

TAKING A MOLECULAR APPROACH TO A CLINICAL PROBLEM: SPERM  
CAPACITATION AS THE MISSING LINK FOR SUCCESSFUL IN VITRO  
FERTILIZATION IN THE HORSE

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TAKING A MOLECULAR APPROACH TO A CLINICAL PROBLEM: SPERM  
CAPACITATION AS THE MISSING LINK FOR SUCCESSFUL IN VITRO  
FERTILIZATION IN THE EQUINE

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Ejaculated mammalian sperm must reside within the female reproductive tract for a finite period of time before acquiring the ability to fertilize an oocyte. The complex biochemical and physiological changes that occur during this process are collectively termed 'capacitation'. Capacitation comprises the molecular events that prepare the sperm for acrosomal exocytosis and a change in the pattern of flagellar motility called hyperactivation. Both of these processes are required for fertilization.

In several domestic and laboratory species, as well as in humans, these events can be recapitulated in the laboratory by incubating sperm in a defined medium. Two *in vitro* markers of capacitation include time dependent increases in protein tyrosine phosphorylation and high rates of agonist-induced acrosomal exocytosis. In contrast to other species, capacitation of stallion sperm has been extremely difficult to achieve with inconsistent results reported between and within laboratories. Moreover, despite numerous attempts, only two foals have ever been produced via *in vitro* fertilization (IVF) technology. Evidence suggests that this is ultimately due to the inability to appropriately capacitate stallion sperm in the laboratory.

Therefore, the objectives of this research were to define and characterize: 1) the incubation conditions that supported capacitation of stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and agonist-induced acrosomal exocytosis; 2) the molecular pathways involved in both of these capacitation-dependent events; 3) the motion parameters associated with hyperactivated motility; and 4) the incubation conditions that supported successful *in vitro* fertilization of mare oocytes.

We determined that incubation of stallion sperm in modified Whitten's containing 25 mM sodium bicarbonate and 7 mg/ml BSA supported significant time-dependent increases in protein tyrosine phosphorylation and significant percentages of progesterone-induced acrosomal exocytosis. We also demonstrated that the serine/threonine kinase protein kinase A (PKA) plays a critical role in the protein tyrosine phosphorylation pathway and that the guanine nucleotide exchange factor EPAC plays a role in acrosomal exocytosis. Moreover, by characterizing the motion parameters associated with procaine-induced hyperactivation in stallion sperm in juxtaposition with our capacitating conditions, we were able to achieve 60.7% *in vitro* fertilization rates of mare oocytes (0-30% previously reported).

## BIOGRAPHICAL SKETCH

Lori A. McPartlin graduated from North Attleboro High School (North Attleboro, MA) in 1995. She then went on to obtain a Bachelor of Arts degree in Biology at Saint Anselm College (Manchester, NH) in 1999. After working in the biotechnology industry in the Boston area for four years doing cell and tissue culture, she returned to school in 2003 and obtained a Master's degree in Animal Biotechnology and Biomedical Sciences from the University of Massachusetts, Amherst, in 2004. She then re-located to Ithaca, NY, to attend Cornell University to pursue of a Doctorate of Philosophy in Molecular and Integrative Physiology, which she completed in 2010.

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## TABLE OF CONTENTS

Biographical Sketch	iii
Acknowledgements	iv
Table of Contents	v
List of Figures	vi
List of Tables	viii
List of Abbreviations	ix
Chapter 1	1
Chapter 2	21
Chapter 3	52
Chapter 4	86
Chapter 5	118

## LIST OF FIGURES

<b>Figure 1.1</b>	<b>3</b>
Postulated pathways leading to the capacitation-dependent increases in protein tyrosine phosphorylation.	
<b>Figure 2.1</b>	<b>31</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm incubated under non-capacitating and capacitating conditions.	
<b>Figure 2.2</b>	<b>33</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm incubated with increasing concentrations of BSA.	
<b>Figure 2.3</b>	<b>34</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm demonstrating the individual and combined effects of BSA and sodium bicarbonate incubated under an air atmosphere (A) or under nitrogen (B).	
<b>Figure 2.4</b>	<b>36</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm incubated with increasing concentrations of sodium bicarbonate.	
<b>Figure 2.5</b>	<b>37</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm incubated with and without the addition of cAMP modulators dbcAMP and IBMX.	
<b>Figure 2.6</b>	<b>38</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm demonstrating the individual and combined effects of BSA and sodium bicarbonate in combination with cAMP modulators dbcAMP and IBMX.	
<b>Figure 2.7</b>	<b>39</b>
PNA-Alexa 488 fluorescence pattern of: (A) an acrosome intact sperm; (B) an intermediately reacted sperm; and (C) an acrosome reacted sperm.	
<b>Figure 2.8</b>	<b>40</b>
Percent of acrosomal exocytosis of stallion sperm incubated under non-capacitating and capacitation conditions for (A) 0 h; and (B) 6 h.	
<b>Figure 3.1</b>	<b>63</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm incubated with and without the addition of the sAC inhibitor KH7 in stallion (A) and mouse (B) sperm.	
<b>Figure 3.2</b>	<b>65</b>
Immunoblot for protein tyrosine phosphorylation in stallion sperm incubated with and without the addition of cBIMPS and the PKA inhibitor PKI in stallion sperm (A) and with and without KH7 in mouse sperm (B).	
<b>Figure 3.3</b>	<b>66</b>
Representative immunoblot of Epac1 and Epac 2 in stallion (lanes 1 and 3) and mouse (lanes 2 and 4) sperm.	
<b>Figure 3.4</b>	<b>68</b>
Immunofluorescence localization of Epac1 in stallion and mouse sperm.	



