PLASMA MEMBRANE CALCIUM ATPase 4
CO-ORDINATES CALCIUM AND NITRIC OXIDE SIGNALING
IN REGULATING MURINE SPERM MOTILITY AND FERTILITY

by

Kristine E. Olli

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Summer 2014

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Approved: _________________________________
Patricia A. Martin-DeLeon, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _________________________________
Randall L. Duncan, Ph.D.
Chair of the Department of Biological Sciences

Approved: _________________________________
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _________________________________
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education
ACKNOWLEDGMENTS

I am so thankful for my advisor, Dr. Patricia DeLeon, for her constant guidance and encouragement on my research over the past year. With her vision and intellect, I was able to be constantly motivated to complete this incredible project. I am thankful for her inspiring me with her passion for andrology. Thank you, for being more than just an advisor.

I would also like to thank the members of my committee, Dr. Deni Galileo and Dr. Donna Woulfe, for their contribution and constructive evaluation of this work. Thank you to the members of the DeLeon laboratory, especially Rachel Andrews and Amal Al-Dossary, who helped and supported me over the past year.

I want to convey my sincerest gratefulness to my family and friends for their unending faith in me over these past two years. I would especially like to thank my parents, Deann and Bill, for their limitless belief in me. I also want to thank Jeanette, Alena and Emily for providing me with infinite emotional support.
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ABSTRACT

Reduced sperm motility or asthenozoospermia is the primary cause of male infertility. In mice, asthenozoospermia leading to infertility results from a deletion of *Pmca4*, which encodes the highly conserved Plasma Membrane Calcium ATPase 4 (PMCA4), the major calcium efflux pump in murine sperm. However, to date the mechanism underlying the loss of PMCA4, which results in the motility defects, is unknown. In somatic cells PMCA4, in addition to its role in calcium efflux, is known to modulate nitric oxide (NO) signaling by negatively regulating the production of NO via nitric oxide synthases (NOS) in endothelial (eNOS) and neuronal (nNOS) cells. Both eNOS and nNOS are activated by calcium. In the absence of PMCA4 there are elevated levels of intracellular calcium and consequently increased levels of NO, leading to increased amounts of the highly reactive peroxynitrite, which reacts with polyunsaturated fatty acids. Since the sperm membrane is enriched in polyunsaturated fatty acids, it is highly susceptible to attack from peroxynitrite with resulting lipid peroxidation and ultimately a decrease in sperm motility. Therefore, in exploring the potential mechanism for the loss of fertility in *Pmca4* null mice, the goal of this study was to determine if eNOS and nNOS are interacting partners in sperm, and if the degree of interaction is calcium-dependent. Using co-localization, co-immunoprecipitation, and Fluorescence Resonance Energy Transfer (FRET) assays, we investigated the association
of PMCA4 and the NOSs in uncapacitated and capacitated (where levels of calcium are elevated) sperm. PMCA4 was shown to co-localize with eNOS and nNOS on the cell membrane of sperm. Co-immunoprecipitation assays detected an association between PMCA4 and eNOS and nNOS, with the interaction being stronger in capacitated versus uncapacitated sperm. FRET analysis showed an energy transfer efficiency of 35% in capacitated sperm versus 23% in uncapacitated sperm, revealing that PMCA4 and eNOS are less than 10 nm apart in both capacitated and uncapacitated sperm. The analysis showed an efficiency rate of 24% in capacitated and only 6% in uncapacitated sperm for PMCA4 and nNOS, revealing an intimate interaction between PMCA4 and nNOS only in capacitated sperm. These results identify eNOS and nNOS as interacting partners with PMCA4 in sperm. The data support the hypothesis that PMCA4 is closely associated with the NOSs under high concentrations of intracellular calcium. During its efflux activity, PMCA4’s association with eNOS and nNOS would expose them to a local environment of decreased calcium, therefore negatively regulating them. Our findings show that co-ordination and integration of calcium and nitric oxide signaling are involved in maintaining normal sperm motility and fertility. Analysis of Pmca4 null sperm motility assay showed that L-NAME, a known inhibitor of NOSs, decreased motility in capacitated Pmca4 null sperm from 1.15% in the untreated sample to 0.07% when treated with low levels of L-NAME and
0.15% at high levels of L-NAME. In uncapacitated sperm there is a dose 
dependent decrease in motility when treated with L-NAME (14.8%, 5.31%, and 
3.20%). In testes of Pmca4 nulls TUNEL assays revealed that the rate of 
apoptosis is significantly (p < 0.001) higher than that in wild-type, consistent 
with earlier findings of increased levels of nitric oxide synthase activity in these 
mutants. Importantly, while apoptotic activity did not differ significantly (p > 
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germ cells.
Chapter 1

INTRODUCTION

1.1 The Sperm Cell and Its Equipment for Motility

Sperm are specialized cells that are designed to accomplish a single task, that of fertilizing an oocyte. Mammalian sperm consist of a head and a tail also known as a flagellum. The entire cell is covered by a plasma membrane. Specifically, the murine sperm head has a hook-shape. Also in rodent’s sperm, there is a total lack of a sperm centrosome and centrioles. Within the sperm head is the nucleus with DNA condensing core. Protamines are responsible for the hypercondensation of the nucleus into a compact hydrodynamic structure that enables sperm motility and the ability to penetrate the oocyte. There is a reduced nuclear envelope, lacking nuclear pore complexes, encompassing the nucleus. Supplementary protection of the nucleus is from the perinuclear theca or perinuclear matrix, composed of disulfide bond-stabilized proteins to create a rigid shell.

The sperm tail, which is responsible for motility, has a unique 9+2 arrangement of microtubules, 9 peripheral, symmetrically positioned microtubule doublets linked doublet to doublet, by dynein arms, to the sheath of a central pair of microtubules by radial spokes. There are 3 specific segments within the tail. The section closest to the head is the midpiece. It is covered by the mitochondrial sheath, with a helix of roughly 75-100 mitochondria to produce energy for motility. The principal piece is
distinguished from the midpiece by the annulus, a ring of dense material, found distal to the mitochondrial sheath. Finally, the tip of the tail is the end piece. Figure 1.1 shows the morphology of murine sperm.

![Figure 1.1](image.png) 

Figure 1.1  Differential interference contrast image of murine sperm, with the morphology labeled.

During the passage through the epididymis sperm develop the ability to swim. Upon ejaculation the sperm mix with the seminal plasma, gaining sugars, among other macromolecules, that are key sources of ATP, which provide energy to enable them to swim for an extended period of time. (Jonge & Barratt, 2006)

### 1.2 Nitric Oxide, Oxidative Stress, & Reduced Sperm Motility

Studies show that non-physiological levels, both low and high, of nitric oxide (NO) lead to a decrease in sperm motility, thus suggesting that NO is one of the causes of asthenozoospermia (Nobunaga et al., 1996). Asthenozoospermia is a condition where sperm are non-motile or have reduced motility, but the concentration and morphology are normal (Yun, Park, Song, & Lee, 2008). When sperm are exposed to elevated levels of NO their respiration is impacted. Studies have shown that elevated levels of NO decrease the amount of ATP in sperm by preventing the ATP-generating
ability of the enzymes required for ATP synthesis (Weinberg, Doty, Bonaventura, & Haney, 1995). While sperm are terminally differentiated and are unable to proliferate they still require large quantities of ATP for motility (Mitchell, Nelson, & Hafez, 1976).

Men with asthenozoospermia have lower levels of NO produced by their sperm and ultimately a decrease in sperm motility (Lewis et al., 1996). Nitric Oxide Synthases release NO from a guanidine nitrogen from L-arginine in the presence of oxygen with the help of nicotinic acid adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), or flavin mononucleotide (FMN) with L-citrulline as a by-product (Sobolewska-Stawiarz et al., 2014); (Donnelly, Lewis, Thompson, & Chakravarthy, 1997). A primary effector of NO is peroxynitrite (OONO⁻), which is produced when NO combines with superoxide (O₂⁻). OONO⁻ plays a physiological role in sperm capacitation and hyperactivated sperm motility (described in more detail in 1.3 Capacitation, Hyperactivation, the Acrosome Reaction and Nitric Oxide).

