian zona

pellucida matrix consists of three or four glycoproteins depending on the species. The mouse zona pellucida is composed of three glycoproteins designated ZP1, ZP2, and ZP3 while the zona pellucida of pig, cow, and dog have a different set of three glycoproteins: ZP2, ZP3, and ZP4. The zona pellucida of rats, hamsters, and humans are composed of four glycoproteins: ZP1, ZP2, ZP3, and ZP4. All of the heavily glycosylated zona pellucida glycoproteins have N- and O-linked glycans which contribute to the spermatozoa zona pellucida interaction and induction of the acrosome reaction [76]. The zona pellucida is the initial binding site of sperm to the oocyte, the main barrier to interspecies fertilization, and is the barrier to polyspermy.

ZP3 is the primary binding site of capacitated and acrosome-intact sperm while ZP2 provides a secondary attachment site by binding to the inner acrosomal membrane. ZP2 holds the equatorial region of the sperm while ZP3 binding initiates the acrosome reaction. The acrosome reaction enables penetration of the zona pellucida by releasing the acrosomal contents and exposing the inner acrosomal membrane [77, 78].

Numerous proteins located on the sperm surface have been identified as possible candidates in primary and secondary binding to the zona pellucida. Proteins involved in primary binding to the zona pellucida will be lost from the sperm as a consequence of acrosomal exocytosis because they are located on the principal segment of the plasma membrane overlying the sperm's acrosome [74]. Sp56, spermadhesions, and galactosyltransferase (GalT) have all been identified as sperm proteins involved in primary zona pellucida binding with GalT satisfying most of the criteria expected of a ZP3 receptor [79].

Sp56 is a 56-kDa peripheral membrane protein originally identified in mouse

sperm because of its ability to become covalently associated with purified mouse ZP3 [80] or with ^{125}I -labeled ZP3 glycopeptides [81]. Studies utilizing monoclonal antibodies specific for sp56 have localized the protein to the dorsal region of the mouse sperm head. In addition, purified sp56 binds to zona pellucida of mouse eggs, and inhibits sperm-egg binding *in vitro* [74]. Additional sequence and localization studies indicated sp56 is present in the acrosomal contents [82]; therefore, sp56 appeared to not have an appropriate cell surface localization to participate in acrosome intact sperm binding to the zona pellucida. However, it was discovered that some acrosomal components, including sp56, are released from the acrosomal matrix and become associated peripherally with the spermatozoa plasma membrane where it is available to interact with ZP3 [83-86].

Spermadhesins are a family of small (12-16kDa) sperm-associated proteins that bind zona pellucida glycoproteins and are characterized mainly in boar sperm. The majority of spermadhesin molecules are lost from the surface of sperm during capacitation, but many are retained and have been shown to bind to the zona pellucida [74]. However, further studies have shown spermadhesins to be located in the acrosomal contents and/or acrosomal membrane [87], thus not having an appropriate cell surface location to participate in acrosome-intact sperm binding to the zona pellucida [67].

β 1-4 Galactosyltransferase (GalT) was the first sperm protein reported as a primary zona pellucida binding candidate. GalT is found on the dorsal, anterior aspect of the sperm head and behaves as an integral membrane protein. GalT is masked by epididymally secreted glycoconjugates on cauda epididymal sperm. These glycoconjugates are shed from the sperm surface during capacitation, thus exposing

GalT and making it available to bind to its ligand on the zona pellucida. Reagents that block GalT or the GalT-recognition site on the zona pellucida inhibit sperm-egg binding and blocking or removing the GalT-binding site on the zona pellucida destroys sperm binding activity [79]. Gene knockout studies resulting in GalT-null sperm show substantially reduced binding of soluble ZP3 and no ZP3-induced acrosome reaction. These findings implicate GalT as an essential ZP3 binding protein, thus functioning in ZP3 induced signaling [67].

Sperm must remain transiently attached to the zona pellucida before penetration as they undergo the acrosome reaction. Secondary binding involves ZP2 binding to specific receptors that are present on the inner acrosomal membrane. At least two proteins found on the sperm have been proposed as candidates and they are PH-20 and acrosin [74].

Acrosin is the sperm's trypsin-like serine protease and is found within the acrosome of mammalian sperm. Due to its location, acrosin is most likely to participate in secondary binding following the acrosome reaction. The zona pellucida seems to regulate the activation of proacrosin (acrosin's inactive form) to the biologically active form of acrosin [88]. Because of its demonstrated ability to bind to the zona pellucida and proteolytic activities [89-91], acrosin has been believed to be active in both sperm binding and penetration of the zona pellucida.

Spermatozoa Acrosome Reaction

The mammalian acrosome is a sac-like (vesicle) structure covering the anterior portion of the spermatozoa nucleus and consists of an inner acrosomal membrane (IAM) and an outer acrosomal membrane (OAM). The contents of the acrosome consist of

glycohydrolases, proteinases, esterases, sulfatases, phosphatases, and phospholipases C and A2 [13]. The binding of the spermatozoa to ZP3 results in a sustained increase of calcium between the plasma membrane and the OAM of capacitated spermatozoa by activating calcium channels and other second messengers including cAMP and IP3 [13, 52, 92 1996, 93, 94]. The increase in cAMP production results in activation of protein kinases (cAMP-dependent kinase, calcium and phospholipid kinases) while the increase in intracellular calcium concentration initiates a series of events resulting in the fusion of the sperm plasma membrane with the OAM and exocytosis of the acrosomal contents [13].

It is also noteworthy that enzymes located in the acrosome, phospholipase C and A2, are activated by calcium and are believed to have a critical role in acrosomal exocytosis. The influx of calcium activates phospholipase C and phosphatidylcholine (PC)-specific phospholipase C, resulting in an increase in the production of diacylglycerol (DAG) which activates DAG-dependent protein kinase C. The interaction of a spermatozoa receptor with an agonist in combination with the calcium influx results in the activation of phospholipase A2. The hydrolytic product of phospholipase A2 serves as a precursor in the generation of other second messengers leading to membrane fusion [13, 95].

Multiple isotypes of the PLC family have been shown to play a role in acrosomal exocytosis. The phospholipase C- β 1 (PLC- β 1) signaling cascade and a tyrosine kinase receptor coupled to PLC- γ have been shown to interact with a G-protein coupled receptor located on spermatozoa plasma membrane during zona pellucida induced acrosomal exocytosis [76, 96]. The spermatozoa from PLC- δ 4 knockout male mice are

unable to initiate the acrosome reaction resulting in small litters or males being completely sterile [97]. Increased levels of 1-phosphatidylinositol-3 (PI3) kinase are seen in response to zona pellucida binding resulting in accumulation of phosphatidylinositol-(3,4,5)-triphosphate (PIP3), which serves as a binding site for 3-phosphoinositide-dependent protein kinase (PDK1) and mediates the activation of its downstream targets, AKT (protein kinase B) and PLC- ζ . Both AKT and PLC- ζ were shown to be essential for acrosome reaction in the mouse [21, 98]. In addition to releasing the acrosomal contents, the acrosome reaction also results in the exposure of a new set of antigens on the surface of the spermatozoa head.

Spermatozoa-Oocyte Plasma Membrane Interactions

Candidate Sperm Ligands

Primakoff et al. (1987) used a library of monoclonal antibodies (MAbs) directed against the guinea pig sperm plasma membrane and identified a sperm surface protein that is potentially required for sperm-egg fusion. The PH-30 MAb recognized fertilin/PH-30 and was able to inhibit fusion. The PH-30 MAb also immunoprecipitated a heterodimeric protein composed of two subunits: fertilin α (ADAM1) and fertilin β (ADAM2) [99-101]. These two subunits were sequenced and revealed three regions of interest: 1) a metalloprotease domain in the fertilin α subunit, 2) a disintegrin domain in both subunits, and 3) an amphipathic alpha helix region in the fertilin α subunit which is similar of the fusion peptides of some viruses [102, 103].

Both subunits were later identified in other mammalian species: mouse [104], monkey [105], rabbit [106], and cattle [100], although some species (humans) may lack the fertilin α subunit [107]. Cyritestin (ADAM3) was later identified in the mouse and

monkey [104, 108, 109]. Many other members of this new family of proteins were discovered and named the ADAM (A Disintegrin and A Metalloprotease domain) family because of the presence of a disintegrin and a metalloprotease domain [104].

Over 30 members of the ADAM family have been identified in vertebrates while being expressed in a wide range of tissues and cell types. Proteins of the ADAM family have a distinct domain structure: a signal sequence, prodomain, metalloprotease domain, disintegrin-like domain, cysteine-rich domain, an epidermal growth factor (EGF)-like repeat, and a transmembrane segment with a short cytoplasmic tail. The disintegrin-like domains have homology to snake venom ligands for the integrin family of cell adhesion molecules, which suggest these sperm proteins have a role in cell adhesion [110-112]. Many of the snake disintegrin peptides were shown to have a putative binding site consisting of a short loop formed by disulfide bonds at the base and an RGD (Arg-Gly-Asp) sequence at its tip [113] and have been shown to induce parthenogenetic development in the bovine system [114].

In addition to the work performed by Primakoff et al., 1987, several functional studies have provided evidence that ADAM1, ADAM2, and ADAM3 participate in the sperm-oocyte interaction. Antibodies to these proteins bind to sperm and inhibit fertilization in IVF assays [115, 116]. Recombinant forms of ADAM1, ADAM2, and ADAM3 bind to the mouse oocytes plasma membrane, and inhibit sperm-oocyte binding resulting in a reduction of fertilization [117-122]. Peptides corresponding to the ADAM2 disintegrin loop reduce fertilization by inhibiting sperm binding in the mouse [116, 123-127], and guinea pig [128]. The fertility of ADAM1, ADAM2, and ADAM 3 knockout mice is all dramatically reduced due to the inability of sperm to migrate

through the oviduct and decreased sperm binding to the zona pellucida and oocyte plasma membrane [129-133].

CRISP glycoproteins are cysteine-rich epididymal secretory proteins absorbed onto the surface of the spermatozoa and have been proposed to be involved in capacitation and sperm-oocyte binding [134, 135]. Epididymal protein DE (CRISP1) is localized on the posterior region of the spermatozoa head and appears to have 2 different affinities. The CRISP1 population that is weakly bound to the spermatozoa head is removed during capacitation and is hypothesized to play a role in this process by regulating protein tyrosine phosphorylation [136]. The CRISP1 population that is strongly bound is localized on the dorsal region of non-capacitated spermatozoa head and is involved in sperm binding to the zona pellucida prior to capacitation. Once zona pellucida mediated capacitation occurs, CRISP1 migrates to the equatorial segment where it mediates sperm-oocyte fusion through the interaction of its S2 motif with an unknown partner on the oocyte plasma membrane. CRISP1 knockout mice are fertile but suffer from reduced zona pellucida penetration and fusion [137] while mice immunized against CRISP1 have reduced fertility [138]. In addition to CRISP1 it appears that additional CRISP proteins could be involved in mediating fertilization. The intra-acrosomal testes specific CRISP2 protein also has been shown to play a role in sperm-oocyte fusion when incubation of antibodies against CRISP2 resulted in the accumulation of spermatozoa in the perivitelline space of mouse oocytes during *in vitro* fertilization. Zona-free fertilization studies showed that CRISP1 and CRISP2 compete for binding sites on the oocyte plasma membrane, indicating either a redundant or cooperative role in sperm-oocyte fusion [139, 140].

IZUMO is a testis specific member of the immunoglobulin superfamily cell adhesion molecules (CAMs) and is located on the anterior acrosome and equatorial regions of acrosome reacted mouse and human spermatozoa [134, 141]. CAMs are known for their role in the immune response and are able to bind identical or similar members of the immunoglobulin family [142]. Male IZUMO knockout mice are infertile due to the inability of their sperm to fuse with oocytes and it appears that IZUMO plays a similar role in human gamete fusion as well [143]. IZUMO does associate with itself and other non-IZUMO proteins including tetraspanins in the formation of homoprotein and multiprotein membrane complexes and could function to stabilize such complexes on the spermatozoa membrane [141].

The postacrosomal sheath WW domain-binding protein (PAWP) is an alkaline extractable protein localized to the postacrosomal sheath region of the perinuclear theca [144]. Microinjection of recombinant PAWP into bovine, porcine, and xenopus oocytes resulted in meiotic resumption and pronuclear formation while co-injection of a competitive PAWP derived peptide inhibited these same events [144]. Microinjection of recombinant PAWP in xenopus oocytes resulted in calcium release from intracellular stores in a manner similar to sperm-induced intracellular calcium release while both calcium release mechanisms were prevented by co-injection of PAWP derived competitive peptides or antibodies [144, 145].

Candidate Oolemma Receptors

Integrins are a large family of heterodimeric cation-dependent transmembrane proteins composed of non-covalently linked α and β subunits. Each subunit contains a large N-terminal extracellular domain, a transmembrane domain, and a short C-terminal

cytoplasmic domain. There are eighteen α and eight β subunits identified and these subunits combine to form 23 heterodimers. The major function of integrins is to mediate cell-to-cell interactions and cell-to-substratum attachment even though they may mediate a variety of other cellular functions [146]. Integrins associate and interact with cytoskeletal proteins through the cytoplasmic domain of the β subunit and aggregate as focal adhesion sites [147]. These focal adhesion sites also contain signaling complexes involving focal adhesion kinase (FAK), integrin-linked kinase (ILK), molecules of the MAP kinase pathway, small GTPases (ras and rho), lipid kinases (PIP 5-kinase and PI3 kinase), PIP2, PLC- γ , serine/threonine kinases, phosphatases, and activation of focal adhesion sites can result in changes in intracellular pH and calcium concentration [148-151].

Numerous integrin subunits, including α_2 , α_3 , α_4 , α_5 , α_6 , α_V , α_8 , α_9 , α_{11} , α_M , β_1 , β_2 , β_3 , β_5 , and β_7 have been detected in mammalian oocytes at the protein or mRNA level [123, 124, 152-159]. The interaction of human and hamster sperm with zona pellucida-free hamster and human oocytes by RGD peptides implicated the involvement of the RGD-binding subfamily of integrins ($\alpha_5\beta_1$ $\alpha_8\beta_1$ $\alpha_V\beta_1$ $\alpha_V\beta_3$ $\alpha_V\beta_5$ $\alpha_V\beta_6$ $\alpha_V\beta_8$ $\alpha_{11}\beta_3$) [160, 161]. Even though RGD peptides do have an inhibitory effect on the binding of recombinant fertilin β to mouse oocytes, they do not have a substantial inhibitory effect on mouse sperm-oocyte interactions [123, 124]. However, cyclic RGD-containing peptides were found to inhibit sperm-oocyte interaction in the mouse model in addition to stimulating the activation of PKC and cortical granular exocytosis [162]. Also in the mouse model, Baessler et al., 2009 illustrated that the β_1 integrin subunit is intimately involved with the initial adhesion of the spermatozoa to the oocyte plasma membrane in

addition to mediating the subsequent attachment and fusion. The involvement of the $\beta 1$ and $\alpha 9$ integrin subunits in murine fertilization was also shown using RNA interference and function blocking antibodies [163]. Bovine sperm-oocyte interactions have been shown to involve integrins, in particular the αV , $\alpha 5$, $\beta 1$ and $\beta 3$ subunits and may be mediated through cell surface receptors that contain RGD recognition sequences [164]. Biochemical analysis has implicated αV and $\beta 1$ integrins subunits on the pig oocytes in the recognition of pig sperm membrane proteins [157]. RGD containing peptides have also been shown to inhibit or block fertilization in amphibians [165]. There is evidence that integrins may not be sufficient for a full oocyte activation response [162]; however, they cannot be excluded as playing a critical role in mediating sperm-oocyte fusion and binding.

Glycosyl phosphatidylinositol (GPI)-anchored protein is a glycolipid that anchors proteins by their C-terminal tail to the extracellular side of the plasma membrane. The O-side of a GPI associates with DAG in the membrane via DAG's inositol residue. They lack a cytoplasmic tail so their extracellular domain dictates their membrane targeting properties. They participate in signaling cascades but it is unclear how they are able to transduce signals [166]. A glycosyl phosphatidylinositol (GPI)-anchored protein may be required for fertilization, because removal of GPI-anchored proteins treated with phosphatidylinositol-specific phospholipase C (PI-PLC) results in oocytes greatly inhibited in their ability to support sperm adhesion and fusion [167]. GPI knockout female mice also resulted in highly reduced infertility further implicating GPI involvement in sperm-oocyte interactions [168].