<b>Figure 3.5</b>	<b>69</b>
Immunofluorescence localization of Epac2 in stallion and mouse sperm.	
<b>Figure 3.6</b>	<b>70</b>
Immunoblot for protein tyrosine phosphorylation in stallion (A) and mouse (B) sperm incubated with and without the addition of the Epac specific cAMP analogue 8pCPT.	
<b>Figure 3.7</b>	<b>71</b>
Percentages of acrosomal exocytosis in stallion (A) and mouse (B) sperm incubated under capacitating conditions prior to exposure to DMSO (solvent control), calcium ionophore A23187 (Cal), progesterone (P4), or the Epac-specific cAMP analogue 8pCPT.	
<b>Figure 3.8</b>	<b>73</b>
Average membrane potential ( $E_m$ ) of non-capacitated stallion sperm (NC), capacitated stallion sperm (Cap) and stallion incubated under capacitating conditions in the presence of 8pCPT (8P).	
<b>Figure 3.9</b>	<b>77</b>
Hypothesized pathway involved in plasma membrane hyperpolarization and zona pellucida-induced acrosomal exocytosis.	
<b>Figure 4.1</b>	<b>98</b>
Hyperactivation induced by procaine. Representative motion tracks of control sperm at 0 h (A) and 6 h (C), and sperm treated with 5 mM procaine at 0 h (B) and 6 h (D).	
<b>Figure 4.2.</b>	<b>100</b>
Representative images from Supplemental Movies 1-4 (available online at <a href="http://www.biolreprod.org">www.biolreprod.org</a> ) of control and hyperactivated stallion sperm.	
<b>Figure 4.3.</b>	<b>100</b>
Immunoblot for protein tyrosine phosphorylation of stallion sperm incubated in non-capacitating or capacitating conditions with and without the addition of 5 mM procaine.	
<b>Figure 4.4.</b>	<b>104</b>
Percentages of acrosomal exocytosis in sperm incubated under capacitating conditions and exposed to calcium ionophore A23187, 5 mM procaine, or DMSO (vehicle control) at 0 h (A) and 6 h (B).	
<b>Figure 4.5.</b>	<b>105</b>
Representative <i>in vitro</i> fertilized mare oocytes. (A) Hoechst 33258 staining showing 2 pronuclei (18 h post IVF); (B) light microscopy of 2 cell embryo (24 h post IVF); (C & D) light microscopy of 8 cell embryos (Day 3).	
<b>Figure 5.1</b>	<b>123</b>
Postulated pathways leading to capacitation-dependent increases in protein tyrosine phosphorylation, plasma membrane hyperpolarization and acrosomal exocytosis.	

## LIST OF TABLES

<b>Table 2.1</b>	<b>30</b>
Total and progressive motility data for stallion sperm incubated in three different capacitating media.	
<b>Table 4.1</b>	<b>97</b>
CASA measurements before and after the addition of 5 mM procaine to stallion sperm at Time 0 hour.	
<b>Table 4.2</b>	<b>88</b>
CASA measurements before and after the addition of 5 mM procaine to stallion sperm at Time 6 hour.	

## LIST OF ABBREVIATIONS

BSA: bovine serum albumin

BWW: Biggers, Whitten, and Whittingham

cAMP: cyclic adenosine monophosphate

CASA: computer assisted semen analysis

cBIMPS: Sp-5,6-DCI-cBIMPS (cAMP analogue)

cccp: Carbonyl cyanide m-chlorophenylhydrazone

CFTR: cystic fibrosis transmembrane conductor

dbcAMP: dibutyryl cyclic adenosine monophosphate (cAMP analogue)

DiSC<sub>3</sub>(5): 3,3'-dipropylthiadicarbocyanine iodide

E<sub>m</sub>: membrane potential

ENaC: epithelial sodium channel

EPAC: guanine nucleotide exchange factor

FBS: fetal bovine serum

FSH: follicle stimulating hormone

IBMX: isobutylmethylxanthine

K<sup>+</sup><sub>ATP</sub>: potassium adenosine triphosphate channel

KH7: soluble adenylyl cyclase inhibitor

LVA: low voltage activated T-type calcium channel

MW: modified Whitten's

PKA: protein kinase A

PKI: myristolated protein kinase A inhibitor

sAC or SACY: soluble adenylyl cyclase

sp-TALP: sperm TALP

8pCPT: 8-(*p*-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic  
monophosphate (cAMP analogue)

Motility Analysis Abbreviations:

VAP: average path velocity (the average velocity of sperm along the smoothed  
cell path in  $\mu\text{m}/\text{sec}$ )

VCL: curvilinear velocity (the average rate of travel of the sperm head  $\mu\text{m}/\text{sec}$ )

VSL: straight line velocity (the average velocity measured in a straight line  
from beginning to end  $\mu\text{m}/\text{sec}$ )

BCF: beat cross frequency (frequency that the head crosses the sperm track  
in Hz)

STR: straightness ( $\text{VSL}/\text{VAP} \times 100$ )

LIN: linearity ( $\text{VSL}/\text{VCL} \times 100$ )

## CHAPTER 1

# LITERATURE REVIEW: CURRENT UNDERSTANDING OF SPERM CAPACITATION AND THE STATE OF *IN VITRO* FERTILIZATION IN THE HORSE

### INTRODUCTION

When mammalian sperm are first ejaculated, they are fertilization incompetent. They must reside within the female reproductive tract for a finite period of time before they acquire the ability to fertilize an oocyte [1]. The complex biochemical, physiological and cellular changes that occur during this process are collectively termed 'capacitation'. The concept that sperm must undergo a maturational process within the female reproductive tract to acquire the ability to fertilize an oocyte was proposed by both Austin and Chang in 1952 [2-4] and with this discovery came the advent of *in vitro* fertilization (IVF).