Elevated levels of NO lead to elevated levels of OONO⁻, a highly Reactive Oxygen Species (ROS), which cause molecular damage in the cell (Jonge & Barratt, 2006); (Radi, Beckman, Bush, & Freeman, 1991). Sperm are highly susceptible to oxidative stress because the plasma membrane is rich in polyunsaturated fatty acids and sperm normally produce ROS. They are especially susceptible to lipid peroxidation since the removal of hydrogen in the plasma membrane is accelerated by the presence of double bonds in unsaturated fatty acids. Hence, there is lipid peroxidation of the membrane. It has been shown in human sperm that after introducing extracellular ROS there
was lipid peroxidation and a directly linked loss of sperm motility (Gomez, Irvine, & Aitken, 1998). In addition to lipid peroxidation of the plasma membrane, sperm are also susceptible to collateral DNA damage resulting from ROS (Bianco et al., 2013).

When lipid peroxidation occurs sperm are not able to restore the membrane to its original state because they lack the cytoplasmic organelles and catalase necessary to defend against the toxic effects of ROS (Sharma & Agarwal, 1996). Consistent with this finding, data show that in the presence of oxygenated medium, sperm rapidly lost motility. The mitochondria in sperm increase the production of ROS, once the cell begins to undergo apoptosis, in a response to the presence of free unsaturated fatty acids. With increasing unsaturation, the mitochondria generate more ROS (Aitken, Smith, Jobling, Baker, & De Iuliis, 2014). This elevated level of NO is also known to cause apoptosis of the germ cells (Taneli et al., 2005). There are data showing cell shrinkage, nuclear condensation, and membrane blebbing suggesting that elevated levels of NO play a key role in the apoptosis of germ cells (Marmar, 2001). It has been shown that oxidative stress once initiated is self-activating and will eventually lead to oxidative damage, a loss of function, and finally apoptosis of the sperm cells (Aitken, et al., 2014). However, despite the potential damaging effects of elevated levels of NO, physiological levels are required in order for sperm to become fully mature and to be functional.
1.3 Capacitation, Hyperactivation, the Acrosome Reaction and Nitric Oxide

Austin who stated that the sperm must undergo a physiological change before it is able to penetrate the oocyte, first introduced the term capacitation in 1952 (Austin, 1952). This term encompasses all the changes that the sperm undergoes in preparation for penetrating the oocyte. Capacitation is regulated by the oxidative properties of ROS, including NO (Leclerc, deLamirande, & Gagnon, 1997). ROS are necessary for the stimulation of NADPH and for cAMP to be involved in the process of capacitation. It has been shown that NO impacts sperm motility by accelerating the capacitation process. In order for sperm to have the capability of fertilizing oocytes at the appropriate time, it is necessary for them to undergo a process in the female reproductive tract known as capacitation. There are two main changes that the sperm undergoes during capacitation: hyperactivation and the acrosome reaction (AR).

Calcium plays a vital role in both phases of capacitation. Early research showed evidence for the presence of a calmodulin-stimulated calcium ATPase in sperm that resembles an enzyme in somatic cells (Adeoya-Osiguwa & Fraser, 1996). Calcium is also required for hyperactivation (Herrero, de Lamirande, & Gagnon, 1999). Hyperactivation results in altered flagellar beating and is necessary for the sperm to reach the oocyte and to penetrate the multiple layers around the oocyte in order to effect fertilization in vivo. Post-hyperactivation, the sperm is able to generate increased forces in response to the viscous resistance present in the female reproductive tract. This gives the sperm a mechanistic advantage to successfully fertilize the oocyte (Suarez, Katz, Owen, Andrew, & Powell, 1991). Freshly ejaculated
murine sperm have a low amplitude and a symmetric beating pattern, however once hyperactivation has occurred the sperm have increased amplitude with a lower frequency of beating (deLamirande, Leclerc, & Gagnon, 1997). In a low viscosity environment, in vitro, hamster sperm that have undergone hyperactviation have asymmetrical beating with a large amplitude and curvature. Conversely, hamster sperm that have been taken from fresh ejaculate move with a more symmetrical beat going in a straight path. Therefore, the event of hyperactivation enables the sperm tail to produce increased forces when in a highly viscous environment (Suarez, et al., 1991). The hyperactivated motility seen at the site of fertilization is non-progressive, vigorous, and, frantic with an increase in the velocity and a decrease in lateral head movement (deLamirande, et al., 1997). This type of motility is necessary to free the sperm from the crevasses in the oviductal lining (Coy, Garcia-Vazquez, Visconti, & Aviles, 2012).
The use of the L Nitroarginine Methyl Ester (L-NAME), a nitric oxide synthase inhibitor, was employed to determine the importance of NO in capacitation (Herrero, et al., 1999). While it has been shown that physiological levels of NO are necessary for sperm to undergo capacitation, if levels
become elevated there is a decrease in sperm motility (Zini, Delamirande, & Gagnon, 1995). At the toxic levels, NO decreases the progressive movement of sperm in humans (Rosselli, Dubey, Imthurn, Macas, & Keller, 1995). The nitric oxide inhibitors, L-NAME (Herrero, et al., 1999) and hemoglobin (Donnelly, et al., 1997), are able to quench the nitric oxide.

An increase in the level of calcium is required for the docking of the acrosome during the AR. The AR inducers progesterone, calcium ionophore A23187, and Epigenetic Growth Factor (EGF) allow for the introduction of calcium at the inter-membrane space and the docking of the acrosome to the apical plasma membrane (Gadella & Luna, 2014). It has been shown in mammals that the AR involves multiple fusions and vesiculation of the plasma membrane of the sperm head and the underlying outer acrosomal membrane. This enables vesicles containing lytic and adhesion molecules to be discharged and the exposure of the inner acrosomal membrane (Wolf, et al., 1986); (Chang, 1984). The AR is required for the sperm to be able to penetrate the zona pellucida of the oocyte (Leclerc, et al., 1997).

Capacitation allows the sperm to have a heightened sensitivity toward calcium signaling. This change can be seen by a decrease in the cholesterol content within the sperm plasma membrane, the inhibition of membrane ATPase activity, a slight increase in intracellular calcium levels, and a rise in the intracellular level of cAMP. The concentration of calcium increases in the flagellum during hyperactivation and in the head during the AR (deLamirande, et al., 1997), after entering the sperm via CatSper channel on the proximal principal piece (Brenker et al., 2012; deLamirande, et al., 1997).
Capacitation and the accompanying AR take place in the female genital tract, however they can be stimulated in vitro by specific media, such as HTF (Human Tubal Fluid). One of the artificial stimuli of the AR is the calcium ionophore A23187 (deLamirande, et al., 1997). Other natural stimuli include follicular fluid, progesterone, and the zona glycoprotein ZP3. All of these allow for the necessary rapid calcium uptake into the acrosomal region of the sperm head. Capacitation includes the removal of the glycoprotein coat from the sperm cell, thus exposing the AR. The acrosome reaction then allows the release of hydrolytic enzymes in order for the sperm to penetrate the zona pelucida (Chang, 1984).

In addition to chemical factors, sperm must be exposed to specific oxygen levels and temperatures within the oviduct in order to undergo capacitation. Important changes occur at the membrane level during capacitation. At the start of capacitation there is a rapid disorganization of lipids. In the membranes of the acrosome region, midpiece, and flagellum there is an increase in the rate of diffusion after capacitation (deLamirande, et al., 1997).

1.4 Plasma Membrane Calcium ATPase 4

After the influx of calcium required for capacitation and AR, there are mechanisms to restore calcium to resting levels. The major calcium efflux pump in murine sperm is Plasma Membrane Calcium ATPase 4 (PMCA4), a P-type ATPase (Wilhelm, Brandenburger, Post, & Aumuller, 2008). In order to function, these proteins require ATP to pump ions with a single catalytic
subunit and mid-cycle there is a phosphorylated reaction. PMCAs in animal cells are activated by calmodulin (Palmgren & Axelsen, 1998).