Members of the tetraspanin superfamily have been shown to form membrane

complexes with adhesion receptors from the integrin family [169, 170]. Tetraspanins CD9, CD53, CD63, CD81, CD82, and CD151 form integrin-tetraspanin complexes with numerous integrins [171]. The absence of the tetraspanin protein CD9 on the oocytes of knockout mice resulted in sperm bound to the oocyte membrane; however, the sperm had lost almost all of its ability to fuse [172-174]. Zona pellucida-free oocytes treated with anti-CD9 MAbs were reported to have reduced number of bound sperm [175], and also showed reduced levels of binding of the sperm ligands, ADAM3 [119], ADAM1 [120], and ADAM2 [122]. However, CD9 knockout mice did conceive and give birth to pups on rare occasions [122]. Recently it was discovered that oocytes release CD9 exosome-like vesicles that are transferred to the spermatozoa head and assist in spermatozoa-oocyte fusion [51, 176-178]. CD81 was localized in the zona pellucida of mouse oocytes with the deletion of the CD81 gene resulting in an inability of oocytes to fuse with sperm [178, 179]. Ohnami et al., 2012, concluded that CD9 and CD81 are both involved in spermatozoa-oocyte fusion and behave independently of one another. Jegou et al., utilized a recently developed biophysical approach to measure the strength of interaction between two live cells and found that CD9 was able to generate the strongest adhesion sites for spermatozoa.

Oocyte Activation

Membrane fusion triggers cellular responses in the oocyte, and prevents additional sperm that have penetrated the zona pellucida from fusing with the oocyte plasma membrane. The resulting signal transduction events “activate” the eggs and include the initiation of intracellular calcium oscillations, resumption of meiosis, and cortical granule exocytosis [180, 181]. It is accepted that the majority of these activation

events are directly related to calcium signaling cascades and the subsequent changes in particular protein kinase activities [56].

The release of sequestered calcium in response to fertilization involves an IP₃ evoked release of calcium from the endoplasmic reticulum. The increase in IP₃ production is believed to be a result of the activation of a member of the PLC family of enzymes which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce two common secondary messengers, DAG and IP₃ [182]. DAG is able to activate PKC while IP₃ binds to an IP₃R channel on the endoplasmic reticulum, resulting in a conformational change to the receptor and a significant release of intracellular calcium. A variety of hypotheses have been proposed in attempt to explain the initiation of these calcium oscillations including: 1) the contact (receptor-mediated) hypothesis or 2) the fusion (sperm factor) hypothesis.

The contact or receptor mediated hypothesis predicts the interaction of a sperm ligand with a receptor on the oocyte plasma membrane activates a PLC isoform endogenous to the oocyte to generate the IP₃. If an oocyte specific PLC is involved, a characterization of the upstream pathway would paint a clearer picture of oocyte activation. In many non-mammalian species PLC- γ is activated by Src family kinases shortly after fertilization and appears to be required for fertilization induced calcium oscillations in these species [182]; however, this has not been established in mammalian fertilization [56]. It would be worthwhile to review the Src Family Kinases in addition to the PLC family of enzymes in order to get a better understanding of potential players in the oocyte activation signaling cascade in response to ligand-receptor binding, including experimental evidence supporting or refuting their specific involvement in

mammalian fertilization.

Src Family Kinases

SFKs are rapidly activated following integrin-ligand interactions and subsequently activate downstream kinases and adapters [183-185]. SFKs are able to bind directly to β -integrin tails in a tail and SFK specific manner [183, 186, 187] in addition to binding and phosphorylating FAK and FAK-binding proteins.

SFKs include ten non-receptor tyrosine kinases (Abl, Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk) that are similar in structure and function. Src, Fyn, and Yes are ubiquitously expressed while the others show a more restricted pattern of expression [183, 188-191]. SFKs have a N-terminal myristoylation signal and a modular architecture consisting of 5 domains: the kinase unique N-terminal domain, the SH3 and SH2 protein interaction domains, the catalytic domain, and the C-terminal regulatory domain [188]. The myristoylation signal is critical for membrane localization and the unique N-terminal domain is the only non-conserved region within the SFKs. Two tyrosine phosphorylation sites, an autophosphorylation site at Tyr417 in the catalytic domain and a Tyr527 site in the C-terminal domain regulate the catalytic activities of SFKs positively and negatively. The SH3 domain binds to proline rich sequences and in so doing contributes to substrate recruitment, while also playing a critical role in the regulation of kinase activity [183, 190 1993, 192-196]. The SH2 domain also assists with protein-protein interactions by binding phosphotyrosine-containing sequences [183, 190].

Species that fertilize externally display intense activation of SFKs immediately after fertilization [188, 197-199]. The specific member of oocyte SFKs involved in this

activity vary from species to species and include Fyn in zebrafish and sea urchins [197, 200], and Src in sea urchins, starfish, and *Xenopus* [198, 201, 202]. The SFK activation begins at a localized site of sperm binding and indicates that gamete interactions (ligand-receptor mediated) and cell fusion initiate the SFK activation [188]. Sperm binding and fusion activates oocytes SFKs that phosphorylate and activates PLC- γ in echinoderms and ascidians [203, 204], but the involvement of SFKs in mice activation does not appear to be essential [205]. Injection of recombinant c-Fyn into mouse oocytes resulted in oocyte activation and is believed to interact with the truncated c-kit tyrosine kinase of sperm to activate PLC- γ [206, 207]. However, it appears that this pathway is different than the activation pathway that occurs in mice [182]. Abl [208], Src [197, 199], and Yes [209] are all active in vertebrate oocytes; however, correlating activities of vertebrate SFKs with invertebrate SFKs has proven to be challenging because of sequence diversity [188].

FAK is a conserved, ubiquitously expressed 125 kDa scaffold protein that recruits cytoskeletal and signaling molecules. FAK contains an N-terminal FERM domain, a central kinase domain, proline rich regions, and a C-terminal focal-adhesion-targeting (FAT) domain. The FERM domain facilitates a signaling linkage from receptor tyrosine kinases in addition to binding to and promoting integrin and FAK mediated activation of non-receptor tyrosine kinases [210]. Two proline-rich regions are found in the C-terminal domain that serve as binding sites for Src homology (SH)3 domain containing proteins. Also located in the C-terminal domain is the FAT region which promotes the colocalization of FAK with integrins at focal adhesions via binding of integrin associated proteins paxillin and talin.

When FAK is autophosphorylated at Tyr397, a motif forms that is recognized by proteins with SH2 domains including SFKs, PLC- γ , and PI3K. Of the SFKs, Src binding is promoted after FAK Tyr397 autophosphorylation that results in a conformational activation of Src and a dual activated FAK-Src signaling complex. Within the complex Src then phosphorylates FAK at Tyr861 resulting in additional SH3-domain mediated binding of the adaptor protein p130Cas to the proline rich regions in the C-terminal domain. Activated Src also phosphorylates FAK at Tyr925, thus creating an SH2 binding site for the GRB2 adapter protein, which leads to activation of Ras and the ERK2/MAPK signaling cascade. However, Src mediated transphosphorylation of FAK within the activation loop at Tyr576 and Tyr577 results in the maximum FAK catalytic activation [210].

FAK and closely related PYK2 are found to be rapidly phosphorylated/activated after fertilization in the zebrafish and could serve as targets of SFKs in perpetuating the activation signaling cascade [211]. Activated FAK as also been found in localized pools in response to fertilization in zebrafish [188].

Phospholipase C family of enzymes

In response to activation of receptors by neurotransmitters, hormones, growth factors and other molecules, phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP₂) to generate two second messengers: inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Both second messengers initiate further signal transduction cascades with IP₃ activating intracellular calcium release while DAG activates protein kinase C (PKC) [212-216]. A variety of PLCs with different molecular masses, isoelectric points, and calcium dependencies

have been discovered in a variety of different tissues and categorized into 6 classes based on structure and activity: PLC- β (1-4), PLC- γ (1 and 2), PLC- δ (1, 3, and 4) PLC- ϵ , PLC- ζ , and PLC- η (1 and 2) [216, 217]. Each isozyme family contains conserved domains as well as isozyme specific domains. All PLC isozymes contain catalytic X and Y domains in addition to isozyme specific regulatory regions including the pleckstrin homology (PH) domain, the C2 domain, and the EF-hand motif. The isotype specific domains allow for subtype specific regulatory mechanism including the Ras-associating domain and Ras-GTPase exchange factor-like domain in PLC- ϵ and the src homology (SH) domain in PLC- γ [216]. Numerous alternative splicing variants for each PLC isotype have been found across multiple species, indicating an extremely complex level of additional regulation [217].

The family of PLC- β isozymes consists of 4 enzymes (1-4) and is characterized by the presence of an elongated C-terminus that contains many of the required sequences for G protein interaction, membrane binding, and nuclear localization. PLC- β isozymes are regulated by heterotrimeric GTP-binding proteins and have a high ability to stimulate GTPase. Typically PLC- β isozymes function in the cytoplasm as effector enzymes for transmembrane proteins containing seven transmembrane segments. PLC- β isozymes are differentially expressed in a variety of tissues with PLC- β 1 being the most abundant. Overexpression of PLC- β 1 in mice had a large effect on sperm-induced calcium oscillations by greatly reducing the total amount of calcium released and even a modest reduction in PLC- β 1 protein levels significantly reduced the amplitude of the calcium oscillations in mice [218]. The authors stated that they only focused on PLC- β 1 and their results could not address or exclude the involvement of other oocyte derived

PLCs [218].

Two mammalian PLC- γ isozymes (1 and 2) have been identified and are characterized by the presence of two tandem Src homology 2 (SH2) domains and a Src homology 3 (SH3) domain flanked by a split PH domain. PLC- γ isozymes are activated by growth factors, immune receptors, and integrins [216, 217]. In response to growth factor stimulation, the SH2 domains of PLC- γ 1 mediate binding to the autophosphorylated tyrosine regions of the intracellular region of the receptor and are critical for membrane recruitment and tyrosine phosphorylation of PLC- γ 1. After recruitment to multi-molecular signaling complexes, both PLC- γ isozymes are phosphorylated by non-receptor tyrosine kinases from the Src, Syk, and Tec kinase families. Both PLC- γ 1 and PLC- γ 2 have similar expression patterns, but appear to perform independent functions [217]. The involvement of PLC- γ in the oocyte activation signaling cascade in echinoderms and ascidians in addition to potential implications in the mouse model was stated earlier and will not be repeated here.

The PLC- δ isozyme family (1, 3, and 4) has the simplest structure of all of the PLC isotype families and contains a PH domain, EF hand motif, X and Y domains, and a C2 domain which could explain the high homology among the three family members. The PLC- δ isozyme family is the most sensitive to intracellular calcium concentrations suggesting its activity to be tightly regulated by intra-cellular calcium levels. PLC- δ 1 appears to play a role in cell cycle regulation [217, 219] in addition to have anti-oncogene activity [220], and both PLC- δ 1 and PLC- δ 3 are essential for proper trophoblast development during formation of the placenta [221, 222]. PLC- δ 4 in sperm plays a critical role in fertilization and oocyte activation as PLC- δ 4 knockout mice suffer

from male infertility. Spermatozoa from PLC- δ 4 knockout mice result in fewer activated oocytes with those oocytes being activated having delayed or no calcium oscillations at all [97]. In addition, the sperm from PLC- δ 4 mice were unable to initiate the acrosome reaction demonstrating that PLC- δ 4 functions in the zona pellucida induced acrosome reaction during mammalian fertilization [223, 224]. This was further evidenced by the ability of solubilized mouse zona pellucida to generate a sustained intracellular calcium increase in wild type sperm but was only able to generate a minimal calcium increase in PLC- δ 4 null mice sperm [225]. These data indicate PLC- δ 4 plays a pivotal role in the generation of the calcium response in the sperm during the zona pellucida induced acrosome reaction.

PLC- ϵ is the largest PLC isozyme and contains 2 domains not found in other PLC isozymes. It contains RA domains in the C-terminus and a CDC25 homology domain located in the N-terminus. The RA domains mediate the GTP dependent interaction with Ras family small G-proteins [226, 227] and the CDC25 homology domain functions as a guanine nucleotide exchange factor for one of the Ras family small G-proteins [228]. Based on these structural features, the role of PLC- ϵ is believed to mediate the interplay between Ras-mediated and PLC-dependent pathways. PLC- ϵ is activated by growth factors, in particular EGF and PDGF, and is recruited into the plasma membrane by activated RAS through the R2 domain [226]. PLC- ϵ plays a role in the development of some organs and is involved in cell proliferation and tumor formation [229-234].

Recently, two PLC- η isozymes (1 and 2) were identified in mice and humans [230, 235, 236] and this family contains the PH domain, four EF-Hand motifs, catalytic

X and Y domains, and the C2 domain. The function(s) of both PLC- η isozymes have yet to be elucidated; however, both PLC- η isozymes are found in neuron enriched areas of the brain indicating their potential involvement in neural regulation [217].

PLC- ζ was identified as a sperm specific PLC as a result of analyzing EST sequences from human and mouse testis. It is the smallest mammalian PLC isozyme with a molecular weight of 70kDa and consists of the EF-hand motif, catalytic X and Y domains, and the C2 domain. PLC- ζ has a high degree of sequence homology (64%) with PLC- δ 1 in addition to sharing catalytic residues in the X domain, which indicates that the catalytic activation of PLC- ζ is similar to PLC- δ 1. PLC- ζ does not contain a PH domain like the other PLC isozymes so it is unclear how PLC- ζ can target PIP₂, its membrane bound substrate [217]. PLC- ζ has been proposed to be the testis-specific, sperm borne activating factor (SOAF) that initiates oocyte activation after gamete fusion in support of the fusion hypothesis [237].

Microinjection of PLC- ζ cRNA or recombinant protein into mouse oocytes results in intracellular calcium oscillations, activation and development [237, 238]. Sperm from male mice with decreased expression of PLC- ζ due to RNA interference are able to fuse with oocytes and initiate calcium oscillations; however, the number of oscillations was reduced and no transgenic offspring were born [239]. PLC- ζ was also shown to be defective due to reduced protein levels or mutated forms in men suffering infertility [240, 241].

For a spermatozoa protein to be a viable SOAF candidate, many criteria must be met: 1) Protein specific to male germ cells and more particular is only expressed in elongating spermatids and spermatozoa; 2) Triggers cortical granule exocytosis,

pronuclear development, and cleavage; 3) Ability to induce repetitive calcium oscillations; 4) Not species specific; 5) During the early stages of fertilization should be localized to the postacrosomal sheath of the spermatozoa perinuclear theca; 6) Soluble and released into cytoplasm of oocyte upon fusion [242, 243]. Unfortunately PLC- ζ fails to fulfill all of these requirements so its function as the SOAF needs to be taken into question.

PLC- ζ expression has also been shown not to be testes specific, as PLC- ζ expression has been detected in mouse brain [244] in addition to the ovary and brain of the puffer fish [245]. Aarabi et al., [246] recently demonstrated that PLC- ζ is actually secreted by the epididymis as a component of the acrosome during mouse spermatogenesis. Nevertheless, a novel function of PLC- ζ might be required for spermatogenesis, as PLC- ζ knockout mice seem unable to complete spermatogenesis with spermatocytes failing to develop beyond elongation [247], although the specificity of these effects needs to be evaluated in more detail.

By removing all sperm membranous and acrosomal components with the non-ionic detergent, Triton X-100, the SOAF has been shown to be localized to the perinuclear theca (PT) [248-250], and actually more precisely localized to the postacrosomal sheath of the perinuclear theca (PAS-PT) which is the region of the PT that is first solubilized on sperm entry into the ooplasm [242]. Consequently the SOAF should be non extractable by non-ionic detergents and localized to the PT, two requirements that the 74 kDa catalytically active form of PLC- ζ failed to meet [242].

PLC- ζ was originally believed to be localized to the part of the spermatozoa head that first enters the egg [251]; however, it was recently shown that PLC- ζ disappears

from the sperm head when it fuses with the oocyte plasma membrane and subsequent incorporation into the oocyte [242]. Reports also suggest that PLC- ζ undergoes dynamic changes in its pattern of localization in the spermatozoa of mice [252], hamster [252], and humans [253]. For example, it was demonstrated that PLC- ζ displays variable localizations in the sperm head, including the equatorial segment and the post-acrosomal regions, and it was suggested that these distinct localizations may reflect a different functional status of the sperm, i.e. capacitation and acrosome reaction, and that PLC- ζ may be involved in some of these physiological steps. However, to date, there is no supporting data to implicate PLC- ζ in these steps.