The molecular events involved in capacitation prepare the sperm for acrosomal exocytosis and lead to a change in the pattern of flagellar motility. In several species this can be recapitulated in the laboratory by incubating sperm in a defined medium; in contrast to other species, capacitation of sperm in the horse has been extremely difficult. This is evidenced by the inability to achieve *in vitro* markers of capacitation. For example, only low levels of protein tyrosine phosphorylation [5, 6] and poor acrosomal exocytosis percentages [7-10] in addition to very low rates of homologous IVF have been reported in this species [11-17]. Moreover, despite numerous attempts, IVF has only produced two foals [18]. Evidence suggests that all of these deficiencies are ultimately due to the inability to appropriately capacitate stallion sperm in the laboratory.

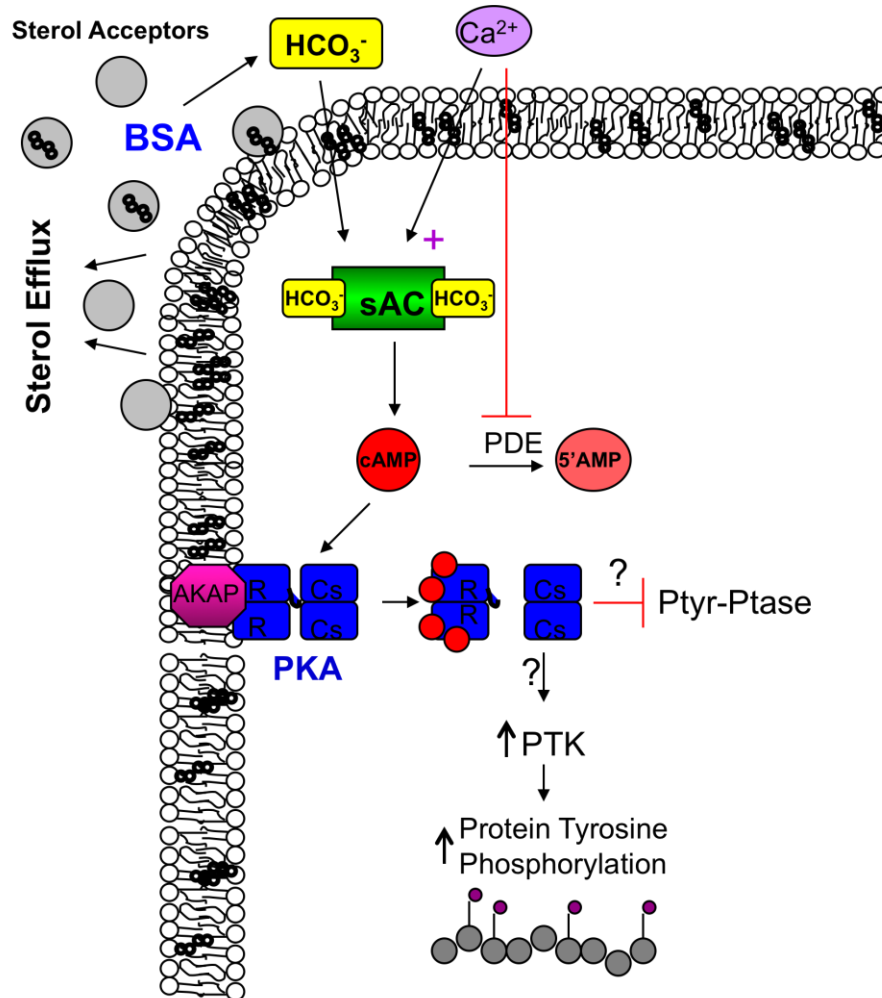
This chapter, therefore, reviews the current understanding of stallion sperm capacitation and IVF in this species based on the numerous previous attempts at these *in vitro* techniques.

## **CAPACITATION-DEPENDENT PROTEIN TYROSINE PHOSPHORYLATION**

Although the molecular and biochemical changes leading to sperm capacitation are still under investigation, it has been shown that this process is associated with changes in sperm plasma membrane fluidity, intracellular ion concentrations, metabolism and motility [19-26]. Changes in membrane fluidity are postulated to occur as a result of sterol efflux from the plasma membrane; in this regard BSA can be used as a sterol acceptor during *in vitro* capacitation [27-29]. These changes then impact enzymatic function and/or ion transport. For example, calcium and bicarbonate enter the cell and activate soluble adenylyl cyclase (sAC or SACY), amongst other functions, with a resulting increase in intracellular cAMP levels. Altogether, these events lead to an increase in the activity of the serine/threonine kinase, protein kinase A (PKA). Through a series of intermediates and pathways that have yet to be identified, activation of PKA is transduced into a series of protein tyrosine phosphorylation events (Figure 1.1) [19, 30].

While this is presently the accepted pathway leading to protein tyrosine phosphorylation during sperm capacitation, the discovery of another cAMP-regulated downstream regulator may profoundly change the previous assumptions of cAMP signaling. The recently discovered family of guanine nucleotide exchange factors, called EPAC, mediate signal transduction pathways via the second messenger cAMP through PKA-independent pathways. There are two known isoforms of EPAC (EPAC1 and EPAC2).