PMCA4 is a 10-pass transmembrane protein and is ubiquitously expressed in eukaryotic cells (Wilhelm, et al., 2008); (Guerini, Garcia-Martin, Zecca, Guidi, & Carafoli, 1998). It has a molecular mass between approximately 125 and 140 kDa (Strehler, Filoteo, Penniston, & Caride, 2007).
Figure 1.3 A schematic representation of PMCA4 showing the 10-pass transmembrane protein and indicating the N-terminus, both of the cytoplasmic loops and the C-terminus (adapted from (Axelsen & Palmgren, 1998)).
The role of PMCA family members is to control the movement of calcium from the cell in order to maintain homeostasis of calcium within the cell and to create a calcium gradient across the plasma membrane. As seen in Figure 1.4, there are two splice variants of PMCA4, PMCA4a and PMCA4b, distinguished by the length and sequence of the C-terminal tail where PMCA4b has a PDZ ligand. The inactivation of PMCA4b is slower than that of PMCA4a, indicating that PMCA4b is involved with slow changes in calcium (Strehler, et al., 2007). PMCA4b is known to be involved in sperm signal transduction and the DeLeon Lab has shown that in murine sperm it interacts with Calcium-dependent Serine Kinase (CASK) (Aravindan et al., 2012).

![Figure 1.4](image)

Figure 1.4  A schematic of the alternative splicing that results in the isoforms PMCA4a and PMCA4b. The linear model at the top shows the 10 transmembrane regions as well as both the C- and N-terminal ends. (Adapted from (Patel, et al., 2013))
PMCA4 is predominantly present on the principal piece on the tail of the sperm and on the acrosome (Prasad, Okunade, Liu, Paul, & Shull, 2007). This pump is present in very low quantities consisting of approximately 0.1-0.3% of the total membrane protein (Wilhelm, et al., 2008); (Knauf, Proverbi.F, & Hoffman, 1974).

*Pmca4* null mutants have no histological abnormalities and are able to grow and develop to adulthood normally (Okunade et al., 2004); (Schuh et al., 2004). However, *Pmca4* null male mice are infertile, although they exhibited normal spermatogenesis and mating behavior. The sperm were unable to achieve hyperactivation and were therefore unable to penetrate the oocyte due to a lack of progressive and hyperactivated motility (Wilhelm, et al., 2008).

Studies performed on the sperm showed that there was a surge in mitochondrial condensation indicating that there was an excess of calcium within the cell due to the absence of the pump. The mitochondria were swollen, contained dense floccular inclusions and were repositioned closer to the membrane (Okunade, et al., 2004). Very few of the sperm were able to travel successfully within the female reproductive tract.

Before capacitation, the *Pmca4* null murine sperm are similar to wild-type murine sperm; once capacitated, the *Pmca4* null sperm were unable to be hyperactivated (Jonge & Barratt, 2006). A motility test of the *Pmca4* null murine sperm showed that the average path velocity, progressive velocity, and track speed were all decreased (Schuh, et al., 2004).

In the DeLeon Lab, studies show that PMCA4 is present in the epididymal luminal fluid (ELF). The ELF contains important molecular
components that aid in the 5-10 day maturation process of epididymal sperm (Patel, et al., 2013); while in the female reproductive tract sperm are able to acquire PMCA4 via oviductosomes (Al-Dossary, Strehler, & Martin-DeLeon, 2013; Patel, et al., 2013). Both isoforms of PMCA4 are expressed in tissues collected from all three regions of the female reproductive tract during estrus. This may aid in capacitation of sperm in the female tract (Al-Dossary, et al., 2013). When the pump was absent from cells, in addition to the lack of motility in sperm, it was shown to cause apoptosis in somatic cells (Okunade, et al., 2004). PMCA4 is known to interact with other proteins in somatic cells, notably among these proteins are Nitric Oxide Synthases (Cartwright, Oceandy, & Neyses, 2009; Schuh, Uldrijan, Telkamp, Rothlein, & Neyses, 2001), which are known to be present in human sperm (Zini, Obryan, Magid, & Schlegel, 1996).

1.5 Nitric Oxide Synthases and Their Regulation by PMCA4

Nitric Oxide Synthases (NOS) are heme-containing enzymes that catalyze reactions including a hydroxylation reaction and NADPH reduction. All types of NOS have a bidomain structure with an N-terminal oxygenase domain that contains a heme group and a tetrahydrobiopterin that can bind to L-arginine and a C-terminal reductase domain that can bind to FAD, FMN, and NADPH (Venema, Ju, Zou, Ryan, & Venema, 1997). There are two constitutive variants based on the location of which they were first identified; neuronal NOS (nNOS) found in the nervous system and endothelial NOS (eNOS).
However, these enzymes have been found outside of these specific systems (Herrero & Gagnon, 2001). NOS activity has been shown in mammalian testes, epididymides, prostates, and seminal vesicles and is activated by calmodulin and calcium (O'Bryan, Zini, Cheng, & Schlegel, 1998); (Herrero, et al., 1999).

Surprisingly, both eNOS and nNOS have been observed in both mouse and human sperm, although in the mouse they have not been localized (Herrero, Martinez, Viggiano, Polak, & deGimeno, 1996; O'Bryan, et al., 1998). When NOS was stained in humans, it was localized to the head and tail with the most intense fluorescence being just below the midpiece. After capacitation the presence of NOS in the head decreases, indicating NOS playing a role in capacitation (Lewis, et al., 1996); (Venema, et al., 1997); (Herrero, et al., 1996). According to the Santa Cruz Biotechnology Incorporated fact sheet for anti-eNOS antibody, eNOS has a molecular mass of approximately ~140 kDa, although others have reported a molecular weight of 130 kDa (Zini, et al., 1996). Staining for eNOS in human sperm showed that abnormal ones generate an excess of NO compared to the normal counterpart, which generate regulated amounts of NO (O'Bryan, et al., 1998).

1.6 Interaction of Plasma Membrane Calcium ATPase 4 and Membrane Proteins

PMCA4 is known to regulate calcium within the cell and regulate signal transduction networks. The NH₂ terminus of nNOS contains a PDZ domain and PMCA4 is known to bind to proteins at their PDZ domain via a ligand at the COOH- terminal region (Kone, Kunczewicz, Zhang, & Yu, 2003). By way of
immunoprecipitation, it was shown in somatic cells that PMCA4 and nNOS are interacting partners (Schuh, et al., 2001). This showed that PMCA4 is able to regulate the production of NO by controlling the calcium necessary to activate nNOS.

Studies have shown that in murine sperm PMCA4b interacts with Calcium/CAM-dependent Serine Kinase (CASK), which is located on the principal piece of the tail at the PDZ domain (Aravindan, et al., 2012). Co-immunoprecipitations showed that both PMCA4a and PMCA4b can interact with CASK, suggesting that a heterodimer of 4b and 4a is involved in the interaction since 4a does not have a PDZ ligand and cannot bind to CASK (Patel, et al., 2013). This interaction of CASK and PMCA4 has been shown to be dependent on the level of calcium within the cell; in uncapacitated sperm the interaction is more intense than in capacitated sperm (Aravindan, et al., 2012).