Microinjection of PLC- ζ cRNA into oocytes to overexpress PLC- ζ protein has been a common technique used to illustrate the ability of PLC- ζ to induce fertilization-like calcium oscillations and oocyte activation. There is an inherent flaw with this technique in that non-physiological amounts of protein are expressed, so how can one rule out the response is not non-physiological? Igarashi et al. [218] found that microinjecting PLC- β 1 cRNA into mouse eggs significantly altered the sperm-induced oscillations and the authors wondered that if PLC- ζ was the sole activating factor, how could an oocyte specific PLC affect the pattern. This provides evidence that a PLC endogenous to the oocyte might also be involved in initiation of calcium oscillations.

Retrospective studies involving men with globozoospermia [240, 241] concluded their infertility was due to reduced or defective PLC- ζ protein levels. As stated in Aarabi et al. [246] many other sperm proteins are also affected or absent in these patients, so in this case there is no direct experimental evidence indicating the reduced levels or mutated forms of PLC- ζ are the actual cause of the infertility. Association does

not mean causation.

The studies that used transgenic RNAi methods to decrease the amount of PLC- ζ in male mice, concluded that since no transgenic offspring were found, none of the PLC- ζ deficient sperm were able to activate oocytes [239]. In light of the recent findings that PLC- ζ might play a role in the acrosome reaction and is absent from the sperm head at fertilization, it is possible to conclude that no transgenic offspring were born because none of the PLC- ζ deficient sperm were able to undergo the acrosome reaction.

Summary

Much as been said regarding the molecular players involved in mediating spermatozoa-oocyte interactions and I believe there is yet much, much more to be said on the matter. Some facts are certain, in particular, the vital role calcium plays as a secondary messenger in many of the numerous events of mammalian fertilization leading up to and culminating in the formation of a zygote. However, it appears that for every experiment implicating the involvement of a spermatozoa or oocyte membrane protein, there is another experiment or set of experiments proving why the previously implicated protein is no longer “the” candidate or essential for binding or fusion or oocyte activation. The same can be said regarding the proposed mechanism(s) by which fertilization initiates calcium oscillations and embryonic development. Successful fertilization most likely includes the involvement of a multitude of protein complexes with multiple layers of redundancy, both on the oocyte and spermatozoa, to ensure a high probability of success. Additional characterization of some of the implicated molecular players will add a little more clarity to the complex picture of mammalian fertilization in particular, gamete binding, fusion, and oocyte activation.

Research Goals and Possible Applications of Project

The focus of my research included three primary objectives in characterizing the molecular factor(s) involved in bovine fertilization. They are:

- 1) Characterize which integrin subunits on the oocyte plasma membrane are involved in spermatozoa binding and fusion in the bovine model.
- 2) Elucidate the involvement of FAK as a signaling molecule downstream of integrin signaling in bovine fertilization.
- 3) Determine if any of the PLC enzymes endogenous to the bovine oocyte are involved in the oocyte activation signaling cascade.

A more complete understanding of the molecular factors involved in fertilization could have beneficial impacts for human fertility and assisted reproduction.

Overcoming human infertility problems due to the failure of spermatozoa-oocyte interactions [134] in addition to targeting the involved proteins for use in contraceptive therapy are highly plausible outcomes.

Another potential outcome is improved efficiency associated with animal somatic cell nuclear transfer (scNT). Currently the nuclear donor cell is fused with the oocyte plasma membrane via electrofusion and the resulting nuclear transfer couplet is subjected to chemical activation. Both electrofusion and chemical activation are non-physiologic events and could have long lasting negative impacts on embryo survival. A better understanding of spermatozoa-oocyte fusion and the oocyte activation signaling cascade could lead to a more natural method of fusing the donor cell with the oocyte, in addition to a more natural way of inducing oocyte activation and embryonic development. The technology of somatic cell nuclear transfer has a great potential of

producing transgenic animals for biopharmaceutical production and use as animal models for human disease, rescuing endangered species, and producing stem cells to name a few. Improving the efficiency of scNT will allow for increased advancements and benefits of the technology.

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CHAPTER 2
THE INVOLVEMENT OF INTEGRINS, IN PARTICULAR THE α V AND β 1
SUBUNITS, ON THE BOVINE OOCYTE VITELLINE
MEMBRANE IN BOVINE FERTILIZATION

Abstract

Integrins are heterodimers composed of two subunits, alpha (α) and beta (β), and facilitate cell migration and attachment to the extra-cellular matrix, mediate cell-cell adhesion, and act as two-way signaling molecules. Integrins located on the vitelline membrane of bovine oocytes have been implicated in mediating the sperm-oocyte interaction [164, 254] and the identity of the specific integrin subunits present on the bovine oocyte has also been determined (α V, α 2, α 4, α 5, α 6, β 1, and β 3) [255, 256]. Anti-integrin function blocking antibodies and immunofluorescence were utilized to reveal which specific integrin subunits are involved in mediating the sperm-oocyte interaction during bovine fertilization. Zona-free oocytes were pre-incubated with function blocking antibodies specific for the integrin subunits located on the vitelline membrane of bovine oocytes and subsequently fertilized and cultured in vitro according to our standard laboratory procedures [257]. The oocytes pre-incubated with the function blocking antibodies for the α V and β 1 subunits had significantly lower cleavage rates, pronuclei formation and oocytes exhibiting calcium transients ($p < 0.05$) compared to all other treatment groups. Immunofluorescence also confirmed the recruitment of α V and β 1 and not α 2, α 4, α 5, α 6, or β 3 integrin subunits to the site of spermatozoa binding in bovine oocytes in response to sperm binding to the vitelline membrane. However, despite the fact that the integrin α 5 subunit was not shown to aggregate in the oocyte

plasma membrane at the site of sperm binding, it was only localized to the equatorial segment of acrosome reacted sperm during membrane binding. These data are compelling evidence that the αV and $\beta 1$ integrin subunits are essential for fertilization in the bovine and could form the integrin heterodimer involved in the sperm-oocyte interaction.

Introduction

Fertilization is an essential step for all life and the process by which cells from two different parents combine to create unique offspring. In order for fertilization to occur, a single sperm cell must penetrate the oocyte, their DNA must combine, and a cascade of intracellular reactions must occur to initiate division and growth. The oocyte is surrounded by a protective structure called the zona pellucida (ZP). The sperm first binds to the ZP that initiates the acrosomal reaction and as a result releases hydrolases and other digestive enzymes that create a channel through the ZP for the sperm to enter. Once the sperm enters the perivitelline space it fuses with the oocyte plasma membrane and is engulfed by the oocyte.

Fusion of the gametes is marked by the resumption of meiosis, transient calcium [Ca^{2+}] oscillations, and expulsion of the second polar body. In all species studied thus far transient Ca^{2+} release from intracellular stores is mandatory for resumption of the cell cycle [182]. Once these processes have occurred the oocyte is “activated” and becomes a developing zygote.

The factors responsible for fusion as well as activation of the oocyte have not been identified; however, several families of proteins have been implicated as possible players, and one such group is the integrin family. Integrins are heterodimeric

transmembrane proteins composed of an alpha (α) and beta (β) subunit and are located on the vitelline membrane of mammalian oocytes [258]. Integrins facilitate cell-cell recognition and have been found to mediate oocyte activation in the bovine model [164].

Five α subunits ($\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and αV) and two β subunits ($\beta 1$ and $\beta 3$) have been found on the bovine oocyte [255, 256]. It is possible that one or both subunits mediate the fusion and/or activation of the oocyte. Bovine sperm-oocyte interactions have been shown to involve integrins, in particular the αV , $\alpha 5$, $\beta 1$ and $\beta 3$ subunits and may be mediated through cell surface receptors that contain RGD recognition sequences [164]. The deactivation of subunits through the use of functional blocking antibodies has been used to study the importance of integrins in other species, and this study uses a similar approach to demonstrate the involvement of integrins in bovine fertilization.

Materials/Methods

Oocyte Collection and In Vitro Maturation (IVM)

All reagents were purchased from ICN Biomedicals Inc. (Irvine, CA) unless otherwise stated. All procedures were performed according to published methods routinely used in this laboratory [1]. Bovine oocytes were collected from a local abattoir (E.A. Miller, Hyrum, UT). Oocytes from follicles 3-8 mm in size were aspirated into 50-ml centrifuge tubes using an 18-gauge needle attached to a vacuum pump. Oocytes with uniform cytoplasm and intact multiple layers of cumulus cells were selected and washed with PB1+ (phosphate-buffered saline with Ca^{2+} and Mg^{2+} plus 5.55 mM glucose, 0.32 mM sodium pyruvate, 3 mg/ml BSA). Oocytes were transferred into 500 μL of maturation medium, M199, containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 0.5 $\mu\text{g}/\text{ml}$ FSH (Sioux Biochemicals, Sioux City, IA), 5

$\mu\text{g/ml}$ LH (Sioux Biochemicals), 100 units/ml penicillin (Life Technologies, Grand Island, NY), and 100 $\mu\text{g/ml}$ streptomycin (Life Technologies) in four-well culture dishes (Nunc, Milwaukee, WI) and cultured at 39°C in a humidified atmosphere of 5% CO₂ and air for 18 h. After the maturation period, oocytes were vortexed for 4 min in a 15-ml conical centrifuge tube containing 1 ml PB1+ stock (without BSA) medium containing 10 mg/mL hyaluronidase to completely remove cumulus cells. After cumulus cell removal, zonae pellucidae were removed from the cumulus-free mature oocytes by incubation in 600 μL drops of 0.33% pronase (Fluka, Milwaukee, WI) in PB1+ Stock on a shaker at 100 RPM for 15 minutes. Zona-free oocytes were collected from the drop, washed thoroughly in PB1+, and returned to 500 μL of fresh maturation medium for 6 hours at 39°C in a humidified atmosphere of 5% CO₂ and air to recover.

In Vitro Fertilization

Oocytes were collected and matured according to methods previously described. Twenty-four-hour IVM bovine oocytes were subjected to our standard *in vitro* fertilization protocol [257]. Cryopreserved bovine semen (Hoffman AI, Logan, UT) was thawed and live sperm were separated by centrifugation on a 45%/90% layered Percoll gradient. Sperm was capacitated with heparin and acrosome reacted with lysophosphatidylcholine according to published procedures [259]. Treatment groups included a 2-hour incubation prior to fertilization with no antibody, and either the $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αV , $\beta 1$, and $\beta 3$ function blocking antibodies at a concentration of 5 $\mu\text{g/ml}$. Zona free *in vitro* matured oocytes were randomly separated into the treatment groups and fertilized *in vitro* for 18-20 h at 39°C in 5% CO₂ and air. After the fertilization period, oocytes were vortexed for 2 minutes 40 seconds in a 15-ml conical centrifuge tube

containing 1 ml PB1+ medium to completely remove unbound sperm. Presumptive embryos were co-cultured on a monolayer of bovine cumulus cells in CR1aa medium containing 3% FBS at 39°C in 5% CO₂ and cleavage was determined 24 hours after removal of sperm.

Analysis of Pronuclei Formation

Bovine oocytes were fertilized *in vitro* according to protocols described above with the appropriate inhibitor and control. After 5 hours, unbound sperm were removed by vortexing vigorously for 2 minutes 40 seconds. Embryos were rinsed well through drops of PB1+ and stained in a solution of PB1+ containing 1 µg/mL Hoechst 33342 for 20 minutes at 37°C. After staining, embryos were rinsed again through 8 drops of PB1+ and observed by fluorescence microscopy to evaluate pronuclear formation.

Calcium Indicator Fura-2 AM Loading

Oocytes were loaded with Ca²⁺ indicator by incubation in 2 µM Fura-2 AM ester (Molecular Probes Inc., Eugene, OR) and 0.02% Pluronic F-127 (Molecular Probes Inc.) in Ca²⁺- and Mg²⁺- free phosphate buffered saline (Hyclone Laboratories) containing 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA, and 100 µM EGTA at 39°C in darkness for 45 minutes. After loading indicator, oocytes were washed extensively with PB1- (phosphate-buffered saline without Ca²⁺ and Mg²⁺ plus 5.55 mM glucose, 0.32 mM sodium pyruvate, 3 mg/ml BSA) and maintained in this solution at 39°C until use.

Measurement of intracellular Ca²⁺ transients

Fura-2 indicator was loaded, as described above, and Ca²⁺_i levels were measured

after fertilization. Sperm were prepared as described above, after which a 50- μ L drop of Fert-talp containing PHE, heparin, and sperm was made in the center of a 35-mm dish (Beckton Dickinson, Franklin Lakes, NJ) and overlaid with mineral oil. Microinjected oocytes were visualized in groups of 8-10. The plate was placed in an incubator at 39°C in a humidified atmosphere of 5% CO₂ and air for 5 hours. At this point the dish was removed from the incubator and visualized over a 2-hour period to observe Ca²⁺ transients on a Nikon Diaphot inverted microscope, coupled with a Hamamatsu ORCA-ER digital camera and analyzed on Metafluor Imaging Series 7.6 software (Universal Imaging Corp, Downingtown, PA).

Immunofluorescence

Oocytes were placed in PBS with 4% formaldehyde (fixative) for 30 minutes at 4°C, washed in PBS with 0.05% Tween 20 (PBST) for 30 minutes at room temperature, permeabilized in PBS with 1% Triton X-100 for 30 minutes at room temperature, and transferred to blocking buffer consisting of PBST with 1% BSA and incubated overnight at 4°C. Oocytes were incubated with the appropriate primary antibodies for 3 hours at 37°C, washed with PBST for 30 minutes at room temperature, incubated with the appropriate secondary antibodies for 2 hours at 37°C, washed with PBST for 30 minutes at room temperature, and oocytes were transferred to slides with drops of Vectashield Hard Set mounting medium containing DAPI and covered with a cover slip. All samples were viewed on a Zeiss Axioobserver equipped with a Vivatome module and an Axioacam MRm camera.

Statistical Analysis

Data were pooled from three replicates per group. Chi-square analysis was used to determine differences in cleavage, pronuclei formation, and intracellular calcium release. Unless otherwise noted, a probability of $p < 0.05$ was considered statistically significant.

Results

Effect of Integrin Function Blocking Antibodies on Bovine In Vitro Fertilization

The results indicate the involvement and importance of the αV and $\beta 1$ integrin subunits in bovine fertilization. Compared with all other treatment groups, there was a significant reduction ($p < 0.05$) in cleavage for the oocytes incubated with the αV or $\beta 1$ function blocking antibodies (Fig. 2-1). Blocking the $\alpha 4$, $\alpha 5$, and $\beta 3$ integrin subunits also resulted in a significant reduction in cleavage compared to the IVF control, but not to the extent of the αV or $\beta 1$ subunits ($p < 0.05$).

Effect of Integrin Function Blocking Antibodies on Pronuclei Formation

Oocyte incubation with αV or $\beta 1$ function blocking antibodies resulted in a significant decrease ($p < 0.05$) in pronuclei formation, consequently providing additional evidence that the αV and $\beta 1$ integrin subunits are involved in the fusion of sperm and oocyte in bovine fertilization (Fig. 2-2). The $\alpha 5$ and $\beta 3$ function blocking antibodies also resulted in a significant decrease ($p < 0.05$) in pronuclei formation compared to the IVF control, but had less of an effect than the αV or $\beta 1$ function blocking antibodies.