There is currently no membrane permeable antagonist for EPAC [31]; however, the development of EPAC-selective cAMP analogues that do not activate PKA has enabled the discovery of novel PKA-independent mediated pathways. Studies in somatic cells indicate that EPAC is involved in ion channel function, intracellular calcium signaling, ion transport activity, and exocytosis [32].



**Figure 1.1.** Postulated pathways leading to capacitation-dependent increases in protein tyrosine phosphorylation. BSA: bovine serum albumin; HCO<sub>3</sub><sup>-</sup>: sodium bicarbonate; sAC: soluble adenylyl cyclase; PDE: phosphodiesterase; PKA: protein kinase A; PTK: protein tyrosine kinases; AKAP: A-kinase anchoring protein; Ptk-Ptase: protein tyrosine phosphatases.

Additionally, in somatic cells and in mouse sperm, activation of EPAC with the cAMP analogue 8pCPT results in the direct downstream activation of RAP1 [33, 34], and in some cell types, this is followed by ERK1/2 activation [35]. RAP1 activates the Map Kinase (MAPK) family of proteins, including ERK1/2, which are known to be present in mammalian sperm [35, 36].

Interestingly, EPAC1 has been recently identified in ejaculated human and epididymal mouse sperm [34, 37] and EPAC2 has been identified in mouse spermatogenic cells [38]. Given the importance of the aforementioned signal transduction pathways in sperm capacitation, it is important to re-visit the regulation of these pathways, which were previously attributed to PKA-mediated events.

In species other than the horse, protein tyrosine phosphorylation has been correlated with capacitation and thus the ability of sperm to undergo acrosomal exocytosis [28, 30] and/or to fertilize an oocyte *in vitro* [19]. If this hallmark of capacitation is inhibited, sperm do not undergo acrosomal exocytosis and are unable to fertilize [27, 39]. In the stallion, unlike all other species studied thus far [40-42], incubation conditions that support the time-dependent increases in protein tyrosine phosphorylation, and presumably sperm capacitation have not been defined.

Media reported in the literature to capacitate stallion sperm include sperm TALP and Biggers, Whitten, and Whittingham (BWW) [5, 43]. Sperm TALP is used for sperm capacitation and IVF in the bovine species, is a HEPES buffered medium, and contains lactate and pyruvate as energy sources (glucose inhibits capacitation in this species) [40]. Biggers, Whitten, and Whittingham is used for capacitation and IVF with human sperm, is also a HEPES buffered medium, and contains lactate, pyruvate and glucose as energy sources. Despite the fact that



these media are used with great success in their respective species, they do not support stallion sperm capacitation. This is exemplified by the fact that tyrosine phosphorylation in stallion sperm has only been achieved in medium supplemented with reagents that pharmacologically raise intracellular cAMP levels, such as dibutyryl cAMP (dbcAMP) and phosphodiesterase inhibitors (e.g. caffeine), or reactive oxygen species [5, 44, 45]. Use of such chemicals bypasses signaling pathways in response to external stimuli, membrane changes, and the activation of downstream effectors, and as such, is not considered physiological.

Both Sperm TALP and BWW were adopted from protocols of other species without any investigation into the specific physiological or metabolic requirements for stallion sperm. As such, our understanding of the specific requirements for stallion sperm capacitation and its associated pathways are vastly lacking.

## **CAPACITATION-DEPENDENT ACROSOMAL EXOCYTOSIS**

Sperm of all mammalian species contain a secretory vesicle called the acrosome that forms a cap over the anterior portion of the sperm head. The proteolytic enzymes contained within the acrosome are released during the early interaction between a capacitated sperm and an oocyte through a process called acrosomal exocytosis. This is an irreversible calcium-dependent event, which enables the sperm to penetrate through the zona pellucida and fuse with the oolemma [1]. Acrosomal exocytosis is therefore required for successful fertilization, and as such, is used as an *in vitro* marker for capacitation.

The molecular changes that occur during capacitation, including protein tyrosine phosphorylation, are required in order for a sperm to undergo

acrosomal exocytosis upon the proper stimulation. *In vivo*, this includes contact with the zona pellucida and/or progesterone stimulation; *in vitro*, progesterone is also used, as well as the 'non-physiological' inducer calcium ionophore.

Because the incubation conditions used to capacitate stallion sperm have not been ideal (as described above attempts to induce acrosomal exocytosis in stallion sperm incubated with agonists such as porcine zona pellucida, progesterone, heparin and calcium ionophore [7-9, 18, 46, 47], have resulted in variable and less than ideal results when compared to those reported in other species [19, 28, 48], with percentages ranging from 10 - 26% [10, 46].

Christensen et al. reported acrosomal exocytosis percentages as high as 71.8% after a 4 h incubation and subsequent exposure to heparin. The viability of sperm that was reported, as determined propidium iodide staining, however, was identical at 71.8% [7]. These identical percentages indicate that all viable sperm, and only the viable sperm, underwent acrosomal exocytosis, leading one to question the reliability of these results (some sperm will undergo spontaneous exocytosis upon cell death). In contrast, based on studies in other mammalian species, it is generally accepted that only ~ 50% of sperm in a population will be able to undergo agonist-induced exocytosis [49].