Unpublished pilot data in the DeLeon Lab suggests that at low and even more at high calcium concentrations NOS activity is increased in Pmca4 null sperm. Preliminary data also show that there is more OONO' in Pmca4 null murine sperm compared to wild-type. Furthermore, capacitated sperm have a higher concentration of OONO' than uncapacitated sperm in both wild-type and nulls. A model was predicted, based on these data, as seen in Figure 1.5. This shows that in the uncapacitated state PMCA4 is preferentially bound to CASK at its PDZ domain. However, in capacitated sperm when calcium is globally high PMCA4 binds to nNOS at its PDZ domain.
Figure 1.5  This model shows the different calcium environments of sperm and their impact on the production of NO. a) When there are globally low levels of calcium (uncapacitated state) CASK is bound to PMCA4 and minimal NO is produced. b) Globally high concentrations of calcium (capacitated) involves the interaction of PMCA4 with both eNOS and nNOS and NO is being produced because the calcium is available to activate the NOS enzymes. Because the NOSs are tethered to PMCA4, which is extruding calcium, they are held in a microenvironment of decreased cytosolic calcium levels and thus their output of NO is regulated. c) When PMCA4 is absent, there is a globally high environment of calcium causing the NOS enzymes to produce elevated levels of NO in the absence of their regulation.
Based on the evidence from the preliminary data described above, the following hypothesis was proposed (advanced):

**HYPOTHESIS**

In sperm, PMCA4 co-localizes with eNOS and nNOS and negatively regulates them to modulate the production of nitric oxide, thereby regulating the motility of sperm and ultimately fertility.

**AIMS**

1. Use co-immunoprecipitation in order to determine if eNOS and nNOS are interacting partners with PMCA4 and if the interaction levels vary based on the level of cytosolic calcium concentration.

2. Use Fluorescence Resonance Energy Transfer (FRET) in order to determine if eNOS, and nNOS as well as CASK are within 10 nm of PMCA4 depending on intracellular calcium levels.

3. Determine a) the impact of a synthase inhibitor on sperm motility in *Pmca4* knockout (KO) sperm and b) investigate the presence of a germ cell apoptosis in the testes of KO males.
Chapter 2
MATERIALS AND METHODS

2.1 Animals and Reagents

Sperm samples were procured from the cauda epididymides of sexually mature (>3 months old) mice of the C57BL/6, FVBN or ICR backgrounds. *Pmca4* KO males on the FVBN background were generated from the matings of *Pmca4* +/- donated by the Shull laboratory (Okunade, et al., 2004). Testes were collected and immediately frozen in Optimal Cutting Temperature (O.C.T. medium). The studies performed were approved by the Animal Care Committee at the University of Delaware and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health.

All chemicals were purchased from Fisher Scientific Company (Malvern, PA), Santa Cruz Biotechnology Incorporated (Dallas, TX), BioRad Laboratories Incorporated (Hercules, CA), and Millipore Corporation (Billerica, MA) unless otherwise stated.

2.2 General Techniques

2.2.1 Sperm Collection

Sexually mature mice were sacrificed and the cauda epididymal tissue was collected in HTF HEPES with EDTA and Glutamine (HTF medium)
The tissue was then minced and incubated at 37°C for 10 min. The sperm suspensions were separated from the tissue by gravity settling and sperm were recovered by centrifugation 500 x g for 20 min. In order to capacitate samples, the sperm were incubated for 90-120 min in HTF medium at 37°C. The samples were then centrifuged at 500 x g for 15-20 min.

2.2.2 Protein Extraction

The cells were homogenized in 300 µL of either Homogenization Buffer (HB) or 1x RIPA buffer with Protease Inhibitor (PI) (Sigma Aldrich, Cat. No. P2714-1BTL) and rotated overnight at 4°C. The suspension was then centrifuged at 14,000 x g for 5 min, and the protein extract was collected. The protein concentration was then determined using bicinchoninic acid (BCA) assay kit (Pierce, Cat. No. 23225) and spectrophotometry.

2.2.3 Co-Immunoprecipitation (Co-IP)

Fifty microliters of PureProteome Protein G Magnetic Beads (Cat. No. LSKMAGG10) were washed with 1 mL 1x PBS + 0.01% Tween 20 by vortexing and centrifuging at 5,000 x g for 3 min twice. Then, 100 µL 1x PBS (NaCl, KCl, Na₂HPO₄, KH₂PO₄, pH 7.4) with 2 µL of the specific antibody (anti-pPMCA4, anti-eNOS, or anti-nNOS) was added to the beads for 2 h on a rotator at 4°C. The beads were centrifuged at 5,000 x g for 3 min and the supernatant was discarded. Next, 125 µg of protein extract was added to the beads in a total volume of 500 µL of Immunoprecipitation buffer (25 mM Tris, 150 mM NaCl, pH 7.2) and PI, and the samples were rotated overnight at 4°C.
The samples were centrifuged at 5,000 x g for 3 min and the supernatant was collected for SDS-PAGE.

2.2.4 SDS-PAGE and Western Blot Analysis

Western blotting was performed to detect an interaction between nNOS and pPMCA4 and eNOS and PMCA4 in the protein extracts from sperm. All but 20 µL of the IP buffer/PI with protein extract solution was discarded, and was then subjected to the reducing conditions of 100°C for 5 min in the presence of 5x sample buffer (1.5 M Tris pH 8.8, 3.3% Glycerol, 1% SDS, 1% β-Mercaptoethanol, 0.5% Bromophenol Blue). 25 µL of sample was loaded into an 8% agarose gel and separated. Protein was then transferred to nitrocellulose membrane according to standard protocols.

Membranes were probed with either rabbit polyclonal antibodies specific to nNOS (NOS1 (R-20): sc-648) at a 1:500 dilution or eNOS (NOS3 (C-20): sc-654) at a 1:250 dilution or goat polyclonal antibody specific to PMCA4 (PMCA4 (Y-20): sc-22080) at a dilution of 1:500 at 4°C for 24-48 h. Membranes were then washed three times with 1x TBST (NaCl, KCl, Tris Base, Tween 20 pH 8) for 10 min.

Membranes probed with anti-PMCA4 were then probed with 1:5000 biotin donkey anti-goat (Jackson, Cat. No. 705-475-147) for 30 min at RT. Next, the membranes were washed 3 times with 1x TBST for 10 min at RT. A dilution of 1:5000 Streptavidin HorseRadish Peroxidase (SA-HRP) (Jackson, Cat. No. 016-030-084) was added to the membrane for 30 min at RT. Then, the membrane was then washed 3 times with 1x TBST at RT for 5 min followed by double distilled water twice for 5 min at RT. In the dark, Immobilon
Western Chemiluminescent HRP Substrate kit (Cat. No. WBKLS0050) was added to the membrane for 1 min at RT. The membranes were then exposed on Classic X-Ray Film for autoradiography and Chemiluminescence (RPI, Cat No. 248300) and developed using a photograph processor.

Membranes probed with anti-eNOS or anti-nNOS were first washed 3 times with 1x TBST for 10 min at RT, then either probed with Secondary Antibody Solution Alkaline Phosphatase (AK) conjugated anti-rabbit (Part no. 46-7007) or with Multilink (Biogenex, Cat. No. HK268-UKE). If they were probed with AK conjugated anti-rabbit IgG, they were exposed for either 30 min at RT or for 2 h at 4°C. Next, the membranes were washed 5 times with 1x TBST for 15 min at RT. Then, the membranes were rinsed twice with double distilled water for 5 min at RT. In the dark, 3 mL of Immuno-Star AP substrate (Cat. No. 170-5018) was combined with 150 µL of Immuno-Star Enhancer (Cat. No. 170-5019) and added to the membranes for 5 min. Next, the membranes were exposed on Classic X-Ray Film and developed using a photograph processor. If they were probed with Multilink, they were exposed for 30 min at RT. Next, the membranes were then washed 3 times with 1x TBST for 10 min at RT. A dilution of 1:5000 SA-HRP was added to the membrane for 30 min at RT. Then, the membrane was washed 3 times with 1x TBST RT for 5 minutes and then double distilled water twice for 5 min at RT. In the dark, Immobilon Western Chemiluminescent HRP Substrate kit was added to the membrane for 1 min at RT. The membranes were then exposed on Classic X-Ray Film and developed using a photograph processor.
2.2.5 Immunocytochemistry of Proteins in Murine Sperm

Sperm were collected as described above. For capacitated samples, they were incubated in HTF medium for 90 min at 37°C. Sperm extracts were centrifuged at 500 x g and the supernatant was discarded. The pellet was re-suspended in 4% paraformaldehyde and incubated overnight at 4°C for fixation. The samples were centrifuged at 500 x g for 15 min and the supernatant was discarded. The samples were then washed twice with 1x PBS and centrifuged at 500 x g for 15 min. Next, permeabilization reagent (0.1% Triton X-100 in 1x PBS) was added to the pellet and the samples were incubated at RT for 10 min.