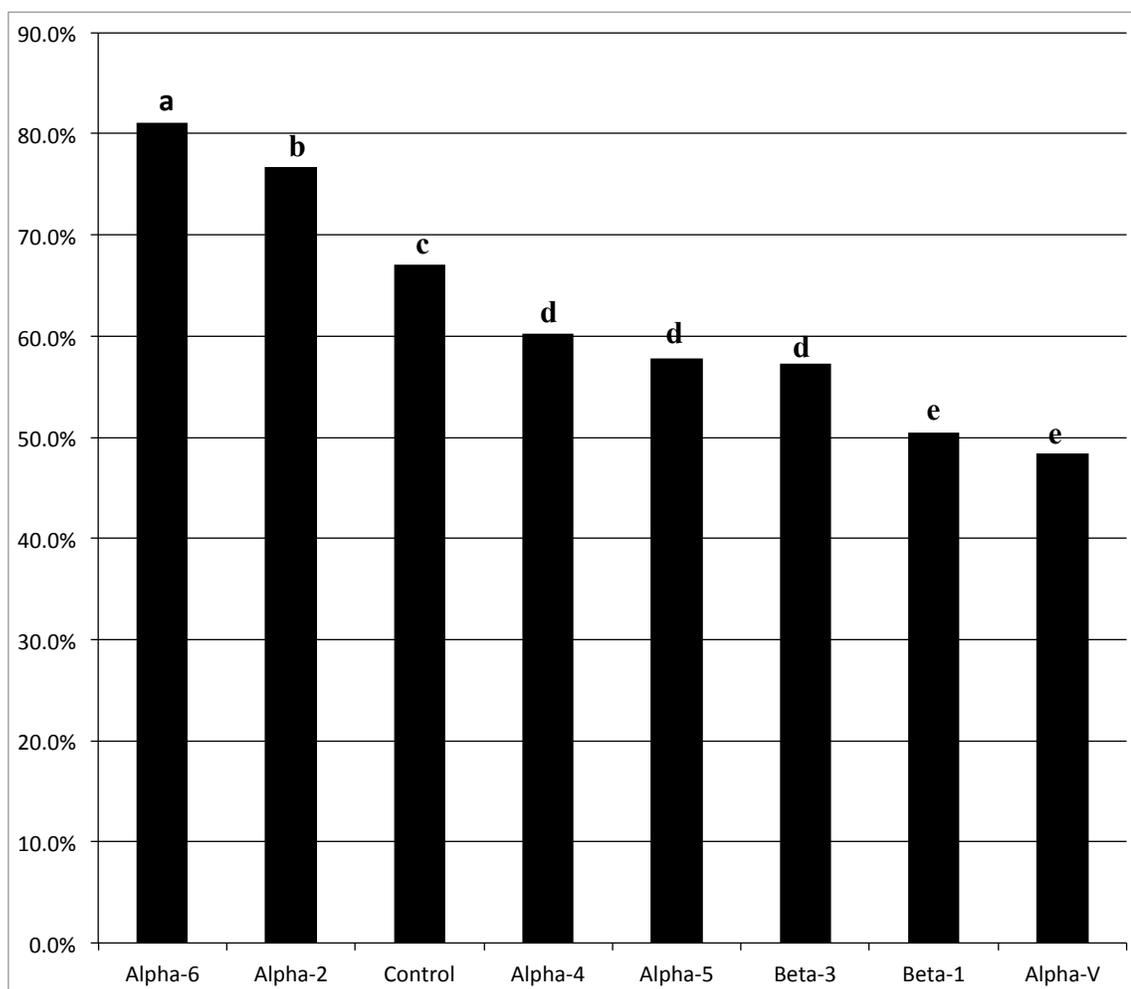


FIG 2-1. Percent cleavage of IVF embryos treated with anti-integrin antibodies and incubated with sperm for 18 hrs. Values represent the mean (n=3) and unlike superscripts are statistically significant $p < 0.5$.

*Effect of Integrin Function Blocking Antibodies
on Intracellular Calcium Release in
Bovine Oocytes*

Incubation of oocytes with anti-integrin αV and $\beta 1$ function blocking antibodies during IVF also resulted in significantly lower numbers of oocytes ($p < 0.05$) responding with calcium transients compared to the IVF and control (Table 2-1).

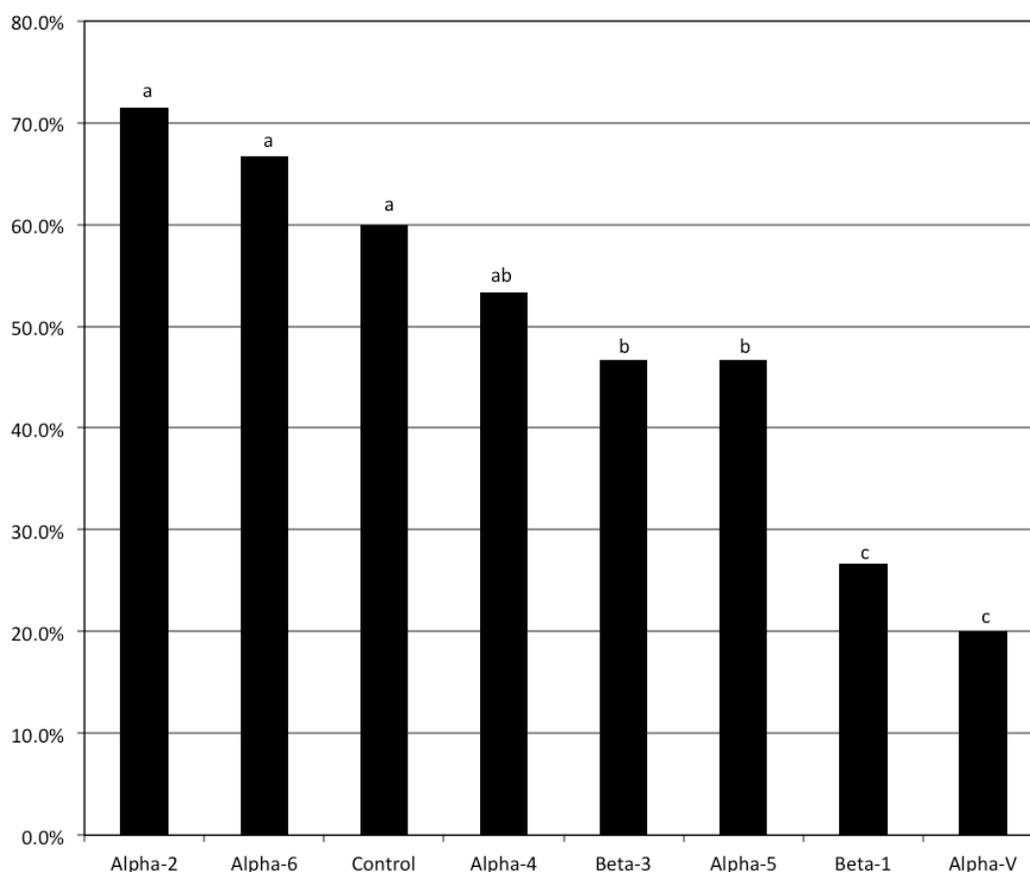


FIG 2-2. Percentage of oocytes with two pronuclei after incubation with anti-integrin antibodies and incubated with sperm for 6 hrs. Values represent the mean (n=3) and unlike superscripts are statistically significant $p < 0.05$.

Table 2-1. Percentage of oocytes responding with intracellular calcium transients after incubation with anti-integrin antibodies and incubated with sperm for 6 hrs. Values represent the mean (n=3) and unlike superscripts are statistically significant $p < 0.05$.

Treatment	Number of oocytes responding with an intracellular calcium release	Total number of oocytes
IVF control	36 (72.0 %)a	50
Alpha-2	29 (69.0%) ^{ab}	42
Alpha-6	27 (67.5%) ^{abc}	40
Alpha-4	20 (50.0%) ^{bcd}	40
Alpha-5	18 (46.2%) ^{cd}	39
Beta-3	17(41.5%) ^d	41
Beta-1	7 (21.2%) ^e	40
Alpha-V	8 (19.0%) ^e	42

*Localization of Integrin subunits of αV and $\beta 1$
to Site of Spermatozoa Binding to Oocyte
Vitelline Membrane*

The localization of αV and $\beta 1$ integrin subunits on the vitelline membrane at the site of spermatozoa binding can be seen in Fig. 2-3A and 2-3B, respectively. Despite the fact that the integrin $\alpha 5$ subunit was not shown to aggregate in the oocyte plasma membrane at the site of sperm binding, it was localized to the equatorial segment of acrosome reacted sperm during membrane binding (Fig. 2-3C). All together these data are strong evidence that integrins are involved in bovine fertilization.

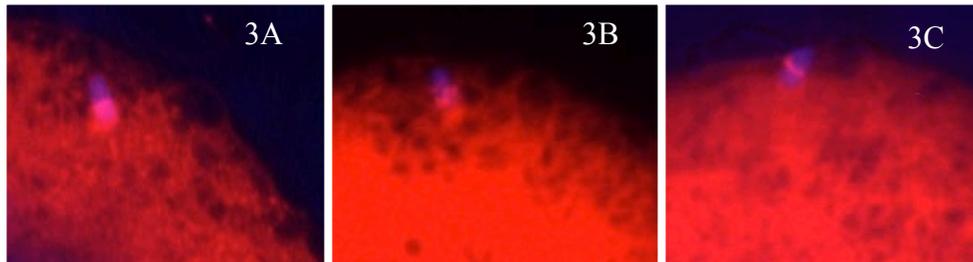


FIG 2-3. Fluorescent images of integrin subunits αV (3A) and $\beta 1$ (3B) at the level of the vitelline membrane localized to the site of spermatozoa binding and $\alpha 5$ (3C) only localized to the equatorial segment of spermatozoa.

Discussion

Integrins are a large family of heterodimeric cation-dependent transmembrane proteins composed of non-covalently linked α and β subunits. Each subunit contains a large N-terminal extracellular domain, a transmembrane domain, and a short C-terminal cytoplasmic domain. There are eighteen α and eight β subunits identified and these subunits combine to form 23 heterodimers. The major function of integrins is to mediate cell-to-cell interactions and cell-to-substratum attachment even though they may mediate a variety of other cellular functions [146]. In the mouse model, Baessler et al., illustrated that the $\beta 1$ integrin subunit is intimately involved with the initial adhesion of

the spermatozoa to the oocyte plasma membrane in addition to mediating the subsequent attachment and fusion [260]. The involvement of the $\beta 1$ and $\alpha 9$ integrin subunits in murine fertilization was also shown using RNA interference and function blocking antibodies [163]. Bovine sperm-oocyte interactions have been shown to involve integrins, in particular the αV , $\alpha 5$, $\beta 1$ and $\beta 3$ subunits and may be mediated through cell surface receptors that contain RGD recognition sequences [164]. Biochemical analysis has implicated αV and $\beta 1$ integrins subunits on the pig oocytes in the recognition of pig sperm membrane proteins [157].

Membrane fusion triggers cellular responses in the oocyte, and prevents additional sperm, that have penetrated the zona pellucida, from fusing with the oocyte plasma membrane. The resulting signal transduction events “activate” the eggs and include the initiation of intracellular calcium oscillations, resumption of meiosis, and cortical granule exocytosis [180, 181]. It is accepted that the majority of these activation events are directly related to calcium signaling cascades and the subsequent changes in particular protein kinase activities [56].

The release of sequestered calcium in response to fertilization involves an IP₃ evoked release of calcium from the endoplasmic reticulum. A variety of hypotheses have been proposed in attempt to explain the initiation of these calcium oscillations including: 1) the contact (receptor-mediated) hypothesis or 2) the fusion (sperm factor) hypothesis. The contact or receptor mediated hypothesis predicts the interaction of a sperm ligand with a receptor on the oocyte plasma membrane activates a PLC isoform endogenous to the oocyte to generate the IP₃.

The incubation of oocytes with anti-integrin function blocking antibodies

decreased the ability of spermatozoa to fertilize the oocytes; however, a complete blockage did not occur. A common interpretation of this result is that integrins are not required for bovine fertilization since some oocytes were successfully fertilized. However, this result could also be explained considering the concept that spermatozoa were able to “out-compete” the function-blocking antibody over time. This theory is supported by the pronuclei data and calcium data illustrating the ability of the function blocking antibodies to block sperm effectively over a shorter time period. The antibody could also easily be denatured over the 18-20 hour IVF period and consequently allow more oocytes to be fertilized. Integrins are known to interact with other integrins in addition to membrane proteins including tetraspanins, Ig superfamily receptors and GPI anchored proteins, which also have been shown to be involved with the process of fertilization in a variety of species [166, 261-263]. From an evolutionary aspect, it would make sense to have redundancy and multiple proteins (ligands and receptors) involved to ensure the highest probability of success for such a critical process leading to propagation of the species.

It is clear that the αV and $\beta 1$ integrin subunits located on the bovine oocyte vitelline membrane contribute to the spermatozoa binding/fusion process. It appears that the $\alpha 5$ integrin subunit is localized to the equatorial region of acrosome reacted sperm, which confirms the findings of Thys et al., 2009. Integrin $\alpha 5$ didn't localize on the oocyte plasma membrane at the site of spermatozoa binding and fusion to the oolemma; however this doesn't preclude a potential role of integrin $\alpha 5$ located on the spermatozoa in initiating the spermatozoa-oocyte binding events [256]. These results provide evidence that spermatozoa interact with integrins, known “outside-in” signaling

complexes, at the level of the vitelline membrane in bovine oocytes. These events could serve as the initiation of signaling cascades associated with bovine oocyte activation and require further investigation.

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CHAPTER 3
EVIDENCE OF INVOLVEMENT OF FOCAL ADHESION KINASE
IN BOVINE FERTILIZATION

Abstract

Focal adhesion kinase (FAK), also known as protein tyrosine kinase 2, is localized to focal adhesions and is a key component of signal transduction pathways mediated by integrins. The clustering of integrins at focal contacts results in activation of FAK and the subsequent assembly of signaling complexes including SRC family kinases. Because integrins have been implicated in bovine fertilization and activation [164, 254], the investigation of the involvement of FAK is an essential step for elucidating the molecular mechanisms involved in bovine fertilization and activation. The presence of FAK at the mRNA and protein levels in bovine oocytes was verified by real time PCR (qPCR) and immunoprecipitation and the localization of FAK at the site of sperm binding to the oocyte plasma membrane was verified using immunohistochemistry. Small interfering RNA (siRNA) duplexes directed against bovine FAK, and known FAK inhibitors FAK I and FAK II, were microinjected into bovine oocytes at various concentrations and the resulting effects on FAK mRNA and protein levels, intracellular calcium release, and embryo development were evaluated. FAK I inhibitor, FAK II inhibitor, all resulted in lower cleavage rates and a decrease in the number of oocytes responding with calcium transients. The sham and scrambled FAK sequence siRNA control microinjections had no effect on fertilization while the microinjection of the FAK siRNA did result in significantly lower cleavage rates and the FAK siRNA treatment group was not significantly different than the no sperm control.

The microinjection of FAK siRNA resulted in lower levels of FAK mRNA transcript while western blot analysis indicated a decrease in the relative levels of FAK protein corresponding to a decrease in mRNA levels after microinjection with the FAK siRNA. Neither the FAK siRNA nor inhibitors acted at the level of the zona pellucida or the plasma membrane, as indicated by sperm-oocyte penetration rates equal to controls. These data are compelling evidence that FAK is involved in bovine activation and fertilization.

Introduction

Integrins are a large family of heterodimeric cation-dependent transmembrane proteins composed of non-covalently linked α and β subunits. Each subunit contains a large N-terminal extracellular domain, a transmembrane domain, and a short C-terminal cytoplasmic domain. There are eighteen α and eight β subunits identified and these subunits combine to form 23 heterodimers. The major function of integrins is to mediate cell-to-cell interactions and cell-to-substratum attachment even though they may mediate a variety of other cellular functions [146]. Integrins associate and interact with cytoskeletal proteins through the cytoplasmic domain of the β subunit and aggregate as focal adhesion sites [147]. These focal adhesion sites also contain signaling complexes involving focal adhesion kinase (FAK), integrin-linked kinase (ILK), molecules of the MAP kinase pathway, small GTPases (ras and rho), lipid kinases (PIP 5-kinase and PI3 kinase), PIP2, PLC- γ , serine/threonine kinases, phosphatases, and activation of focal adhesion sites can result in changes in intracellular pH and calcium concentration [148-151].

The interaction of human and hamster sperm with zona pellucida (ZP)-free

hamster and human oocytes by RGD peptides implicated the involvement of the RGD-binding subfamily of integrins [160, 161]. Even though RGD peptides do have an inhibitory affect on the binding of recombinant fertilin β to mouse oocytes, they do not have a substantial inhibitory effect on mouse sperm-oocyte interactions [123, 124]. However, cyclic RGD-containing peptides were found to inhibit sperm-oocyte interaction in the mouse model in addition to stimulating the activation of PKC and cortical granule exocytosis [162]. Also in the mouse model, Baessler et al., 2009 illustrated that the $\beta 1$ integrin subunit is intimately involved with the initial adhesion of the spermatozoa to the oocyte plasma membrane in addition to mediating the subsequent attachment and fusion [260]. The involvement of the $\beta 1$ and $\alpha 9$ integrin subunits in murine fertilization was also shown using RNA interference and function blocking antibodies [163]. Bovine sperm-oocyte interactions have been shown to involve integrins and may be mediated through cell surface receptors that contain RGD recognition sequences [114, 164, 254].

FAK is a conserved, ubiquitously expressed 125 kDa scaffold protein that recruits cytoskeletal and signaling molecules to focal adhesions at the site of integrin clustering [183]. FAK contains an N-terminal four-point-one, ezrin, radixin, moesin (FERM) domain, a central kinase domain, proline rich regions, and a C-terminal focal-adhesion-targeting (FAT) domain. The FERM domain facilitates a signaling linkage from receptor tyrosine kinases in addition to binding to and promoting integrin and FAK mediated activation of non-receptor tyrosine kinases including Src and phosphatidylinositol 3-kinase (PI3-kinase) [210]. Two proline-rich regions are found in the C-terminal domain that serve as binding sites for Src homology (SH)3 domain

containing proteins. Also located in the C-terminal domain is the FAT region which promotes the co-localization of FAK with integrins at focal adhesions via binding of integrin associated proteins paxillin and talin. FAK and closely related PYK2 are found to be rapidly phosphorylated/activated after fertilization in the zebrafish and could serve as targets of SFKs in perpetuating the activation signaling cascade [211]. Activated FAK has also been found in localized pools in response to fertilization in zebrafish [188].