A key component in the regulation of acrosomal exocytosis is sperm plasma membrane hyperpolarization. Hyperpolarization, which has been demonstrated to occur during capacitation, is required for fertility [50] and in the mouse, represents a change in membrane potential from ~ -30 mV to ~ -60 mV [50]. Currently, cAMP is believed to play a role in hyperpolarization of the sperm plasma membrane through a PKA-mediated pathway [50]. The current model involves increases in intracellular cAMP, the activation of PKA, followed by phosphorylation of the cystic fibrosis transmembrane conductor (CFTR).

Activation of CFTR allows the influx of negatively charged  $\text{Cl}^-$  ions as well as the inhibition of the constitutively active epithelial sodium channels (ENaC), thus preventing the influx of  $\text{Na}^+$  [50]. It has also been demonstrated that the capacitation-associated increase in intracellular pH activates  $\text{K}^+_{\text{ATP}}$  channels, also contributing to membrane hyperpolarization [51]. The resting and hyperpolarized membrane potentials of non-capacitated and capacitated stallion sperm have not been reported.

Evidence suggests that when capacitated-hyperpolarized spermatozoa come in contact with the oocyte's zona pellucida, a rapid depolarization of the plasma membrane occurs, allowing an influx of calcium through low voltage-activated channels [25, 52]. These channels remain in the inactivated state for as long as the sperm remain depolarized (i.e. non-capacitated), preventing premature acrosomal exocytosis [52]. Following capacitation-induced hyperpolarization, however, these channels switch to a closed state, becoming available for activation following the zona pellucida-induced depolarization.

In cerebellar neurons, the activation of EPAC with 8pCPT has been demonstrated to induce hyperpolarization [36]; however, in various other excitable and non-excitable somatic cells, EPAC activation has been observed to have effects on above mentioned ion channels such that a depolarization of the plasma membrane would be expected. For example, in pancreatic beta cells, Kang *et al.* observed an EPAC-mediated inhibition of  $\text{K}^+_{\text{ATP}}$  channels [53]; in rat pulmonary epithelial cells EPAC activation increased the channel open probability of ENaC [54]; and in rat hepatocytes, it stimulated an outwardly rectifying  $\text{Cl}^-$  current [55].

In addition, activation of EPAC is believed to mediate calcium mobilization through cAMP in three hypothesized ways: 1) by interacting directly

with inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R) to release calcium from intracellular stores; 2) by acting through RAP GTPases to activate protein kinases that phosphorylate and regulate the function of calcium release; and/or 3) by stimulation of RAP GTPases that activate phospholipase C (PLC), generating IP<sub>3</sub> [32].

Recent evidence from Branham *et al.* shows that activation of EPAC in permeabilized-human spermatozoa induces acrosomal exocytosis in a calcium-dependent manner [37]. Currently, there are no reports investigating the effect of EPAC activation in intact-mammalian sperm, or its effect on stallion sperm physiology.

## **HYPERACTIVATION**

Penetration of the outer vestments of the oocyte at fertilization also requires a change in the pattern of sperm motility that has been termed hyperactivation [24, 56, 57]. The acquisition of hyperactivated sperm motility has been observed within the oviducts of mammals at the time of fertilization [58-60] and is required for zona pellucida penetration [24, 25]. It is also believed to play a role in the ability of sperm to detach from the oviductal wall [60], navigate the labyrinthine environment of the oviduct [61], and penetrate the viscous mucoid environment encountered in the female reproductive tract [60, 62] (for review see [63]). The precise motility pattern that defines hyperactivation is species-specific; however, it is generally characterized by increases in lateral head displacement, flagellar bend amplitude and beat asymmetry [25, 56, 64].

Capacitation and hyperactivation are often linked together in the continuum of changes that a sperm must undergo to fertilize an oocyte; moreover, both processes require calcium, bicarbonate and the activation of

cAMP synthesis [19, 27, 65, 66]. However, the pathways that regulate these events are considered separable and independent [63, 67, 68]. This has been demonstrated in Catsper null mutant mice whereby disruption of any CATSPER proteins (which form alkaline activated calcium channels) resulted in normal sperm production, protein tyrosine phosphorylation patterns and rates of acrosomal exocytosis; however, the Catsper null sperm failed to hyperactivate, could not penetrate zona-intact oocytes and as a consequence these mice were infertile [24, 57]. In addition, *in vitro*-incubated bovine sperm that exhibit markers of capacitation do not hyperactivate; however, hyperactivation can be exogenously induced in capacitated as well as non-capacitated sperm by exposure to procaine [68], further supporting the hypothesis that capacitation and hyperactivation are independent processes. Although the exact mechanism by which procaine induces hyperactivation is not known, it is believed to stimulate calcium influx by increasing the permeability of the plasma membrane to calcium [67].

Although Rathi *et al.* [43] designated certain motility parameters (as measured by computer assisted semen analysis (CASA)) as indicative of hyperactivated motility in stallion sperm, none of the treatments tested in that study significantly affected the proportions of sperm showing these patterns. In fact, after 5 h of incubation under capacitating conditions, only 35% of sperm were motile, of which 5.3% were considered hyperactivated as defined by a curvilinear velocity (VCL) greater than 180  $\mu\text{m}/\text{sec}$  and an amplitude of lateral head displacement (ALH) greater than 12  $\mu\text{m}$ . Incubation in the presence of 1  $\mu\text{M}$  calcium ionophore to induce hyperactivation had a negative effect on motility such that no sperm were motile after 3.5 h of incubation [43]. Thus, the pattern of hyperactivation and its associated measures of motility have not been defined

in stallion sperm.