Afterwards they were centrifuged at 500 x g for 20 min to collect the cells. After washing in 1x PBS and they were re-suspended in blocking buffer (2% Bovine Serum Albumin in 1x PBS) washed and blocked at RT for 30 min. After blocking, the pellet was reconstituted and aliquotted into two tubes (control and experiment). Equal concentrations (1 µg/mL) of IgG (goat and rabbit or mouse) and antibody (PMCA4 and eNOS or nNOS or CASK) were added to the samples and incubated at 4°C overnight.

After the incubation, cells were collected by centrifugation and washed 3 times with 1x PBS. The secondary antibodies were centrifuged 1,000 x g for 1 min. In the dark, blocking buffer was combined with 1:500 dilution of Alex Fluor donkey anti-goat (Cat. No. A21432) and goat anti-mouse (Cat. No. sc-2010) or Alexa Fluor goat anti-rabbit (Cat. No. A11008) and incubated at RT for 30 min. The samples were then centrifuged at 500 x g for 20 min and the supernatant was discarded.
After the secondary antibodies, the samples were washed 3 times with 1x PBS and centrifuged at 500 x g for 20 min and the pellet reconstituted in 40 µL of 1x PBS. Approximately 20 µL of cell suspension were layered onto a coverslip (Microscope Cover Glass 12-542-B, 22 x 22 - 1) with 50 µL of prolong gold antifade reagent with DAPI (REF P36931) and carefully laid onto a Colorfrost Plus Microscope Slides (Cat. No. 12-550-17) and cured at 4°C for 24 h. The edges of the slide were sealed after being cured. The slides were stored at 4°C and visualized using confocal microscopy.

2.2.6 Co-localization and Fluorescence Resonance Energy Transfer (FRET)

Slides were prepared as discussed in “Immunocytochemistry of Proteins in Murine Sperm” with a few alterations. The primary antibodies were added in the following pairs, PMCA4 and CASK, PMCA4 and eNOS, and PMCA4 and nNOS. Secondary antibodies were also added in sets corresponding to the respective primary antibodies.

Slides were visualized using a C-Aprochromat 63x/1.40 oil objective with a Zeiss LSM 780 confocal microscope and analyzed using ZEN software. The pinhole was set to 109 µm and 405 nm, 488 nm, and 561 nm lasers were used to excite the fluorochromes in addition to a filter of 490-560. A specific Region Of Interest (ROI) was selected to encompass the entire sperm cell and five initial images were taken, followed by a bleaching event consisting of 40 iterations, and 15 more images taken after the bleaching event. The bleaching event bleached out the acceptor (red channel). The average of the initial images (pre-bleach) and the images after the bleaching event (post-bleach)
were calculated. A circular ROI was selected within the original ROI, and the area of the circular ROI was calculated. These values were used to calculate the FRET efficiency (Equation 1). If this value exceeded 20% the two molecules tagged are considered within 10 nm of each other (Bragdon et al., 2009).

\[
\text{FRET Efficiency} = \left( \frac{\bar{x}_{\text{post-bleach}} - \bar{x}_{\text{pre-bleach}}}{\bar{x}_{\text{post-bleach}}} \right) \times 100
\]  

(1)

Figure 2.1  A representative model of the mechanism behind FRET. A) The donor tagged molecule transfers energy to the acceptor. B) The acceptor fluorochrome is bleached out and the donor is no longer able to transfer the energy to the acceptor resulting in an increase in the emission at 519 nm.
2.2.7 Treatment of *Pmca4* Null Sperm with a Competitive Inhibitor of eNOS and nNOS

The cauda epididymes of 3 sexually mature *Pmca4* mice were collected in 1x PBS and minced at 37°C. The minced tissue and solution were incubated at 37°C for 10 min to allow sperm to be released. The sperm suspension was removed from the tissue and incubated in 500 µM (low) or 1 mM (high) L-NAME for 2 h at 37°C.

In order to capacitate the sperm, the samples were first centrifuged at 500 x g for 15 min. The pellet was reconstituted in HTF medium and incubated for 2 h at 37°C. Within each set of replicates, controls with the PBS carrier and the competitive inhibitor were included.

Following incubation, 2 µL of each sample was pipetted into a pre-warmed MicroTool 20-micron fixed depth chamber slide (Cytonix, Beltsville, MD) and the motility was recorded via a video camera (AmScope MU500) using the 10x objective for a period of 30 sec for 5 random field of views per animal with approximately 100-150 sperm per view. The data collected were then analyzed using ImageJ to determine the motility percentage.

2.2.8 TUNEL Assay for the Detection of Apoptosis in *Pmca4* Null Testicular Tissue

Three sexually mature WT and *Pmca4* KO males were sacrificed and the testes were harvested. The tissue was placed into Tissue-Tek Cryomold (Sakura, Torrance, CA Cat. No. 4546) with O.C.T. Compound (Sakura, Torrance, CA, Cat. No. 4583) and placed onto dry ice. The tissues were sectioned (25 µm thickness) using a cryostat.
The slides were post-fixed in pre-cooled ethanol:acetic acid (2:1) for 5 min at -20°C in a coplin jar. Next, the slides were washed twice in 1x PBS for 5 min and subjected to Terminal deoxyribonucleoptidyl transferase-mediated d-UTP Nick End Labeling (TUNEL). To the slides 13 µL of equilibration buffer was added directly on the specimen and incubated for a minimum of 10 sec at RT. The excess fluid was blotted around the tissue and 11 µL of working strength TdT (Terminal deoxynucleotidyl Transferase) enzyme was added to the tissue and incubated in a humidified chamber at 37°C for 1 h. The slides were then treated with working strength stop/wash buffer for 10 min, with the first 15 sec of the slides being agitated. Then, the slides were washed 3 times in 1x PBS for 1 min at RT. The excess liquid was removed and warm working strength anti-digoxigenin conjugate was added to the tissue and incubated in a dark humidified chamber at RT for 30 min. The slides were washed 4 times in 1x PBS for 2 min at RT. A counter-stain of fluorescein was added with 15 µL of DAPI and a 22 x 50 mm coverslip was placed onto the slide. The slides were imaged using a Plan-Aprochromat 20x/0.75 TILE objective and a Zeiss LSM 780 confocal microscope. The pinhole was set to 77 µm and the lasers were 405 nm and 488 nm with a filter of 415-490. The slides were stored at -20°C. The number of apoptotic cells in 100 tubules and their locations were blindly recorded and analyzed for each group.

2.2.9 Statistical Analysis

Results were statistically analyzed using ANOVA and t-tests.
3.1 FRET Analysis of PMCA4 and CASK

Following immunofluorescence assays of fixed samples, confocal microscopic images of sperm were taken and analyzed using Zen software. Figure 3.1 shows an example of uncapacitated sperm and Figure 3.2 is one of the capacitated sperm. The intensities of the images before and after bleaching were calculated at specific regions of interest. Figure 3.3 shows that the pre- and post-bleaching intensities were similar for before and after bleaching for the two groups. When intensities were compared between capacitated and uncapacitated, the latter was significantly greater ($p < 0.01$), indicating that there were more molecules interacting (using ANOVA).

However, the FRET efficiencies were 3.0% in capacitated sperm and 2.6% in uncapacitated sperm. These calculated values for both sets of treatments fall below the 20% threshold FRET efficiency value required to establish interactions (Bragdon, et al., 2009) and therefore PMCA4 and CASK are not within 10 nm of one another in both capacitated and uncapacitated sperm.
Figure 3.1  PMCA4 and CASK are not within 10 nm in uncapacitated sperm
Representative image of the interaction between PMCA4 and CASK before (a) and after (b) a bleaching event, indicating that there are no regions of interest where PMCA4 transfers energy to CASK.