An inquiry into the involvement of FAK in bovine fertilization is merited based on the evidence illustrating integrin involvement in fertilization and the previously characterized recruitment of FAK as a signaling molecule to the site of integrin clustering.

Materials and Methods

All reagents were purchased from MP Biomedicals LLC (Solon, OH) unless otherwise stated.

Oocyte Collection and In Vitro Maturation (IVM)

All procedures were performed according to published methods routinely used in this laboratory [164]. Bovine oocytes were collected from a local abattoir (E.A. Miller, Hyrum, UT). Oocytes from follicles 3-8 mm in size were aspirated into 50-ml centrifuge tubes using an 18-gauge needle attached to a vacuum pump. Oocytes with uniform cytoplasm and intact multiple layers of cumulus cells were selected and washed with PB1+ (phosphate-buffered saline with Ca^{2+} and Mg^{2+} plus 5.55 mM glucose, 0.32 mM sodium pyruvate, 3 mg/ml BSA). Oocytes were transferred into 500 mL of maturation

medium, M199, containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 0.5 mg/ml FSH (Sioux Biochemicals, Sioux City, IA), 5 mg/ml LH (Sioux Biochemicals), 100 units/ml penicillin (Life Technologies, Grand Island, NY), and 100 mg/ml streptomycin (Life Technologies) in four-well culture dishes (Nunc, Milwaukee, WI) and cultured at 39°C in a humidified atmosphere of 5% CO₂ and air until use. Oocytes were vortexed in 1 ml PB1+ containing 10 mg/ml hyaluronidase for 4 min. to completely remove cumulus cells. Oocytes of good quality were selected for use.

Microinjection of FAK siRNA

The FAK siRNA duplexes oligo ribonucleotides were designed with Invitrogen's (Carlsbad, CA) BLOCK-iT™ RNAi Designer using the target sequence of different bovine FAK. Three siRNA duplexes were designed against separate, distinct regions of the bovine FAK mRNA sequence. Each duplex RNA was resuspended in DEPC treated water in order to make a 20-μM stock solution (10mM Tris-HCl, pH8.0, 20 mM NaCl, 1 mM EDTA). Thin-bore borosilicate glass capillaries were pulled into microinjection needles using a Narishige (East Meadow, NY) PB-7 pipette puller. Holding pipettes were similarly pulled and crafted using a Narishige MF-9 micro forge.

For the siRNA injections cumulus cells were removed at 14 hours post maturation and cumulus-free mature oocytes were randomly assigned to each treatment group and microinjected (using a Nikon Diaphot inverted microscope and Narishige IM 300 microinjector) with 20 μM siRNA duplex, water (sham), FAK scramble negative control, or not injected. The approximate volume of a bovine oocyte is 800 pL and the injection volume was calculated to be 8 pL (1% of total volume) so the working concentration in the oocyte of each duplex is 200nM. For the initial screening of the

siRNA duplexes the oocytes were subjected to our standard *in vitro* fertilization protocol [257].

Microinjection of FAK inhibitors

For the inhibitor microinjections, cumulus cells were removed at 20 hours post maturation and cumulus-free mature oocytes were randomly assigned to each treatment group. After a recovery period of two hours in maturation medium in the incubator, the oocytes were microinjected with FAKI inhibitor (EMD Millipore, Billerica, MA) (working concentrations of 10 nM, 100 nM, and 200 nM), FAKII inhibitor (EMD Millipore, Billerica, MA) (working concentrations of 40 pM, 40 nM, and 400 nM), a sham injection of water, or not injected.

In Vitro Fertilization

After microinjection, oocytes were returned to maturation medium and were either subjected to a modified version of our *in vitro* fertilization protocol or snap frozen in groups of 30 for subsequent analysis by real time PCR and Western blot. Briefly, cryopreserved bovine semen (Hoffman AI, Logan, UT) was thawed and live sperm were separated by centrifugation on a 45%/90% layered Percoll gradient. Motile spermatozoa obtained by this method were diluted in fert-TALP to a final concentration of 1.0×10^6 per ml [22]. Capacitation occurred in fert-TALP containing heparin at a concentration of 10 mg/ml. The oocytes were fertilized *in vitro* for 5 hours (24-29 hours post maturation for inhibitor studies, and 27-32 hours post maturation for siRNA studies) at 39°C in 5% CO₂ and air. After the fertilization period, oocytes were vortexed for 2 minutes in a 15-ml conical centrifuge tube containing 1 ml PB1+ medium to completely remove sperm.

Presumptive embryos were cultured in CR1aa medium containing 3% FBS at 39°C in 5% CO₂ and cleavage was determined 48 hours after removal of sperm. Each treatment was performed in three replicates. Data were analyzed with a chi-square analysis for independence in an $r \times 2$ contingency table, where r equals the number of treatments.

Immunofluorescence

For the immunofluorescence studies the oocytes were fertilized for seven hours, after which they were vortexed for 2 minutes in a 15-ml conical centrifuge tube containing 1 ml PB1+ medium to completely remove sperm. Oocytes were placed in PBS with 4% formaldehyde (fixative) for 30 minutes at 4°C, washed in PBS with 0.05% Tween 20 (PBST) for 30 minutes at room temperature, permeabilized in PBS with 1% Triton X-100 for 30 minutes at room temperature, and transferred to blocking buffer consisting of PBST with 1% BSA and incubated overnight at 4°C. Oocytes were incubated with the anti-FAK primary antibody for 3 hours at 37°C, washed with PBST for 30 minutes at room temperature, incubated with the appropriate secondary antibody for 2 hours at 37°C, washed with PBST for 30 minutes at room temperature, and oocytes were transferred to slides with drops of Vectashield Hard Set mounting medium containing DAPI and covered with a cover slip. All samples were viewed on a Zeiss Axioobserver equipped with a Vivatome module and an AxioCam MRm camera.

Sperm Penetration Assay

Microinjected oocytes were fertilized in vitro at 23 hours post maturation. After 6 hours of incubation with sperm, embryos were rinsed through drops of PB1+ and stained in a solution of PB1+ containing 1 mg/mL Hoechst 33342 for 20 minutes at 37°C. After

staining, embryos were rinsed again through 8 drops of PB1+ and observed by fluorescence microscopy to evaluate pronuclear formation. Sperm penetration was determined by positive identification of 2 pronuclei in each embryo.

Calcium Indicator Fura-2 AM Loading

Oocytes were loaded with Ca^{2+} indicator by incubation in 2 μM Fura-2 AM ester (Molecular Probes Inc., Eugene, OR) and 0.02% Pluronic F-127 (Molecular Probes Inc.) in Ca^{2+} - and Mg^{2+} - free phosphate buffered saline (Hyclone Laboratories) containing 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA, and 100 μM EGTA at 39°C in darkness for 45 minutes. After loading indicator, oocytes were washed extensively with PB1- (phosphate-buffered saline without Ca^{2+} and Mg^{2+} plus 5.55 mM glucose, 0.32 mM sodium pyruvate, 3 mg/ml BSA) and maintained in this solution at 39°C until use.

Measurement of intracellular Ca^{2+} transients

Fura-2 indicator was loaded, as described above, and Ca^{2+}_i levels were measured after fertilization. Sperm were prepared as described above, after which a 50- μL drop of Fert-talp containing PHE, heparin, and sperm was made in the center of a 35-mm dish (Beckton Dickinson, Franklin Lakes, NJ) and overlaid with mineral oil. Microinjected oocytes were visualized in groups of 4-6. The plate was placed in an incubator at 39°C in a humidified atmosphere of 5% CO_2 and air for 5 hours. At this point the dish was removed from the incubator and visualized over a 2 hour period to observe Ca^{2+} transients on a Nikon Diaphot inverted microscope, coupled with a Hamamatsu ORCA-ER digital camera and analyzed on Metafluor Imaging Series 7.6 software

(Universal Imaging Corp, Downingtown, PA).

Reverse Transcription and Real Time PCR analysis

Total RNA was extracted from 3 groups of 30 microinjected oocytes for each siRNA duplex using an RNeasy Micro Kit (Qiagen, Valencia, CA). Reverse transcription was performed using a SuperScript III Kit (Invitrogen, Carlsbad, CA) and cDNA was stored at -80°C for later use. Primers for FAK were designed using Primer3 software [264] and qPCR were performed to verify knockdown of the FAK transcript. The delta-delta Ct method ($\Delta\Delta\text{Ct}$) was used for real-time PCR data evaluation [265]. Data was normalized for differing amounts of input cDNA by calculating ΔCt (Ct for the GAPDH housekeeping gene minus Ct for the gene of interest). The $\Delta\Delta\text{Ct}$ was calculated by subtracting the ΔCt of each sample from the ΔCt of a reference cDNA sample. The n-fold increase or decrease in expression levels of each treatment at each time point was calculated using the formula $2^{-\Delta\Delta\text{Ct}}$.

Immunoprecipitation

Total protein was extracted from 100 mature bovine oocytes by placing them in 50 μL of lysis buffer containing 8 M urea, 4% CHAPS, 40 mM Tris, and Complete Protease Inhibitor Cocktail (Roche Diagnostic, Mannheim, Germany). The immunoprecipitation protocol provided by Upstate (Lake Placid, NY) was used. Briefly, the cell lysate was diluted with PBS to roughly 1 $\mu\text{g}/\text{ml}$ total cell protein before beginning the immunoprecipitation, after which 4 μg of monoclonal mouse IgG anti-FAK antibody clone 2A7 (Upstate) was added and gently rocked at 4°C overnight. The immunocomplex was captured by adding 100 μl of washed Protein G agarose bead

slurry and gently rocked at 4°C for 2 hours. The agarose beads were collected by pulsing for 5 seconds in a microcentrifuge at 14,000 x g and draining off the supernatant. The beads were washed three times with ice-cold PBS and resuspended in 60 µl 2X Laemmli sample buffer. The beads were boiled for 5 minutes and collected using a microcentrifuge pulse after which the supernatant was run on a 4-20% gradient polyacrylamide gel (Pierce Precise Protein Gels) while the nitrocellulose membrane and filters were incubated in the transfer buffer at 4°C for 45 minutes. A lysate of 3T3/A31 cells (Upstate) was included as a positive control. After electrophoresis, the gel was washed with transfer buffer for 3 minutes and then the total protein was transferred electrophoretically using a semi-dry blotter (Biorad, Hercules, CA) onto the nitrocellulose membrane for 80 minutes at 20V. After transfer the membrane was washed twice with TBST for 5 minutes each time and then the membrane was blocked overnight at 4°C in TBST with 5% skim milk. To detect bovine FAK the membrane was incubated for 1 hour with polyclonal rabbit IgG primary antibody (Upstate, Lake Placid, NY) washed three times in TBST for 10 minutes each, incubated for 1 hour at room temperature with a goat anti-rabbit HRP-labeled secondary antibody diluted 1:1000 in TBST, and finally processed as described below. The nitrocellulose membrane was washed two times with TBST, 5 minutes each time. For protein detection 3 mls of Luminata Classico Western HRP Substrate (Millipore, Billerica, MA) was poured onto the membrane. The membrane was incubated without agitation in the substrate for 3 minutes, drained of excess substrate, wrapped in a clear plastic wrap, exposed to an x-ray film for 30 seconds and developed.

Western Blot Analysis

The Western blot analysis of bovine FAK was performed according to the procedures used by Sun and Fan, 2004 [266]. Briefly, 30 bovine oocytes were added to 6.5 μ L double-strength sample buffer and 5 μ L ddH₂O in a 0.5 ml Eppendorf tube and frozen at -80°C until use. The samples were boiled for 4 minutes, placed on ice for 5 minutes, and centrifuged at 12,000 x g for 3 minutes. The total proteins were separated by SDS-page on a 4-20% gradient polyacrylamide gel (Pierce Precise Protein Gels) for 45 minutes at 110 V and 115 mA, while the nitrocellulose membrane and filters were incubated in the transfer buffer at 4°C for 45 minutes. After electrophoresis, the gel was washed with transfer buffer for 3 minutes and then the total protein was transferred electrophoretically onto the nitrocellulose membrane for 80 minutes at 20V. After transfer, the membrane was washed twice with TBS for 5 minutes each time and then the membrane was blocked overnight at 4°C in TBST with 5% skim milk. To detect bovine FAK the membrane was incubated for 1 hour with a rabbit polyclonal anti-FAK antibody (sc-558; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:300 in TBST, washed 3 times in TBST for 10 minutes each, incubated for 1 hour at room temperature with a goat anti-rabbit HRP-labeled secondary antibody diluted 1:1000 in TBST, and finally processed as described above.

Statistical Analysis

Data were pooled from three replicates per group. Chi-square analysis was used to determine differences in cleavage and intracellular calcium release. Unless otherwise noted, a probability of $p < 0.05$ was considered statistically significant. For the confirmation of siRNA knockdown experiments, pair-wise comparisons were performed

by utilizing the calculated $\Delta\Delta C_t$ values for each treatment time point and the control were performed using the Student's t-test. A probability of $p < 0.05$ was considered significant.

Results

Immunoprecipitation/Western Blot

Western blot analysis of lysate from mature bovine oocytes immunoprecipitated with a monoclonal anti-FAK antibody revealed that a FAK protein (~125 kDa band) is present in the mature bovine oocyte (Fig. 3-1).

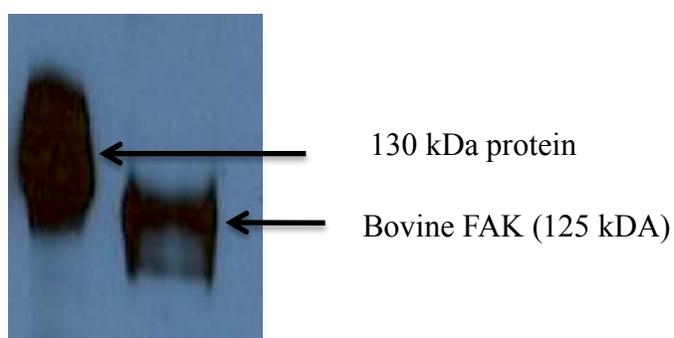


FIG 3-1. Western blot analysis of immunoprecipitated FAK.

Effect of Anti-FAK Agents on Intracellular Calcium Release in Bovine Oocytes

Fak Inhibitors

Microinjection of the FAKI inhibitor at working concentrations of 10 nM ($p < 0.01$), 100 nM ($p < 0.0001$), and 200 nM ($p < 0.0001$) all resulted in significantly lower numbers of oocytes responding with calcium transients compared to the IVF and sham injected controls.

The microinjection of the FAKII inhibitor at the working concentration of 400 nM resulted in significantly lower numbers of oocytes responding with calcium transients ($p < 0.001$). The lower concentrations of 40 nM and 40 pM were not significantly different than the sham injected oocytes.

Anti-FAK siRNA Oligonucleotides

The anti FAK siRNA oligonucleotide microinjected into oocytes resulted in a significantly lower number of oocytes responding with calcium transients ($p < 0.001$). The number of oocytes microinjected with the scrambled FAK siRNA control oligonucleotide responding with calcium transients were not significantly different than the IVF and sham injected controls.

Effect of Anti-FAK Agents on *In Vitro* Fertilization in Bovine Oocytes

Fak Inhibitors

The injection of the FAKI inhibitor resulted in significantly lower cleavage rates at the working concentrations of 200 nM ($p < 0.0001$), 100 nM ($p < 0.0001$), and 10 nM ($p < 0.01$) with the 200 nM and 100 nM concentrations not being significantly different than the no-sperm control.

The injection of the FAK II inhibitor resulted in significantly lower cleavage rates at the working concentration of 400 nM ($p < 0.0001$) and not significantly different than the no sperm control. The concentrations of 40 nM and 40 pM were not significantly different than the IVF and sham injected controls.

TABLE 1. Effect of anti-FAK inhibitors on the intracellular calcium release after incubation with sperm for 6 hrs. Values represent the mean (n=3) and unlike superscripts are statistically significant (p<0.05).