### ***IN VITRO* FERTILIZATION IN THE HORSE**

Only two foals have ever been produced using *in vitro* fertilization (IVF) technology [18] despite decades of work addressing this topic. Though numerous groups have attempted to replicate this result, there has been limited critical investigation into the requirements for horse IVF protocols since the first report was published more than 15 years ago [12-15, 69, 70]. Protocols used were typically derived from bovine IVF protocols, including incubating sperm in the presence of calcium ionophore, heparin or mixed semen samples in which sperm from multiple stallions were incubated together [69], without any investigation into the specific incubation conditions required for this species [71]. Fertilization rates have ranged from 0% to 33% [12] and these results were inconsistent even between studies performed by the same laboratories [13-15].

Protocols with the most success are those in which sperm were incubated in the presence of calcium ionophore, which artificially increases intracellular calcium levels leading to acrosomal exocytosis; fertilization rates of untreated sperm are essentially 0% [12, 18]. Other reports have included partially or completely removing the zona pellucida and zona drilling. These techniques resulted in high rates of polyspermic fertilization, upwards of 65% [70].

It appears that the failure of the sperm to penetrate the zona pellucida is not due to premature hardening of the zona (i.e. cortical granule release) since IVF was equally unsuccessful with *in vivo*-matured and *in vitro*-matured mare oocytes [18], and oviductal transfer of *in vitro*-matured oocytes to inseminated mares results in fertilization rates comparable to those obtained by natural

mating (77%) [11]. Therefore, it is likely that the low sperm penetration rates observed in IVF studies have stemmed from the inability to appropriately capacitate and/or hyperactivate stallion sperm in the laboratory. Altogether, it is clear from the information presented that a critical study into the molecular pathways supporting stallion sperm capacitation, and ultimately IVF, is required.

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## CHAPTER 2

# A DEFINED MEDIUM SUPPORTS CHANGES CONSISTENT WITH CAPACITATION IN STALLION SPERM AS EVIDENCED BY INCREASES IN PROTEIN TYROSINE PHOSPHORYLATION AND HIGH RATES OF ACROSOMAL EXOCYTOSIS<sup>1</sup>

### ABSTRACT

Efficient *in vitro* capacitation of stallion sperm has not yet been achieved as suggested by low sperm penetration rates reported in *in vitro* fertilization (IVF) studies. Our objectives were to evaluate defined incubation conditions that would support changes consistent with capacitation in stallion sperm. Protein tyrosine phosphorylation events and the ability of sperm to undergo acrosomal exocytosis under various incubation conditions were used as endpoints for capacitation. Sperm incubated 4-6 h in modified Whitten's (MW) with the addition of 25 mM NaHCO<sub>3</sub> and 7 mg/ml BSA (capacitating medium) yielded high rates of protein tyrosine phosphorylation. Either HCO<sub>3</sub><sup>-</sup> or BSA was required to support these changes, with the combination of both providing the most intense results. When a membrane permeable form of cAMP and a phosphodiesterase inhibitor (IBMX) were added to MW in the absence of HCO<sub>3</sub><sup>-</sup> and BSA, the tyrosine phosphorylation results obtained in our capacitating conditions could not be replicated, suggesting either effects apart from cAMP were responsible for tyrosine phosphorylation, or that stallion sperm might respond differently to these reagents as compared to sperm from other mammals. Sperm incubation in capacitating conditions was also associated with high percentages ( $P \leq 0.001$ ) of acrosomal exocytosis upon

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<sup>1</sup> McPartlin, LA, Littell, J, Mark, E, Nelson, JL, Travis, AJ, Bedford-Guaus, SJ. *Theriogenology*. 2008 Mar 15;69(5):639-50. License # 2238310704841

exposure to progesterone (44.6%) or calcium ionophore (51.6%), as compared to sperm incubated in medium devoid of BSA and NaHCO<sub>3</sub>. Our results are novel in that we report protein tyrosine phosphorylation in stallion sperm incubated in defined conditions coupled with significant percentages of acrosome reacted sperm. The continuation of these studies might help elucidate the conditions and pathways supporting sperm capacitation in the horse.

## INTRODUCTION

Ejaculated mammalian spermatozoa are not immediately able to fertilize an oocyte but acquire this ability during transit within the female reproductive tract [1]. The process that renders sperm fertilization-competent is termed “capacitation” and in several species it can be mimicked in the laboratory by incubating sperm in defined media and conditions which vary slightly between species [2-5]. Media requirements for *in vitro* capacitation usually include a sterol acceptor (typically BSA), bicarbonate, calcium and energy sources in all species studied to date.

Although the molecular and biochemical changes leading to sperm capacitation are still under investigation, it has been shown that this process is associated with changes in sperm plasma membrane fluidity, intracellular ion concentrations, metabolism and motility [4,6-12]. Changes in membrane fluidity are postulated to occur as a result of sterol efflux from the plasma membrane; in this regard BSA can be used as a sterol acceptor during *in vitro* capacitation [13-15]. Changes in membrane conformation then impact enzymatic function and/or ion transport. For example, calcium and bicarbonate enter the cell and activate soluble adenylyl cyclase (sAC), amongst other functions, with a resulting increase in intracellular cAMP levels. Altogether, these events lead to

an increase in protein kinase A (PKA) activity, which is then transduced into a series of protein tyrosine phosphorylation events [4,16].