Figure 3.2  PMCA4 and CASK are not within 10 nm in capacitated sperm
Confocal image of the interaction between PMCA4 and CASK before (a) and after (b) the bleaching event indicating that there are relatively no regions of interest where PMCA4 transfers energy to CASK.
Figure 3.3  Comparative graph of the intensities of CASK in capacitated and uncapacitated sperm before and after bleaching Analysis of 10 images taken using a confocal microscope shows there is a significant increase in the number of interacting molecules of PMCA4 and CASK in uncapacitated versus capacitated sperm (p < 0.01). However, there was no energy transfer for sperm in either state as the pre- and post-bleaching intensities were almost identical.

3.2 Co-localization of PMCA4 and nNOS

Reciprocal co-immunoprecipitation assays were performed to determine if PMCA4 and nNOS are interacting partners. The proteins were treated with either anti-PMCA4 or anti-nNOS antibodies. The Western blots showed that PMCA4 was able to co-precipitate nNOS and the reciprocal nNOS was able to co-precipitate PMCA4. There is a more intense interaction in capacitated sperm than uncapacitated sperm as seen in Figure 3.4, while the IgG control gave no bands.
3.3 FRET Analysis of PMCA4 and nNOS

Sperm taken from the cauda epididymides were stained with anti-PMCA4 and anti-nNOS antibodies and confocal microscopic images were taken of both capacitated and uncapacitated samples. For uncapacitated (Figure 3.5) and capacitated sperm (Figure 3.6), specific regions of interest were evaluated to determine if there was an energy transfer taking place. The graph in Figure 3.7 compares the intensities before and after bleaching. This shows that there is a significant difference in intensity in capacitated sperm before and after bleaching (p < 0.05), while there is no significant difference when pre- and post-bleach are compared in uncapacitated sperm (using ANOVA).

The FRET efficiencies were calculated using Equation 1, as described in Materials and Methods, for the capacitated and uncapacitated samples, seen in Figure 3.8. Capacitated sperm have a FRET efficiency rate of 24%.
and uncapacitated sperm have an efficiency rate of 6%. The difference between these rates of efficiency is significant \((p < 0.01)\) (using a \(t\)-test). Furthermore, only capacitated sperm have an efficiency rate above the 20% threshold (Bragdon, et al., 2009). Therefore, in capacitated but not uncapacitated sperm, PMCA4 and nNOS are within 10 nm of each other.

Figure 3.5  PMCA4 and nNOS are not within 10 nm in uncapacitated sperm. PMCA4-nNOS interaction before (a) and after (b) bleaching. There are no regions of interest where PMCA4 molecules transferred energy to nNOS molecules.
PMCA4 and nNOS are within 10 nm in capacitated sperm PMCA4-nNOS interaction before (a) and after (b) a bleaching event indicated that there were multiple regions of interest where an energy transfer was taking place, indicating there are multiple regions of interest where PMCA4 and nNOS are within 10 nm.
Figure 3.7  Graphical comparison of PMCA4- and nNOS-immunostained capacitated and uncapacitated sperm before and after bleaching. In capacitated sperm there is a significant difference (p < 0.05) between the intensity in images taken before bleaching compared to those taken after the bleaching event. In uncapacitated sperm the images taken before and after bleaching have relatively the same intensity.
Figure 3.8  FRET efficiency shows PMCA4 and nNOS are within 10 nm in capacitated sperm. Capacitated sperm are over the 20% threshold and therefore PMCA4 and nNOS are within 10 nm of one another. In uncapacitated sperm, the efficiency is below the threshold. There is a significant increase in FRET efficiency in capacitated sperm compared to uncapacitated sperm (p < 0.01).

3.4 Co-localization of PMCA4 and eNOS

Reciprocal co-immunoprecipitation assays were performed in order to investigate if PMCA4 and eNOS are interacting partners. Sperm samples were used in both uncapacitated and capacitated states and lysed to collect membrane proteins. Proteins were treated with the corresponding antibodies, anti-PMCA4 and anti-eNOS. PMCA4 was able to co-precipitate eNOS and the reciprocal, eNOS was able to co-precipitate PMCA4. There is a stronger interaction in both experiments in capacitated sperm compared to uncapacitated sperm as seen in Figure 3.9.
Figure 3.9 Reciprocal co-immunoprecipitations of PMCA4 and eNOS in sperm proteins. Reciprocal co-immunoprecipitations indicate that there is a stronger interaction between PMCA4 and eNOS in capacitated sperm compared to uncapacitated sperm samples using total protein as a positive control and IgG as a negative control. The above is a representative of the results from triplicate experiments.

3.5 FRET Analysis of PMCA4 and eNOS

After immunofluorescence staining with both anti-PMCA4 and anti-eNOS antibodies, confocal microscopic images were taken and then analyzed using Zen software. A FRET efficiency of over 20% provides evidence that the tagged molecules are within 10 nm of one another. Figure 3.10 and Figure 3.11 show uncapacitated and capacitated sperm with regions of interest where PMCA4 and eNOS are interacting. The graph in Figure 3.12 shows there is a significant difference before and after bleaching in both uncapacitated (p < 0.01) and capacitated sperm (p < 0.0001) (using a t-test). The FRET efficiency for capacitated sperm is 35% and uncapacitated sperm have a FRET efficiency value of 24%. The graph in Figure 3.13 compares the FRET efficiency values for both uncapacitated and capacitated sperm. Both have
efficiencies above the 20% threshold value, indicating that PMCA4 and eNOS are within 10 nm of each other (Bragdon, et al., 2009); capacitated sperm have a significantly higher efficiency value (p < 0.01) than uncapacitated sperm (using a $t$-test).

Figure 3.10 PMCA4 and eNOS are within 10 nm in uncapacitated sperm PMCA4-eNOS interaction, revealed via FRET, shows that before (a) and after (b) the bleaching event there are multiple regions of interest where PMCA4 and eNOS are within 10 nm of each other.

Figure 3.11 PMCA4 and eNOS are within 10 nm in capacitated sperm PMCA4-eNOS interaction, revealed via FRET analysis, that when comparing before (a) and after (b) bleaching there is an energy transfer from PMCA4 to eNOS as seen in the regions of interest in warmer colors.
Figure 3.12 Comparative graph of eNOS in capacitated and uncapacitated sperm before and after bleaching. An analysis of 10 confocal images showed there is a significant increase in intensity before and after bleaching in both capacitated (p < 0.0001) and uncapacitated (p < 0.01) sperm.
Figure 3.13 FRET efficiency shows PMCA4 and eNOS interact at a distance of less than 10 nm in capacitated and uncapacitated sperm. Both capacitated and uncapacitated sperm are over the 20% threshold indicating that eNOS is within 10 nm of PMCA4 in both calcium conditions. Capacitated sperm have a significantly higher FRET efficiency (p < 0.01) than uncapacitated sperm.

3.6 Effect of L-NAME on sperm motility in *Pmca4* null mice

A motility assay was performed to determine the number of motile sperm in capacitated versus uncapacitated sperm from *Pmca4* null mice (Figure 3.14). In the capacitated sperm samples there was a motility rate of 1.15% in the control and when treated with L-NAME there was significantly less motility in both the low (0.07%) and high (0.15%) doses (p < 0.00001). In the uncapacitated control sperm samples there was significantly more motility (14.8%) than the low (5.3%) and high (3.3%) treatments (p < 0.000001). These uncapacitated control sperm were swimming resembling progressive
movement. There was a dose-dependent decreasing trend in motility in uncapacitated sperm when treated with L-NAME with the low sample having slightly more motility than the higher dosage.