Treatment	Number of oocytes responding with an intracellular calcium release	Total Number of Oocytes
Non-injected	16 (72.7%) ^a	22
Scrambled FAK siRNA	16 (66.7%) ^a	24
Sham	16 (61.5%) ^a	26
FAK II Inhibitor (40 nM)	14 (58.3%) ^a	24
FAK II Inhibitor (40 pM)	16 (57.1%) ^a	28
FAK I Inhibitor (10 nM)	6 (23.1%) ^b	26
FAK siRNA	6 (20.0%) ^b	30
FAK II Inhibitor (400 nM)	4 (14.3%) ^b	28
FAK I Inhibitor (100 nM)	3 (10.7%) ^b	28
FAK I Inhibitor (200 nM)	2 (7.7%) ^b	26

Anti-FAK siRNA Oligonucleotide

The anti-FAK siRNA oligonucleotide resulted in significantly lower cleavage rates (p<0.001) compared to the IVF and sham injected control and was not significantly different than the no sperm control. The scrambled FAK siRNA control oligonucleotide was not significantly different than the IVF and sham injected controls.

Confirmation of siRNA Knockdown

qPCR

The microinjection of FAK siRNA resulted in lower levels of FAK mRNA transcript at 6, 12 and 24 hours post injection as verified by qPCR. The FAK mRNA levels of the oocytes collected at all three time points for the FAK siRNA groups were the only treatment groups significantly different (P<0.05) than the 14 hour control (Fig. 3-2).

TABLE 3-2. Effect of anti-FAK inhibitors on the cleavage rates of bovine oocytes after incubation with sperm for 6 hrs. Values represent the mean (n=3) and unlike superscripts are statistically significant (p<0.05).

Treatment	Percent Cleavage	Total Number of Oocytes
FAK II Inhibitor (40 pM)	32 (72.7%) ^a	44
FAK II Inhibitor (40 nM)	30 (63.8%) ^a	47
Non-injected	26 (61.9%) ^a	42
Sham	25 (61.0%) ^a	41
Scrambled FAK siRNA	24 (51.1%) ^{ab}	47
FAK I Inhibitor (10 nM)	22 (38.6%) ^{bc}	57
FAK II Inhibitor (400 nM)	14 (29.8%) ^c	47
FAK siRNA	12 (21.8%) ^{cd}	55
FAK I Inhibitor (100 nM)	7 (11.7%) ^{de}	70
No sperm control	5 (8.3%) ^{de}	60
FAK I Inhibitor (200 nM)	1 (1.6%) ^e	61

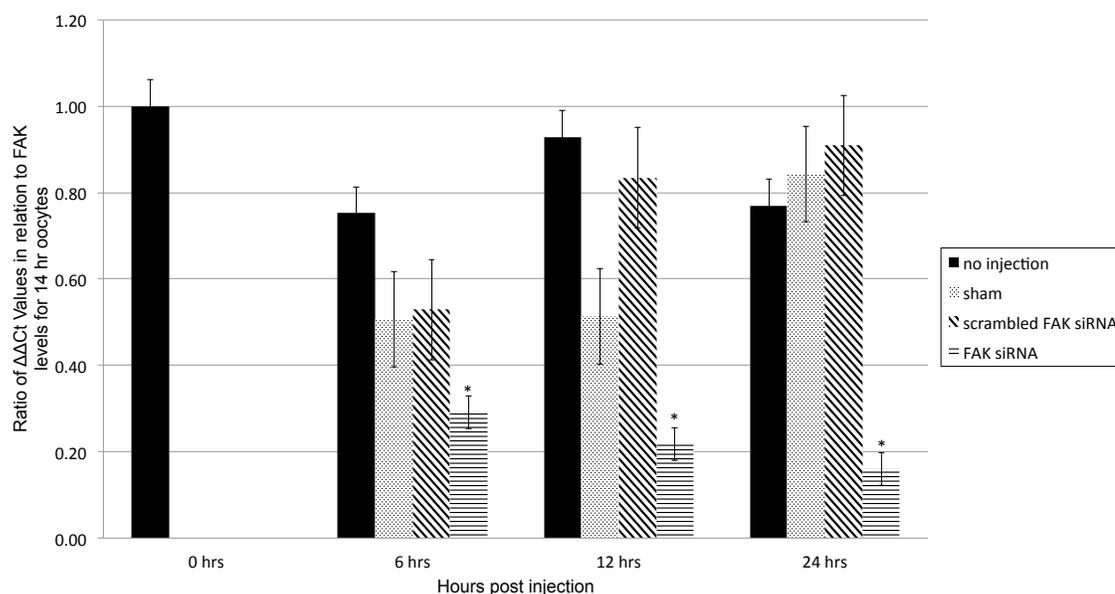


FIG 3-2. Relative FAK mRNA expression levels as compared to 14 hr IVM control oocytes. Values represent the mean \pm SEM (n = 3, *P < 0.05).

Western Blot

Western blot analysis demonstrated a decrease in the relative levels of FAK protein corresponding to a decrease in mRNA levels after microinjection with the FAK

siRNA. As expected the reduction in protein levels occurred at a slower rate than the reduction in FAK mRNA levels with significant reduction in protein levels not occurring until 12 hours post injection (Fig. 3-3).

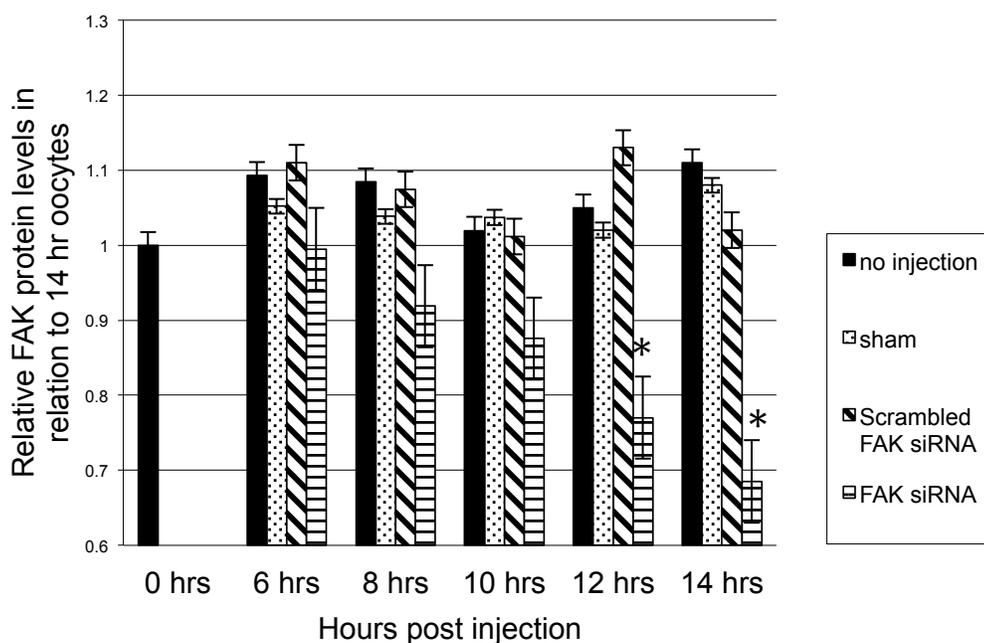


FIG 3-3. Relative FAK protein levels in relation to 14 hr IVM control oocytes. Values represent the mean \pm SEM (n = 3, *P < 0.05).

Immunofluorescence

The results of the immunohistochemistry studies indicate a colocalization of FAK clusters during fertilization at the site of spermatozoa binding to the oocyte plasma membrane (Fig 3-4).

Sperm Penetration Assay

The injected inhibitors and FAK siRNA did not act at the level of the zona pellucida or the plasma membrane, because sperm penetrated the oocytes at levels equivalent to controls and inhibition therefore occurred intracellularly (data not shown).

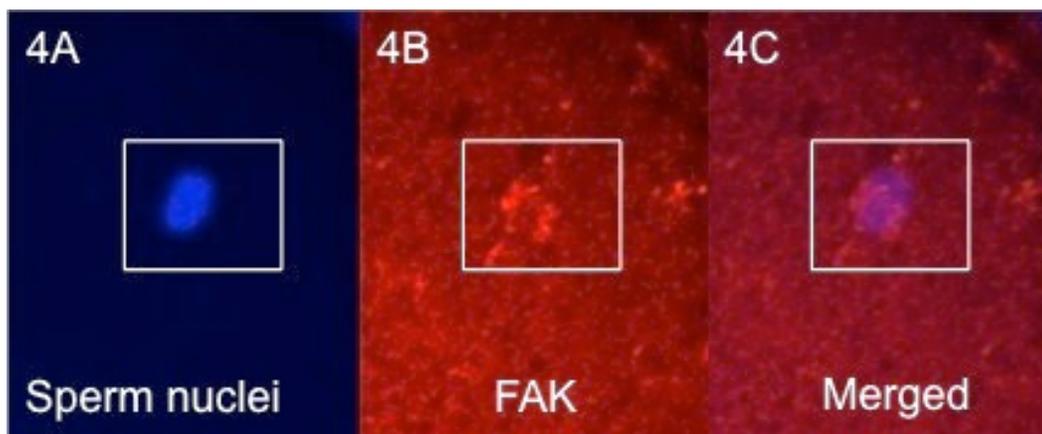


FIG. 3-4. Fluorescent images of FAK localized to the site of spermatozoa binding/fusion during fertilization of a bovine metaphase II arrested oocyte. **A)** Sperm nuclei labeled with Hoechst. **B)** FAK labeled with Alexafluor 594. **C)** Merged image of sperm nuclei and immunolabeled FAK illustrating a localization of FAK at the site of sperm binding/fusion.

Discussion

Focal adhesion kinase (FAK) is a 125 kDa ubiquitous nonreceptor protein tyrosine kinase that localizes to focal adhesions where it functions as a prominent signaling molecule. Upon integrin activation and clustering, FAK is activated via tyrosine phosphorylation in order to interact with other signaling molecules recruited to the focal adhesion including Src and (PI3-kinase) resulting in the transduction of signaling cascades. These focal adhesions involve the recruitment of integrins, transmembrane heterodimers receptor proteins that bind to and respond to the extracellular matrix and consequently act as two-way signaling molecules. FAK has been implicated in mediating the oocyte activation signaling cascade in non-mammalian oocytes following fertilization [188, 211] and integrins have been show to be involved in mammalian fertilization [114, 123, 124, 157, 162-165, 254].

The FAK specific inhibitors, FAK I and FAK II, resulted in significantly lower

cleavage rates and oocytes responding with calcium transients. The sham and the scrambled FAK siRNA control microinjections had no effect on fertilization demonstrating the technique used did not negatively impact normal development. The microinjection of the FAK siRNA did result in significantly lower cleavage rates and the FAK siRNA treatment group was not significantly different than the no sperm control.

The microinjection of FAK siRNA resulted in significantly lower levels of FAK mRNA transcript at all three data points. The FAK mRNA levels of the oocytes collected at 6, 12, and 24 hours post injection for the FAK siRNA groups were the only treatment groups significantly different than the 18 hr control ($p < 0.05$). The siRNA oligonucleotides quickly resulted in a quick reduction in the presence of FAK mRNA, with an almost 80% reduction in FAK mRNA levels in only a 6-hour period

Western blot analysis demonstrated a decrease in the relative levels of FAK protein corresponding to a decrease in mRNA levels after microinjection with the FAK siRNA. The oocytes microinjected with FAK siRNA had the lowest abundance of FAK protein at all three time points. The use of siRNA technology to knockdown FAK protein levels only resulted in an average of a 30-35% decrease in protein levels during the IVF period. Despite only a small reduction in protein levels, it still had a significant impact on cleavage rates and calcium oscillations. The inability to get a greater reduction in protein levels is probably due to the stockpiling of proteins by the oocyte in preparation for fertilization. The bovine oocyte is a challenging system to utilize protein knockdown studies due to the limited window of time to introduce the siRNA oligonucleotides and get significant decrease in protein levels between oocyte collection/maturation and IVF. The beginning time period selected for IVF (27 hours

IVM) corresponded to an ~ 25% decrease in FAK protein levels with the end of the IVF period corresponded to ~40% decrease in FAK protein. Western blots did not distinguish between total protein and active protein so there might actually be a greater reduction in active protein that would help explain the significant decrease in cleavage and oocytes responding with calcium transients.

The release of sequestered calcium in response to fertilization involves an IP3 evoked release of calcium from the endoplasmic reticulum. The increase in IP3 production is believed to be a result of the activation of a member of the PLC family of enzymes which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce two common secondary messengers, DAG and IP₃ [182]. DAG is able to activate PKC while IP₃ binds to an IP₃R channel on the endoplasmic reticulum, resulting in a conformational change to the receptor and a significant release of intracellular calcium. Two hypotheses have been proposed in attempt to explain the initiation of these calcium oscillations including: 1) the contact (receptor-mediated) hypothesis or 2) the fusion (sperm factor) hypothesis.

The contact or receptor mediated hypothesis predicts the interaction of a sperm ligand with a receptor on the oocyte plasma membrane activates a PLC isoform endogenous to the oocyte to generate the IP₃. If an oocyte specific PLC is involved, a characterization of the upstream pathway would paint a clearer picture of oocyte activation. In many non-mammalian species PLC- γ is activated by Src family kinases (SFKs) shortly after fertilization and appear to be required for fertilization induced calcium oscillations in these species [182]; however, this has not been established in mammalian fertilization [56]. SFKs are known to be rapidly activated following

integrin-ligand interactions and subsequently activate downstream kinases and adapters [183 2006, 185]. SFKs are able to bind directly to β -integrin tails in a tail and SFK specific manner [183, 186, 187] in addition to binding and phosphorylating FAK and FAK-binding proteins.

PLC- ζ has been proposed to be the testis-specific, sperm borne activating factor (SOAF) that initiates oocyte activation after gamete fusion in support of the fusion hypothesis [237]. Microinjection of PLC- ζ cRNA or recombinant protein into mouse oocytes results in intracellular calcium oscillations, activation and development [237, 238]. Sperm from male mice with decreased expression of PLC- ζ due to RNA interference are able to fuse with oocytes and initiate calcium oscillations; however, the number of oscillations was reduced and no transgenic offspring were born [239]. PLC- ζ was also shown to be defective, with either reduced protein levels or mutated forms, in men suffering infertility [240, 241].

If PLC- ζ is truly the oocyte activating factor, inhibiting FAK located in the oocyte should have no effect on bovine fertilization and oocyte activation. This is obviously not the case. Inhibiting FAK had a dramatic effect on both calcium oscillations and cleavage in the bovine system, indicating that a signaling pathway endogenous to the oocyte is indeed actively involved during fertilization and oocyte activation. These data indicate the direct involvement of FAK in addition to further implicating the involvement of integrins in the signaling cascade related to bovine fertilization and oocyte activation.

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CHAPTER 4

**THE INVOLVEMENT OF PHOSPHOLIPASE C ISOFORMS ENDOGENOUS
TO THE OOCYTE IN BOVINE FERTILIZATION AND OOCYTE
ACTIVATION**

Abstract

Phospholipase C (PLC) isoforms stimulate the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP₂) to produce diacylglycerol (DAG) and 1,4,5 inositol triphosphate (IP₃), with IP₃ regulating the release of calcium (Ca²⁺) from the endoplasmic reticulum. This release of calcium is essential for oocyte activation and a sperm specific PLC isoform, PLC ζ , has been proposed as the primary agent that initiates the activation process. However, the oocyte contains many endogenous PLC isoforms (PLC β , γ , and δ) that could also be involved in regulating or initiating these calcium oscillations downstream of other initiating events. In order to better elucidate the involvement of endogenous PLC isoforms as well as the specific role of the sperm-specific form, small interfering RNA (siRNA) directed against the specific bovine PLC isoforms (PLC ζ , PLC γ 1, PLC γ 2, PLC δ 1, PLC δ 3, PLC δ 4, PLC β 1, PLC β 3) were microinjected into bovine oocytes and the subsequent effects on PLC mRNA levels and bovine fertilization were evaluated. Real time PCR (qPCR) was used to quantify the levels of PLC message present in bovine oocytes at the time of injection (15 hours post maturation) and 6, 10, and 14 hours post injection. The qPCR results indicated a near complete knockdown of mRNA levels in bovine oocytes 10 hours post injection for the isotypes PLC γ 1, PLC γ 2, PLC δ 3, PLC δ 4, PLC β 1, PLC β 3, but only partial knockdown of PLC δ 1 mRNA. Oocytes microinjected with PLC siRNA were also fertilized and

cultured in vitro according to our standard laboratory procedures [257]. The oocytes microinjected with PLC ζ , PLC δ 1, PLC δ 3, PLC δ 4, PLC β 1, PLC β 3 siRNA resulted in cleavage rates similar to the negative control siRNA, non-injected, and sham injected treatment groups while bovine oocytes microinjected with PLC γ 1 and PLC γ 2 siRNA had significantly lower cleavage rates compared to the controls. Additionally, cRNA for each specific PLC isoform was also microinjected into bovine oocytes to ascertain each isoform's ability to induce parthenogenetic activation. The oocytes microinjected with PLC ζ and PLC γ 2 cRNA resulted in a significantly higher number of oocytes reaching the 2 cell stage compared to all other treatment groups and not significantly different than the Ionomycin/Cycloheximide activation control ($p < 0.05$). These data illustrate the potential involvement of multiple endogenous PLC isoforms PLC γ 1 and PLC γ 2 and not just the sperm-specific PLC ζ isoform in bovine oocyte activation during fertilization.