In species other than the horse, protein tyrosine phosphorylation has been correlated with capacitation and thus the ability of sperm to undergo acrosomal exocytosis [15,16] and/or to fertilize an oocyte *in vitro* [4]. However, in the stallion, evidence for tyrosine phosphorylation of sperm proteins has only been reported for samples incubated in media supplemented with reagents that increase cAMP levels, such as dibutyryl cAMP (dbcAMP) and phosphodiesterase inhibitors (i.e. caffeine), or reactive oxygen species [17,18]. Use of such chemicals bypasses signaling pathways in response to external stimuli, membrane changes, and the activation of downstream effectors.

Additionally, attempts to induce the acrosome reaction in stallion sperm incubated with agonists such as porcine zona pellucida, progesterone, heparin and calcium ionophore [19-24], amongst others, have resulted in variable and generally less than ideal results when compared to those reported in other species [4,15,25]. Moreover, only two foals have ever been produced using *in vitro* fertilization (IVF) technology [19] and these results have not been repeatable as numerous attempts at IVF have resulted in low sperm penetration rates [26-30]. We suspect that this stems from the inability to capacitate horse sperm in the laboratory appropriately. Therefore, the objective of this study was to evaluate critically *in vitro* incubation conditions supporting protein tyrosine phosphorylation and acrosomal exocytosis as endpoints for stallion sperm capacitation.

## MATERIALS AND METHODS

*Chemicals and Reagents.* Four-Bromo-Calcium Ionophore A23187 was obtained from Calbiochem (San Diego, CA, USA). Tween 20 was purchased from BioRad (Hercules, CA, USA). Anti-phosphotyrosine monoclonal antibody (mAB), 4G10, was purchased from Upstate Biotechnology (Lake Placid, NY, USA). PNA-Alexa 488 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

*Culture Media.* Three different media were initially tested for our experiments to determine which supported both the best longevity of sperm motility and the highest levels of protein tyrosine phosphorylation. Media used included: Sperm TALP (spTALP; 100 mM NaCl, 3.10 mM KCl, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 21.6 mM sodium lactate, 2.0 mM CaCl<sub>2</sub>, 0.4 mM MgCl<sub>2</sub>, 10.0 mM HEPES, and 1.0 mM pyruvate) [31]; Biggers, Whitten, and Whittingham (BWW; 91.06 mM NaCl, 4.78 mM KCl, 2.44 mM MgSO<sub>4</sub>, 1.17 mM KPO<sub>4</sub>, 21.0 mM HEPES, 5.55 mM glucose (anhydrous), 0.25 mM sodium pyruvate, 1.71 mM lactic acid hemicalcium salt and 21.55 mM sodium lactate) [17]; and modified Whitten's (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt and 1.0 mM pyruvic acid) [12]. Sperm TALP is typically used to capacitate bovine sperm [32] and has been widely used in attempts to capacitate stallion sperm as reported in the literature [22,33,34]; BWW is a human IVF medium and has also been used for stallion sperm capacitation [17,35]; and MW is a murine sperm capacitation medium [11].

Negative control base medium was always devoid of BSA and HCO<sub>3</sub><sup>-</sup> and served both as transport and non-capacitating medium. Capacitating conditions

were optimized during these experiments, and unless otherwise indicated, capacitation was achieved by adding 25 mM NaHCO<sub>3</sub> and 7 mg/ml BSA to each NC base media. For all media tested, the final pH was brought to 7.25.

*Semen Collection and Preparation.* Semen was collected with an artificial vagina from five adult stallions of proven fertility in compliance with IACUC guidelines. After visual evaluation of sperm motility under light microscopy (200x) on a heated stage and assessment of sperm concentration using a 534B MOD1 Densimeter (Animal Reproduction Systems; Chino, CA, USA), the sperm rich fraction was diluted 2:1 (vol:vol) in the appropriate pre-warmed non-capacitating medium and transported to the laboratory at 37 °C for immediate processing. Samples were centrifuged in 15-ml conical tubes at 100 x g for 1 min (37 °C) to remove particulate matter and dead sperm. The supernatant was then transferred to a 14-ml round bottom centrifuge tube and centrifuged at 650 x g for 5 min (37 °C), and the resultant loosely-packed sperm pellet was resuspended in the appropriate media to a final concentration of 10 x 10<sup>6</sup> sperm/ml. Samples were incubated in 500 µL aliquots in polyvinyl alcohol coated 5-ml round bottom tubes [36] at 37 °C in a humidified air atmosphere, unless otherwise stated.

For experiments in which it was necessary to prevent the formation of HCO<sub>3</sub><sup>-</sup> from CO<sub>2</sub> in surrounding air or trapped within the medium, sperm were incubated under a nitrogen atmosphere. For this purpose, the medium was saturated by bubbling it with nitrogen gas for at least 1 min. Thereafter, all pre-filled tubes were gassed with nitrogen to displace any air; great care was taken to minimize the time of air exposure, and tubes were immediately capped tightly for incubation.

*Sperm Processing.* At different time-points following incubation, percent total and progressive motility was assessed either subjectively using light microscopy on a heated stage (200x) or by using computer assisted semen analysis (CASA; Hamilton Thorne Bioscience, Beverly, MA, USA), depending on individual experiments. Samples were then transferred into siliconized microcentrifuge tubes containing 5  $\mu$ L of sodium ortho-vanadate (2 mM) in phosphate buffered saline (PBS) to inhibit endogenous phosphatases. Samples were then processed for SDS-PAGE as previously described [15]. Briefly, samples were centrifuged at 2,000 x g for 2 min at 4 °C, washed in 1.5 ml of PBS, and the final pellet was resuspended in 30  $\mu$ L PBS plus 8  $\mu$ L of 5X sample buffer [37] containing DTT (40mM), and boiled for 5 min. The samples were then centrifuged at 7500 x g for 2 min at 4 °C and subjected to SDS-PAGE.