![Bar chart showing percentage of motile sperm with p-values](image)

**Figure 3.14** Unexpectedly, control capacitated and uncapacitated *Pmca4* null sperm show significantly greater motility than sperm treated with L-NAME. Sperm treated with L-NAME showed a decrease in motility in both capacitated and uncapacitated sperm. In capacitated sperm there was significantly greater motility ($p < 0.00001$) in the control samples when compared to both the low and high treatments of L-NAME. Uncapacitated sperm samples treated with L-NAME had a trend of decreased sperm motility with increased concentrations of L-NAME.

### 3.7 TUNEL assay in *Pmca4* null murine testes

A TUNEL assay was performed using TUNEL Apoptosis Detection Kit on both wild-type and *Pmca4* knockout mouse testes (n=3). The numbers of
apoptotic cells were counted per tubule using confocal microscopy. Figure 3.15 shows representative images of the tubules in wild-type (a-c) and knockout (d-f) murine testes with the apoptotic cells shown in green and the nucleus of cells in blue. Figure 3.16 compares the number of apoptotic cells per 10 tubules in wild-type and knockout murine testes. There were significantly higher counts of apoptotic cells in *Pmca4* null murine testes compared to wild-type testes. Specifically, in the spermatogonia and spermatids (*p* < 0.0001) and the spermatocytes (*p* < 0.05) there was significantly higher counts of apoptotic cells in the knockout mice.
Figure 3.15 Representative confocal images of testes stained using a TUNEL assay. The nucleus (a), fluorescein stained apoptotic cells (b), and a composite (c) of the testes of wild-type mice show the majority of apoptotic cells in the interstitial space. The nucleus (d), apoptotic cells (e), and a composite (f) of the testes of Pmca4 knockout mice show the majority of apoptotic cells in the spermatogonia. The red arrow indicates an area of apoptosis in the interstitial space in WT mice and the white arrow indicates an area of apoptosis within the spermatogonia in Pmca4 KO mice.
Figure 3.16 There are significantly increased counts of apoptotic cells in the testes of *Pmca4* null mice. Testes stained for apoptosis showed significantly higher counts of apoptosis in the spermatogonia (p < 0.0001), spermatocytes (p < 0.05) and spermatids (p < 0.0001) of *Pmca4* null mice. The total number of apoptotic cells in the testes was also significantly higher (p < 0.0001) in *Pmca4* null compared to the wild-type mice.
Chapter 4

DISCUSSION

4.1 Interaction between PMCA4 and CASK

Previous work in our lab showed via co-immunoprecipitation that PMCA4 and CASK are interacting partners in murine sperm (Aravindan, et al., 2012). In this study, the use of immunofluorescence and confocal imaging showed that PMCA4 and CASK co-localized on murine sperm, specifically in the head and the principal piece. The confocal microscopy and FRET analyses confirmed that PMCA4 and CASK co-localize on the surface of murine sperm. This interaction was significantly greater in uncapacitated, compared with capacitated, sperm. Since the FRET efficiencies in both groups did not reach the threshold value of 20% (Bragdon, et al., 2009), the distance that separates the molecules is greater than 10 nm. These data are not surprising because the bleached regions were localized from the head to the beginning of the principal piece, and CASK is known to be localized mainly to the principal piece. Further investigation should include a study to determine if CASK is able to phosphorylate PMCA4, which would provide evidence of their proximity to one another.

The significant increase in the number of interacting molecules in uncapacitated sperm (Figure 3.3) is consistent with the model. In the capacitated state, CASK has been found to preferentially interact with JAM-A as opposed to PMCA4 (Aravindan, et al., 2012). Thus the data support the model (Figure 1.5) that the PMCA4 PDZ ligand is bound at the PDZ domain of
CASK in the uncapacitated state, when cytosolic calcium concentration is at basal levels (50 nM) (Herrick et al., 2005).

4.2 Localization of nNOS and its Interaction with PMCA4

The enzyme nNOS was localized to the head of murine sperm; to our knowledge, this is the first localization of nNOS in this species. This localization is similar to what has been seen for human nNOS (Herrero, et al., 1996). While in the uncapacitated state, PMCA4’s PDZ ligand is bound to the PDZ domain of CASK, the co-immunoprecipitation and FRET results of this study show that once calcium levels increase during capacitation, the ligand is preferentially bound to the PDZ domain on nNOS (Aravindan, et al., 2012). In somatic cells, it has been found that PMCA4 and nNOS interact preferentially when intracellular calcium levels are elevated (Schuh, et al., 2001) and calcium is required to activate the nitric oxide synthases (Cartwright, et al., 2009; Herrero, et al., 1999; Schuh, et al., 2001). The co-immunoprecipitation results from this investigation indicate that in the high calcium conditions, associated with capacitation, there is a greater interaction between PMCA4 and nNOS compared to the low calcium, uncapacitated state. The triplicate reciprocal co-immunoprecipitations provide evidence for the interaction of PMCA4 and nNOS.

These data were supported by the immunofluorescence findings via confocal microscopy. The images showed co-localization of PMCA4 with nNOS with more molecular interactions in capacitated sperm. FRET analysis showed that only in the capacitated state are PMCA4 and nNOS over the necessary threshold and are therefore considered to be within 10 nm of one
another. Due to the fact that nNOS is activated by calcium, its close proximity to PMCA4 allows it to be in a locally low calcium environment when the pump is extruding calcium and therefore the activation of nNOS is tempered to prevent the production of elevated levels of NO (Herrero, et al., 1999). The uncapacitated sperm did not surpass this threshold, thus PMCA4 and nNOS are not within 10 nm of each other. This finding is consistent with the report that CASK is preferentially bound to PMCA4 in the uncapacitated state (Aravindan, et al., 2012).

When referring to the model (Figure 1.5), these data support what has been proposed. In the uncapacitated condition, the PMCA4 PDZ ligand is bound to CASK and is unable to bind to the PDZ domain of nNOS. However, once capacitated, the PDZ domain of CASK binds to JAM-A (Aravindan, et al., 2012) and the PMCA4 PDZ ligand is able to bind to the PDZ domain on nNOS.

4.3 Interaction between PMCA4 and eNOS

In somatic cells it has been shown that PMCA4 and eNOS are interacting partners (Holton et al., 2010). This nitric oxide synthase requires calcium in order to convert L-arginine to L-citruline producing nitric oxide in the process (Sobolewska-Stawiarz, et al., 2014). Triplicate reciprocal co-immunoprecipitations show that there is a stronger interaction between PMCA4 and eNOS in capacitated sperm compared to uncapacitated sperm. In the capacitated state sperm have elevated calcium levels and return to basal levels requires the efflux activity of PMCA4, which creates a locally low calcium environment which modulates the activity of eNOS molecules which are tethered to the pump.
As the pump is activated under conditions of high cytosolic calcium concentrations ([Ca$^{2+}$]$_c$) and because eNOS is tethered to it, eNOS is present in a locally low [Ca$^{2+}$]$_c$ which inhibits its activity. As a consequence of this, elevated levels of NO are avoided when intracellular concentrations are elevated. In the uncapacitated state, there are low levels of calcium and therefore less of an interaction between PMCA4 and eNOS is required to down-regulate eNOS which would be inhibited by the low [Ca$^{2+}$]$_c$.

These data from co-immunoprecipitation assays are supported by confocal microscopy, which showed co-localization of PMCA4 and eNOS in both uncapacitated and capacitated sperm. FRET analysis of the confocal images showed that in both capacitated and uncapacitated sperm eNOS is within 10 nm of PMCA4, since the FRET efficiency was over the 20% threshold. However, because eNOS is activated by calcium and regulated by PMCA4, it would interact with PMCA4 more in the capacitated state where this is high [Ca$^{2+}$]$_c$. (Holton, et al., 2010). Since eNOS binds to PMCA4 at the ATP binding site in the larger cytosolic loop and unlike nNOS and CASK, eNOS, has no competitive binding for PMCA4 it appears to retain its binding with PMCA4 at low [Ca$^{2+}$]$_c$. It is shown that eNOS binds to CAV-1 at another domain, and this interaction may be able to regulate or prevent the activity of eNOS at low [Ca$^{2+}$]$_c$. (Ju, Zou, Venema, & Venema, 1997). This interaction may also assist eNOS in remaining close to the membrane.