Introduction

In response to activation of receptors by neurotransmitters, hormones, growth factors and other molecules, phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP₂) to generate two second messengers: inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Both second messengers initiate further signal transduction cascades with IP₃ activating intracellular calcium release while DAG activates protein kinase C (PKC) [212-216]. A variety of PLCs with different molecular masses, isoelectric points, and calcium dependencies have been discovered in a variety of different tissues and categorized into six classes based on structure and activity: PLC- β (1-4), PLC- γ (1 and 2), PLC- δ (1, 3, and 4) PLC- ϵ , PLC- ζ , and PLC- η (1 and 2) [216, 217]. Each isozyme family contains conserved

domains as well as isozyme specific domains. All PLC isozymes contain catalytic X and Y domains in addition to isozyme specific regulatory regions including the pleckstrin homology (PH) domain, the C2 domain, and the EF-hand motif. The isotype specific domains allow for a subtype specific regulatory mechanism including the Ras-associating domain and Ras-GTPase exchange factor-like domain in PLC- ϵ and the src homology (SH) domain in PLC- γ [216]. Numerous alternative splicing variants for each PLC isotype have been found across multiple species, indicating an extremely complex level of additional regulation [217].

PLC- ζ was identified as a sperm specific PLC as a result of analyzing EST sequences from human and mouse testis. It is the smallest mammalian PLC isozyme with a molecular weight of 70kDa and consists of the EF-hand motif, catalytic X and Y domains, and the C2 domain. PLC- ζ has a high degree of sequence homology (64%) with PLC- δ 1 in addition to sharing catalytic residues in the X domain, which indicates that the catalytic activation of PLC- ζ is similar to PLC- δ 1. PLC- ζ does not contain a PH domain like the other PLC isozymes so it is unclear how PLC- ζ can target PIP₂, its membrane bound substrate [217]. PLC- ζ has been proposed to be the testis-specific, sperm-borne activating factor (SOAF) that initiates oocyte activation after gamete fusion [237].

Materials and Methods

Oocyte Collection and In Vitro Maturation (IVM)

All reagents were purchased from ICN Biomedicals Inc. (Irvine, CA) unless otherwise stated. All procedures were performed according to published methods routinely used in this laboratory [164]. Bovine oocytes were collected from a local

abattoir (E.A. Miller, Hyrum, UT). Oocytes from follicles 3-8 mm in size were aspirated into 50-ml centrifuge tubes using an 18-gauge needle attached to a vacuum pump. Oocytes with uniform cytoplasm and intact multiple layers of cumulus cells were selected and washed with PB1+ (phosphate-buffered saline with Ca^{2+} and Mg^{2+} plus 5.55 mM glucose, 0.32 mM sodium pyruvate, 3 mg/ml BSA). Oocytes were transferred into 500 μL of maturation medium, M199, containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 0.5 $\mu\text{g}/\text{ml}$ FSH (Sioux Biochemicals, Sioux City, IA), 5 $\mu\text{g}/\text{ml}$ LH (Sioux Biochemicals), 100 units/ml penicillin (Life Technologies, Grand Island, NY), and 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies) in four-well culture dishes (Nunc, Milwaukee, WI) and cultured at 39°C in a humidified atmosphere of 5% CO_2 and air for 24 hours.

Microinjection of PLC siRNA

The PLC siRNA duplexes oligoribonucleotides were designed with Invitrogen's (Carlsbad, CA) BLOCK-iT™ RNAi Designer using the target sequence of different bovine PLC isoforms (PLC ζ , gi|55669158; PLC β 3, gi|119919227, PLC γ 1, gi|31342035, PLC δ 4, gi|114051038, PLC δ 1, gi|125991881; PLC β 1, gi|27807356; PLC γ 2, gi|359075087; PLC δ 3, 300795644). Three siRNA duplexes were designed against separate, distinct regions of each bovine PLC mRNA. Each duplex RNA was resuspended in DEPC treated water in order to make a 20- μM stock solution (10mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA).

Thin-bore borosilicate glass capillaries were pulled into microinjection needles using a Narishige (East Meadow, NY) PB-7 pipette puller. Holding pipettes were similarly pulled and crafted using a Narishige MF-9 micro forge. Cumulus cells were

removed at 17 hours post maturation and cumulus-free mature oocytes were randomly assigned to each treatment group and microinjected (using a Nikon Diaphot inverted microscope and Narishige IM 300 microinjector) with either 20 μ M of each RNA duplex, water (sham), Stealth RNAi negative control, or not injected. The approximate volume of a bovine oocyte is 800 pl and the injection volume was calculated to be 8 pL (1% of total volume) so the working concentration in the oocyte of each duplex was 200nM.

In Vitro Fertilization

After microinjection oocytes were returned to maturation medium and were either subjected to a modified version of our *in vitro* fertilization protocol [257] or snap frozen in groups of 30 for subsequent analysis by real time PCR. Briefly, cryopreserved bovine semen (Hoffman AI, Logan, UT) was thawed and live sperm were separated by centrifugation on a 45%/90% layered Percoll gradient. Motile spermatozoa obtained by this method were diluted in fert-TALP to a final concentration of 1.0×10^6 per ml [257]. Capacitation occurred in fert-TALP containing heparin at a concentration of 10 μ g/ml. The oocytes were fertilized *in vitro* for 5 hrs (24-29 hours post maturation) at 39°C in 5% CO₂ and air. After the fertilization period, oocytes were vortexed for 1 minute in a 15-ml conical centrifuge tube containing 1 ml PB1+ medium to completely remove sperm. Presumptive embryos were cultured in CR1aa medium containing 3% FBS at 39°C in 5% CO₂ and cleavage was determined 48 hours after removal of sperm.

Reverse Transcription and Real Time PCR

Total RNA was extracted from 3 groups of 30 microinjected oocytes for each

siRNA duplex using an RNeasy Micro Kit (Qiagen, Valencia, CA). Reverse transcription was performed using a SuperScript III Kit (Invitrogen, Carlsbad, CA) and cDNA was stored at -80°C for later use. Primers for each PLC isoform were designed using Primer3 software and qPCR was performed to verify knockdown of each isoform's transcript.

PLC Complementary RNA (cRNA) Preparation

A separate vector containing the full length coding sequence of each PLC isoform was linearized with NotI and used as a template for the in vitro synthesis of capped RNA by using the T7 or SP6 mMessage mMachine High Yield Capped RNA Transcription Kit (Ambion, Austin, TX) depending on the promoter present in the plasmid. Briefly, a 20 μL reaction was assembled adding the following in order: 4 μL nuclease free water, 10 μL 2X NTP/CTP, 2 μL 10X reaction buffer, 2 μL linear template DNA, and 2 μL enzyme mix. The tube was flicked, centrifuged briefly, and incubated at 37°C for 2 hours. A poly-A tail was subsequently added to the cRNA by utilizing the Poly (A) Tailing Kit (Ambion). Briefly, the following tailing reagents were added, in order, to a completed 20 μL mMessage mMachine reaction: 36 μL nuclease free water, 20 μL 5X E-PAP buffer, 10 μL 25 mM MnCl_2 , 10 mM ATP, and 4 μL of E-PAP. The reaction was gently mixed, and incubated at 37°C for 1 hour. The poly-A tailed cRNA was purified by utilizing the MEGAclear Purification Kit (Ambion). Briefly, the volume of RNA samples was brought to 100 μL with Elution Solution and mixed gently. Three hundred fifty microliters of Binding Solution Concentrate was added to the samples, followed by gently mixing. After which 250 μL of 100% ethanol was added to the samples and mixed again. The samples were applied to a filter cartridge inserted into a

Collection and Elution tube, centrifuged for 1 minute at 12,000 x g, and the flow through was discarded. The samples were washed twice by adding 500 μ L wash solution and centrifuged for 1 minute at 12,000 x g. After discarding the wash solution, the filter cartridge was centrifuged for another 30 seconds to remove the wash solution. The cRNA was eluted from the cartridge by placing the Filter Cartridge into a new Collection/Elution Tube, applying 50 μ L of Elution Solution and incubated for 7 minutes at 67 °C. The eluted cRNA was recovered by centrifugation for 1 minute at 12,000 x g at room temperature. The cRNA was stored in single use aliquots at -80 °C until use.

Microinjection of PLC cRNA

Cumulus cells were removed at 20-22 hrs post maturation and cumulus-free mature oocytes were randomly assigned to each treatment group and microinjected with the appropriate concentration of cRNA (0.1, 0.5, or 1.0 μ g/ μ l), water (sham), or not injected.

Microinjection of PLC siRNA and cRNA

Cumulus cells were removed at 14 hours post maturation and cumulus-free mature oocytes were randomly assigned to each treatment group and microinjected with the appropriate concentration of siRNA, water (sham), or not injected and placed back into maturation medium. The same oocytes were microinjected at 22-24 hours post maturation with the correct corresponding cRNA (0.1, 0.5, or 1.0 μ g/ μ l).

Calcium Indicator Fura-2 AM Loading

Oocytes microinjected with only cRNA of cRNA/siRNA were loaded with a

Ca²⁺ indicator by incubation in 2 μ M Fura-2 AM ester (Molecular Probes Inc., Eugene, OR) and 0.02% Pluronic F-127 (Molecular Probes Inc.) in Ca²⁺ - and Mg²⁺ - free phosphate buffered saline (Hyclone Laboratories) containing 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA, and 100 μ M EGTA (PB1-) at 39°C in darkness for 30 min. After loading indicator, oocytes were washed extensively with PB1- and maintained in this solution at 39°C until use.

Intracellular Calcium Monitoring

The Fura-2 loaded oocytes were transferred to a 30- μ L drop of PB1- medium containing one and covered with mineral oil. An additional control of electroporation with 250 nM inositol (1,4,5)-triphosphate (IP3; Molecular Probes Inc.) was included with each replication to ensure that the oocytes were of good quality and capable of intracellular Ca²⁺ transients. Intracellular Ca²⁺ monitoring and electroporation conditions performed according to published methods [267-270].

Parthenogenetic Activation

At either 14 hours or 20 hours after the initiation of maturation, oocytes were vortexed in 1 ml TL-HEPES containing 10 mg/ml hyaluronidase for 4 minutes to completely remove cumulus cells. Oocytes exhibiting an extruded first polar body and of good quality were selected for use. Those oocytes prepared at 14 hours post maturation, were microinjected with siRNA and placed back in maturation medium to be injected with cRNA at 22-24 hours post maturation followed by incubation in 7.5 μ g/ml cytochalasin B for 5 hours, or not injected and exposed to 5 μ M ionomycin for 5 minutes immediately followed by 10 μ g/ml cycloheximide for 5 hours.

Oocytes prepared at 20 hours post maturation were injected with only cRNA, injected with cRNA followed by an incubation in 7.5 $\mu\text{g/ml}$ cytochalasin B for 5 hours, or not injected and exposed to 5 μM ionomycin for 5 minutes immediately followed by 10 $\mu\text{g/ml}$ cycloheximide for 5 hours. After the various treatments were completed, the oocytes were washed in CR1aa medium plus 10% FBS and cultured in the CR2-cumulus cells co-culture system [257]. Cleavage was recorded on Day 2. The treatment groups were compared to in vitro-fertilized oocytes according to our standard laboratory protocol [257].

Statistical Analysis

Data were pooled from three replicates per group. Chi-square analysis was used to determine differences in cleavage, parthenogenetic activation, and intracellular calcium release. Unless otherwise noted, a probability of $p < 0.05$ was considered statistically significant. For the confirmation of siRNA knockdown experiments, pairwise comparisons were performed by utilizing the calculated $\Delta\Delta\text{Ct}$ values for each treatment time point and the control were performed using the Student's t-test. A probability of $p < 0.05$ was considered significant.

Results

Confirmation of siRNA Knockdown

qPCR

The qPCR results indicated a near complete knockdown of mRNA levels in bovine oocytes by 10 hours post injection for the isotypes PLC γ 1, PLC γ 2, PLC δ 3, PLC δ 4, PLC β 1, PLC β 3, but only partial knockdown of PLC δ 1 mRNA (Figure 4-1).

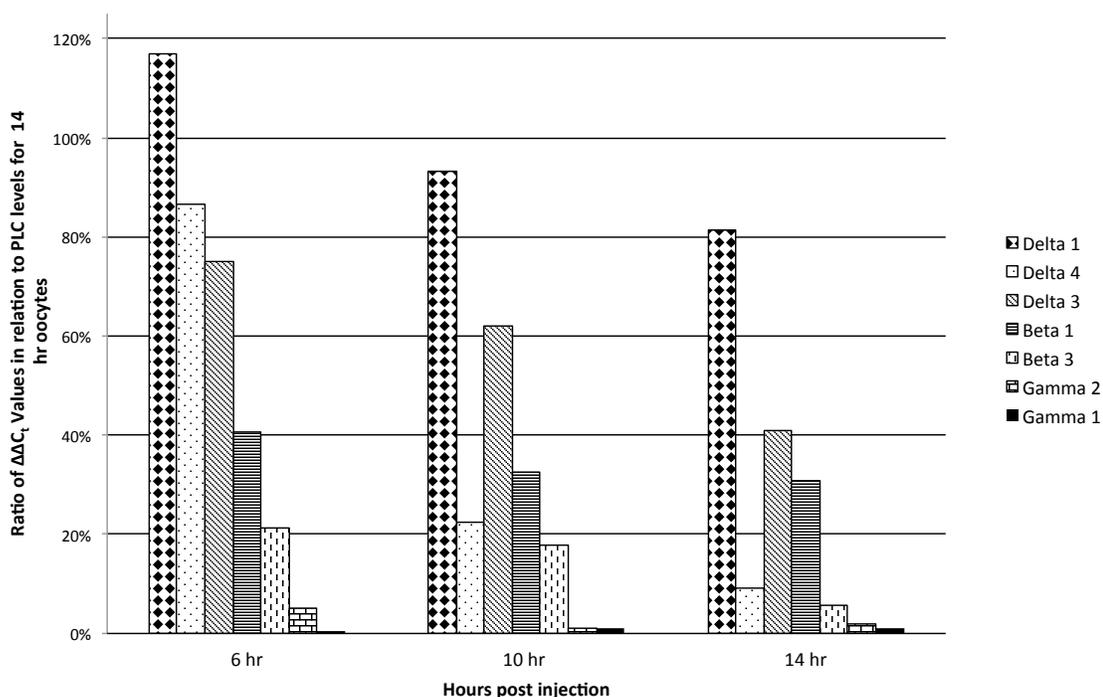


FIG 4-1. Relative PLC mRNA Expression Levels as Compared to 14 hr IVM control oocytes. Values represent the mean (n=3).

Effect PLC siRNA on *In Vitro* Fertilization in Bovine Oocytes

The oocytes microinjected with PLC ζ , PLC δ 1, PLC δ 3, PLC δ 4, PLC β 1, PLC β 3 siRNA resulted in cleavage rates similar to the negative control siRNA, non-injected, and sham injected treatment groups while bovine oocytes microinjected with PLC γ 1 and PLC γ 2 siRNA had significantly lower cleavage rates ($p < 0.05$) compared to the controls (Fig. 4-2).

Effect of PLC cRNA on Parthenogenetic Development Rates in Bovine Oocytes

The oocytes microinjected with PLC ζ and PLC γ 2 cRNA resulted in a significantly higher number of oocytes ($p < 0.05$) reaching the 2 cell stage compared to all

other treatment groups and not significantly different than the Ionomycin/Cycloheximide activation control (Fig. 3-3). For clarity, only the highest cleavage rate out of the three different concentrations of cRNA microinjected for each PLC is represented in Figure 4-3 (0.1 $\mu\text{g}/\mu\text{l}$ for $\delta 1$, $\delta 4$, and ζ ; 0.5 $\mu\text{g}/\mu\text{l}$ for $\beta 3$ and $\delta 3$, and 1.0 $\mu\text{g}/\mu\text{l}$ for $\beta 1$, $\gamma 1$, and $\gamma 2$).