The model depicted in Figure 1.5 shows that eNOS remains bound to PMCA4 in the low calcium, uncapacitated state, and the high calcium,
capacitated state. This is concurrent with the data that show that eNOS is within 10 nm of PMCA4 regardless of the calcium condition.

4.4 Motility of sperm when treated with L-NAME

In an attempt to understand the mechanistic basis of the loss of motility in the absence of PMCA4, Pmca4 null mice were studied. They were generated by the Shull Lab (Okunade, et al., 2004) and donated as a gift to the DeLeon Lab. These mice have elevated levels of intracellular calcium. Unpublished data from our Lab has shown that in the absence of PMCA4’s efflux activity, there are elevated levels of NOS activity and peroxynitrite levels in Pmca4 null sperm. This is depicted in the model in Figure 1.5. To test the model and to determine if oxidative stress is responsible for the absence of motility, a NOS inhibitor, L-NAME, which inhibits both eNOS and nNOS was used to treat Pmca4 null sperm in the uncapacitated and capacitated states to assess its effect on motility.

Unexpectedly, instead of an increase in motility rates, uncapacitated sperm had a dose-dependent decrease in motility when treated with L-NAME. Similarly, the rate of motility decreased in capacitated sperm after treatment with L-NAME. The motion of the uncapacitated control samples involved an average of 14.8% sperm exhibiting progressive motility. Because L-NAME inhibits NOS activity, it was expected that the elevated levels of NOS activity in Pmca4 nulls would have been tempered and the decrease in activity would have been reflected in an increase in motility. Normally, NO decreases the ATP of the cell by inhibiting the ATP-synthases. With a decrease in production of NO, there is a subsequent increase in ATP and an increase in motility.
(Weinberg, et al., 1995). However, our results did not show an increase in motility, but rather reflected the opposite scenario.

The fact that capacitated sperm remained essentially motionless regardless of the dosage of L-NAME, with the control having an average motility of 1.2% indicates that the calcium toxicity after capacitation had severe deleterious effects on the nulls. The high calcium level associated with capacitation without the presence of PMCA4’s efflux activity, generated unregulated NOS activity producing elevated levels of NO and the subsequent high levels of OONO⁻. The latter is known to cause lipid peroxidation of the sperm plasma membrane (Hellstrom, Bell, Wang, & Sikka, 1994; Weinberg, et al., 1995). Our results suggest that NO levels might best be tempered by the administration of NO quenchers rather than by NOS inhibitors (Blair, Shaul, Yuhanna, Conrad, & Smart, 1999), and this should be pursued in future studies.

4.5 Apoptosis Assay in Pmca4 Null Murine Testes

Previous work has shown that cells under oxidative stress linked to NO have a higher instance of apoptosis (Taneli, et al., 2005). Prior work in the DeLeon Lab indicated that Pmca4 null mice have elevated levels of NO compared to wild-type mice. A TUNEL assay was performed to investigate if a lack of PMCA4 would cause an increase in apoptosis in the testes of Pmca4 null mice. The results showed that there were significantly greater numbers of apoptotic cells in the spermatogonia, spermatocytes, and spermatids of Pmca4 null mice testes compared to wild-type testes. These locations are the areas where germ cells are becoming increasingly mature. Apoptosis is linked
to elevated levels of NO, therefore, the testes of *Pmca4* KO mice are in a state of oxidative stress.

Interestingly, the level of apoptotic cells in the interstitial spaces was not impacted by the absence of PMCA4, as there were no significant differences for the rates in wild-type and null testes (Figure 3.16). This is in contrast to the recently reported findings of significant rates of apoptotic cells in the interstitial spaces, compared to the germ cells, of mouse testes exposed to titanium dioxide nanoparticles, which cause oxidative stress (Smith, A.S., Michael, S.R., Aravindin, R.G., Dash, S., Shah, I., Galileo, D.S., Martin-DeLeon, P.A., *in press* 2014; Joshi, S.C. & Kaushik, U., 2013). The difference is explained by the finding that the interstitial spaces are populated with blood vessels, which are likely to express a member of the PMCA family other than PMCA4 or a different combination including PMCA4 (Freeman, Howard, Bentsen, Legon, & Walters, 1995), and the fact that the toxicity in these cells is a result of nanoparticles that are obtained from the site after administration in the animal (Brini & Carafoli, 2011). Interestingly, Okunade reported that there was apoptosis in the liver of *Pmca4* null males (Okunade, et al., 2004), consistent with the expression of PMCA4 expression in the liver (Howard, Barley, Legon, & Walters, 1994; Okunade, et al., 2004).

Since the absence of PMCA4 results in apoptosis in both the liver and testis of *Pmca4* nulls as shown in Figure 3.15, and since apoptosis is linked to elevated levels of NO and oxidative stress which have been demonstrated to be present in *Pmca4* null sperm via elevated levels of NOS
activity and peroxynitrite levels (unpublished DeLeon Lab data), it follows that the motility loss in null sperm is a result of oxidative stress.

These data support the hypothesis that the testes of Pmca4 null mice are under oxidative stress resulting from an unregulated production of NO by eNOS and nNOS. The elevated levels of NO cause increased levels of OONO\(^-\), resulting in oxidative stress, which leads to lipid peroxidation (Aitken, et al., 2014), a key mechanism in loss of motility (Gomez, et al., 1998).
Chapter 5

CONCLUSIONS

The following conclusions can be made based on the results of the present study:

1. To date this is the first Co-IP and FRET data to reveal that PMCA4 and eNOS and PMCA4 and nNOS are interacting partners in sperm.
2. The finding that more CASK molecules interact with PMCA4 under uncapacitated conditions provides support for the notion that CASK maintains inactivation of the pump after it is auto-inhibited possibly by phosphorylating it.
3. In capacitated sperm, both eNOS and nNOS are within 10 nm of PMCA4, supporting the model.
4. In uncapacitated sperm, PMCA4 and nNOS are not interacting, whereas eNOS is still within 10 nm, supporting the model.
5. Maintenance of optimal levels of NO is dependent on the interaction of eNOS and nNOS with PMCA4, and therefore in the absence of PMCA4, the resulting loss of motility results from elevated levels of NO.
6. Unexpectedly, capacitated Pmca4 null murine sperm have a decrease in motility when treated with L-NAME, an inhibitor of the eNOS and nNOS.
7. There is an increase in apoptosis in Pmca4 null murine testes compared to wild-type testes and this increase comes predominantly
from DNA damage in germ cells rather than cells in the interstitial spaces.

8. The mechanism by which Pmca4 null mice lose their motility is likely due to the sperm undergoing oxidative stress due to elevated levels of NO and its effector, OONO$^-$$^\text{.}$

9. Future studies should be aimed at using NO quenchers such as oxyhemoglobin and oxidized low density lipoprotein, oxLDL, to determine if motility levels can be increased in Pmca4 null sperm.
REFERENCES


Appendix

APPROVAL PAGES TO USE ANIMALS IN RESEARCH

Title of Protocol: Mechanism of sperm motility defects seen in JAM-A/PMCA4 null mice

AUP Number: 1181-2014-0

Principal Investigator: Patricia A. DeLeon

Common Name: Mouse

Genus Species: Mus Musculus

Pain Category: (please mark one)

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<tr>
<th>USDA PAIN CATEGORY: (Note change of categories from previous form)</th>
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<td>B</td>
<td>Breeding or holding where NO research is conducted</td>
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<td>C</td>
<td>Procedure involving momentary or no pain or distress</td>
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<td>D</td>
<td>Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)</td>
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<td>E</td>
<td>Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation</td>
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Official Use Only

IACUC Approval Signature: [Signature]

Date of Approval: 3/14/15
**Principal Investigator Assurance**

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.

2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).

3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.

4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.

5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.

6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.

7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.

8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.

9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.

10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.

11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*

12. I understand that by signing, I agree to these assurances.

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# NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

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