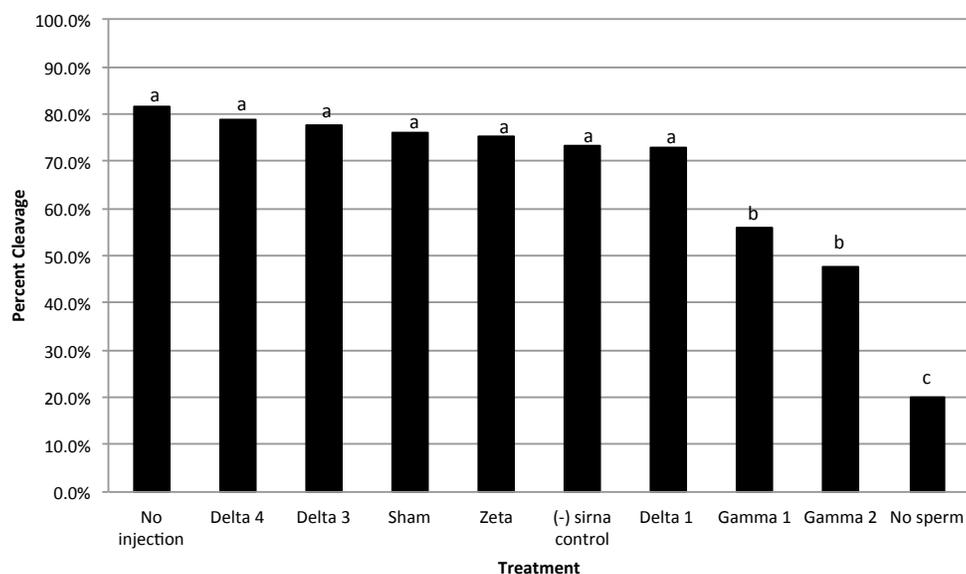


FIG. 4-2. Effect of microinjection of PLC specific siRNA's on cleavage rates of bovine oocytes after incubation with sperm for 5 hours. Values represent the mean (n=3) and unlike superscripts are statistically significant.

Effect of PLC cRNA on Intracellular Calcium Release in Bovine Oocytes

The number of oocytes responding with calcium transients after the microinjection of PLC- ζ (8/13) and PLC- $\gamma 2$ (7/12) cRNA were at rates similar to the *in vitro* fertilized controls (7/10). The activating effects of any of the cRNA's were negligible if the oocytes were microinjected with the corresponding siRNA prior to microinjection with cRNA (data not shown). These data illustrate the potential involvement of an endogenous PLC isoform and not just the sperm-specific PLC- ζ

isoform in bovine oocyte activation during fertilization.

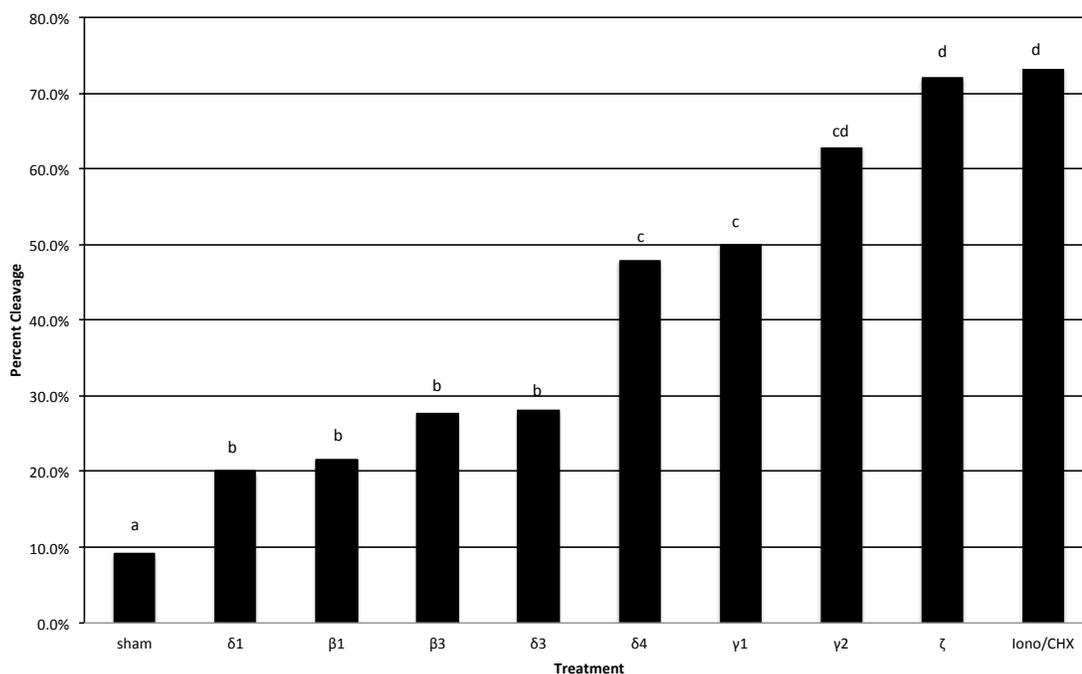


FIG 4-3. Effect of microinjection of PLC cRNA on parthenogenetic development rates in bovine oocytes. Values represent the mean (n=3) and unlike superscripts are statistically significant (p<0.05).

Discussion

Microinjection of PLC- ζ cRNA or recombinant protein into mouse oocytes results in intracellular calcium oscillations, activation and development [237, 238]. Sperm from male mice with decreased expression of PLC- ζ due to RNA interference are able to fuse with oocytes and initiate calcium oscillations; however, the number of oscillations was reduced and no transgenic offspring were born [239]. PLC- ζ was also shown to be defective with either reduced protein levels or mutated forms in men suffering infertility [240, 241].

For a spermatozoa protein to be a viable SOAF candidate, many criteria must be met: 1) Protein specific to male germ cells and more particular is only expressed in

elongating spermatids and spermatozoa; 2) Triggers cortical granule exocytosis, pronuclear development, and cleavage; 3) Ability to induce repetitive calcium oscillations; 4) Not species specific; 5) During the early stages of fertilization should be localized to the postacrosomal sheath of the spermatozoa perinuclear theca; 6) Soluble and released into cytoplasm of oocyte upon fusion [242, 243]. Unfortunately PLC- ζ fails to fulfill all of these requirements so its function as the SOAF needs to be taken into question.

PLC- ζ expression has been proven to not be testes specific, as PLC- ζ expression has been detected in mouse brain [244] in addition to the ovary and brain of the puffer fish [245]. Aarabi et al., [18] recently demonstrated that PLC- ζ is actually secreted by the epididymis as a component of the acrosome during mouse spermatogenesis. Nevertheless, a novel function of PLC- ζ might be required for spermatogenesis, as PLC- ζ knockout mice seem unable to complete spermatogenesis with spermatocytes failing to proceed beyond elongation [247], although the specificity of these effects needs to be evaluated in more detail.

By removing all sperm membranous and acrosomal components with the non-ionic detergent, Triton X-100, the SOAF has been shown to be localized to the perinuclear theca (PT) [248-250], and actually more precisely localized to the PAS-PT which is the region of the PT that is first solubilized on sperm entry into the ooplasm [242]. Consequently the SOAF should be non extractable by non-ionic detergents and localized to the PT, two requirements that the 74 kDa catalytically active form of PLC- ζ failed to meet [242].

PLC- ζ was originally believed to be localized to the part of the spermatozoa head

the that first enters the egg [251]; however, it was recently shown in mice and humans that PLC- ζ disappears from the sperm head when it fuses with the oocyte plasma membrane and subsequently incorporation into the oocyte [242]. Reports also suggest that PLC- ζ undergoes dynamic changes in its pattern of localization in the spermatozoa of mice [252], hamster [252], and humans [253]. For example, it was demonstrated that PLC- ζ displays variable localizations in the sperm head, including the equatorial segment and the post-acrosomal regions, and it was suggested that these distinct localizations may reflect a different functional status of the sperm, i.e. capacitation and acrosome reaction, and that PLC- ζ may be involved in some of these physiological steps. However, to date, there is not supporting data to incriminate PLC- ζ in these steps.

Microinjection of PLC- ζ cRNA into oocytes to overexpress PLC- ζ protein is a common technique used to illustrate the ability of PLC- ζ to induce fertilization-like calcium oscillations and oocyte activation. There is an inherent flaw with this technique in that non-physiological amounts of protein are expressed, this makes it difficult to conclude that this response is physiologic. Therefore, an active purified recombinant form of the protein that could be microinjected at physiologic concentrations would be required to ascertain whether the effect is physiologic [271]. Igarashi et al. [32] found that microinjecting PLC- β 1 cRNA into mouse eggs significantly altered the sperm-induced oscillations and the authors wondered that if PLC- ζ was the sole activating factor, how could an oocyte specific PLC affect the pattern. In this study we found that the microinjection of cRNA for any of the PLC enzymes endogenous to a bovine oocyte resulted in some embryonic development, albeit at low rates, but the physiological relevance of the technique of cRNA injection might be questioned as a result.

Microinjection of cRNA for PLC- γ 2 had similar results to the microinjection of cRNA for PLC- ζ , so this provides evidence that a PLC endogenous to the oocyte might also be involved in initiation of calcium oscillations.

The microinjection of PLC-specific siRNA's also implicated the involvement of PLC- γ 2 and PLC- γ 1 with both resulting in significantly lower cleavage rates compared to the IVF control and all of the other PLC's. Interestingly, siRNA specific for PLC ζ failed to reduce cleavage when treated bovine oocytes were fertilized. PLC ζ RNA has been found in ejaculated spermatozoa of mice and humans, and has been proposed to potentially contribute to the overall pattern of intracellular calcium oscillations [271]. If this is also the case in the bovine model, inactivation of spermatozoa introduced PLC ζ RNA by preloading the oocyte with PLC ζ specific siRNA should have a negative impact on cleavage and intracellular calcium release; however, this did not occur.

The retrospective studies involving men with globozoospermia [240, 241] concluded their infertility was due to reduced or defective PLC- ζ protein levels. As stated in Aarabi et al. [18] many other sperm proteins are also affected or absent in these patients, so in this case there is no direct experimental evidence indicating the reduced levels or mutated forms of PLC- ζ are the actual cause of the infertility. Association does not mean causation.

Studies that used transgenic RNAi methods to decrease the amount of PLC- ζ in male mice, concluded that since no transgenic offspring were found, none of the PLC- ζ deficient sperm were able to activate oocytes [239]. In light of the recent findings that PLC- ζ might play a role in the acrosome reaction and is absent from the sperm head at fertilization, it is possible to conclude that no transgenic offspring were born because

none of the PLC- ζ deficient sperm were able to undergo the acrosome reaction.

Intracytoplasmic sperm injection (ICSI) in humans has proven to be a very successful technology for overcoming infertility issues; however, the application of this technology in domestic livestock species has proven to be challenging. The success of ICSI in cattle is very low and typically requires an additional chemical activation step for improved development [272, 273]. If spermatozoa do indeed contain the SOAF, why does ICSI not result in activation and embryonic development in the bovine model? It is clear that ICSI and bypassing the gamete membrane interaction in the bovine model fails to activate the oocyte and that spermatozoa-oocyte binding and fusion is requisite for activation.

These data illustrate the potential involvement of multiple endogenous PLC isoforms, in particular PLC γ 1 and PLC γ 2, and not just the sperm-specific PLC ζ isoform in bovine oocyte activation during fertilization. Knocking down the mRNA levels of PLC γ 1 and PLC γ 2 had the greatest impact on reducing cleavage rates while overexpressing PLC ζ and PLC γ 2 resulted in the highest parthenogenetic activation rate. Embryonic development and calcium transients were observed in oocytes microinjected with a variety of the cRNA's even though the knockdown of mRNA levels for these same PLC's did not have an impact on fertilization. These findings highlight the difficulty of interpreting the results of protein over expression experiments and whether the response is physiological. That being said, however, the combined results of mRNA knockdown and protein overexpression of PLC γ 1 and PLC γ 2 reinforce each other and indicate the involvement of these two PLC isoforms endogenous to the oocyte in bovine fertilization and oocyte activation.

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CHAPTER 5

SUMMARY

Mammalian fertilization is an extremely complex series of events and has garnered the interest of numerous diverse research groups for many years. Much has been learned, but there is still much more remaining to be discovered pertaining to the molecules and mechanisms involved in fertilization. There is some conservation to an extent in the molecules and mechanisms of fertilization, but there is an inherent danger in making far reaching generalizations between phyla as diverse as echinoderms and mammals or between species as similar as murine and bovine. However, regardless of the animal system, the questions remain the same. What are the carbohydrates and glycoproteins involved in zona binding and penetration? What spermatozoa ligands are interacting with what receptors on the oocyte vitelline membrane during membrane binding and fusion? What are the initiating event(s) and pathways involved in eliciting the calcium release from the oocyte's intracellular stores resulting in embryonic activation? I believe each species is unique requiring individual attention to get to the heart of oocyte activation.

As I stated earlier it seems that for every experiment implicating the involvement of a spermatozoa or oocyte membrane protein, there is another experiment or set of experiments proving why the previously implicated protein is no longer "the" candidate or essential for binding or fusion or oocyte activation. Why does there have to be just "one" essential player and why is it so far fetched that multiple proteins be involved? Due to the complexity of life it is practically impossible to eliminate all of the other confounding variables so that we can get to the basic question of whether a single

protein is involved and essential or non-essential and not involved.

The experiments in this dissertation confirm that integrins located on the vitelline membrane of bovine oocytes are involved in mediating the sperm-oocyte interaction. Anti-integrin function blocking antibodies and immunofluorescence revealed that the αV and $\beta 1$ integrin subunits are involved with some aspect of fertilization in the bovine model and could form the integrin heterodimer involved in the sperm-oocyte interaction.

Integrins associate and interact with cytoskeletal proteins through the cytoplasmic domain of the β subunit and aggregate as focal adhesion sites. Focal adhesion kinase (FAK) is localized to these focal adhesions and is a key component of signal transduction pathways mediated by integrins. Since integrins are involved in bovine fertilization, elucidating the downstream signaling molecules would be a logical step. The presence of FAK in bovine oocytes was verified by real time polymerase chain reaction and immunoprecipitation and the localization of focal adhesion kinase at the site of sperm binding to the oocyte plasma membrane was verified using immunohistochemistry. The inhibition of FAK resulted in fewer cleaved embryos in addition to a reduction in the number of oocytes responding with calcium transients; therefore we concluded that FAK is also involved in the intracellular signaling cascades resulting from sperm binding and fusion.

Phospholipase C isoforms regulate the release of calcium from the endoplasmic reticulum and are known to interact with integrins and focal adhesion kinase. The experiments reported in this dissertation explored the involvement of PLC isoforms endogenous to the oocyte in mediating the calcium release associated with fertilization. Reduction in PLC mRNA levels for the phospholipase C isoforms $\gamma 1$ and $\gamma 2$ resulted in

significantly lower cleavage rates compared to the controls. Maximizing protein levels for the phospholipase C isoforms ζ and $\gamma 2$ resulted in a significantly higher number of oocytes reaching the 2 cell stage compared to all other treatment groups and not significantly different than the activation control. The microinjection of PLC-specific siRNA's also implicated the involvement of PLC- $\gamma 2$ and PLC- $\gamma 1$ with both resulting in significantly lower cleavage rates compared to the IVF control and all of the other PLC's. Interestingly, siRNA specific for PLC ζ failed to reduce cleavage when treated bovine oocytes were fertilized. These data illustrate the potential involvement of an endogenous PLC isoform and not just the sperm-specific PLC- ζ isoform in bovine oocyte activation during fertilization.

PLC- ζ has been proposed to be the testis-specific, sperm borne activating factor (SOAF) that initiates oocyte activation after gamete fusion; however, PLC- ζ fails to fulfill all of the SOAF requirements, so its primary function as the SOAF needs to be taken into question. PLC- ζ expression has been proven to not be testes specific, it is not localized to the region of the sperm head where the SOAF candidate resides, it undergoes dramatic relocalization during the acrosome reaction, and recently was found to target intracellular stores of calcium and not those associated with the endoplasmic reticulum [274]. I find it fascinating that every time PLC- ζ is found to contradict the well established SOAF requirements, it only gains another "novel" function instead of being eliminated as "the" activating agent. As the saying goes, "Two wrongs don't necessarily make a right."

Together these data illustrate the involvement of integrins, FAK, and PLC isoforms endogenous to the oocyte in bovine fertilization and activation. Further work

needs to be done in evaluating the other downstream signaling molecules and cascades involved in bovine oocyte activation.

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- Immunohistochemistry
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