*SDS-PAGE and Immunoblotting.* The total volume of extract corresponding to equal numbers of sperm in each sample ( $5 \times 10^6$  sperm) was loaded on 10% SDS gels. Separated proteins were blotted onto Immobilon P membranes (Millipore, Inc.; Billerica, MA, USA), Ponceau stained to confirm even protein load and blocked for 1 h with 1:10 (vol:vol) dilution of cold water fish skin gelatin in TBS with 1% Tween 20. This solution was used for all subsequent antibody incubations and washes. Immunodetection of proteins was performed at room temperature using a monoclonal antibody against phosphotyrosine at a 1:10,000 dilution for 1 h, washed extensively (1 h, four exchanges) and incubated for 30 min with goat anti-mouse horseradish peroxidase-coupled IgG. After extensive washing of blots, immunoreactivity was visualized using enhanced chemiluminescence detection with an ECL kit (Amersham Corp.; Piscataway, NJ, USA) according to the manufacturer's

directions. Experiments shown are representative of at least three replicates with at least three different stallions tested for each experiment.

For densitometry analysis, three immunoblots from three different stallions were scanned and the relative intensity of two proteins (~ 150 kD and ~ 114 kD) was determined within each experiment using a gel-scanning macro for ImageJ software (<http://rsb.info.nih.gov/ij/index.html>).

*Acrosome Reaction Assay.* At 0 and 6 h of incubation, sperm samples in different media conditions were further incubated for 30 min with 3.2  $\mu$ M progesterone ( $P_4$ ), 5  $\mu$ M calcium ionophore A23187 (Cal), or an equal volume of vehicle control (2.5  $\mu$ l DMSO; 0.5% vol:vol ) to induce the acrosome reaction. At the end of each incubation period the percentages of total and progressively motile sperm were assessed with light microscopy on a heated stage (200x). Acrosomal status of non-fixed, non-permeabilized, live sperm was assessed with the addition of PNA-Alexa 488 (final concentration 0.024 mg/ml) [22] to a 125  $\mu$ L aliquot of the sperm suspension; 100  $\mu$ L was then pipetted onto a glass slide on a heated stage. Sperm were allowed to settle for 1 min and excess medium was removed by pipetting. The sample was then gently washed with warm PBS, coverslipped and scored (600x) for acrosomal status using an upright fluorescent Zeiss Imager ZI microscope with Green Fluorescent Protein (GFP) filters. One hundred morphologically normal sperm were counted per slide and evaluation was performed blindly of treatment status.

*Statistical Analysis.* Statistical analysis was performed using SigmaStat software (San Jose, CA, USA). Data for percent motility were analyzed by repeated measures ANOVA. Statistical analysis for the acrosome induction experiment was performed by repeated measures four-way ANOVA for each of the three possible outcomes (intact, intermediate, reacted) with all stallion,

treatment, time and inducing agent, as well as their interactions, as main effects. Data for relative densitometry for each of two immunoblotted protein bands (~ 150 kD and ~ 114 kD) was analyzed by two-way ANOVA, with both treatment and incubation time, as well as their interaction, as main effects. When significant differences were detected ( $P < 0.05$ ) the Student-Newman-Keuls test was applied to assess all pairwise multiple comparisons.

## **RESULTS**

### *Media Comparison*

Our first objective was to identify an incubation medium that would support sperm motility for the duration of the 6 h incubation period while yielding time-dependent increases in protein tyrosine phosphorylation. For this purpose we compared three different media: spTALP, BWW and MW, using four different stallions during both the breeding season and the winter months. While all three media yielded some increase in protein tyrosine phosphorylation in a time-dependent manner, MW most consistently supported marked levels of tyrosine phosphorylation independent of stallion and season. As shown in Table 2.1, MW also supported the highest percentage of total and progressively motile sperm, with differences appearing at 4 h and continuing throughout the 6 h incubation when compared to sp-TALP ( $P < 0.03$ ). For these reasons, MW was chosen for all subsequent experiments. Shown in Figure 2.1 is a representative immunoblot of the tyrosine phosphorylation levels achieved under capacitating versus non-capacitating conditions with MW. Protein tyrosine phosphorylation levels differed ( $P < 0.01$ ) between the two incubation conditions for both protein bands analyzed (~ 150 kD and ~ 114 kD), both at 2 and 4 h.



### *Effects of BSA and NaHCO<sub>3</sub> in the Capacitation Medium*

In all species studied to date, sperm must be capacitated in a medium containing a sterol acceptor, typically BSA, bicarbonate and calcium [2,3,13,38,39]. Therefore, as a first step, we incubated sperm in medium containing 1, 5, 7 and 10 mg/ml BSA, in the presence of 25 mM HCO<sub>3</sub><sup>-</sup>, for 0, 2 and 4 h to establish the optimal concentration requirement of BSA in regards to tyrosine phosphorylation. Sperm incubated in medium devoid of BSA and NaHCO<sub>3</sub> served as our negative control. All concentrations of BSA tested supported higher levels of tyrosine phosphorylation than the non-capacitating condition (Figure 2.2; P<0.05) for the protein band at ~ 150 kD. However, concentrations of BSA between 5-10 mg/ml were superior to 0-1 mg/ml at supporting sperm longevity of motility (data not shown). Based on these results we chose the medium range concentration of 7 mg/ml BSA for our capacitating conditions thereafter.

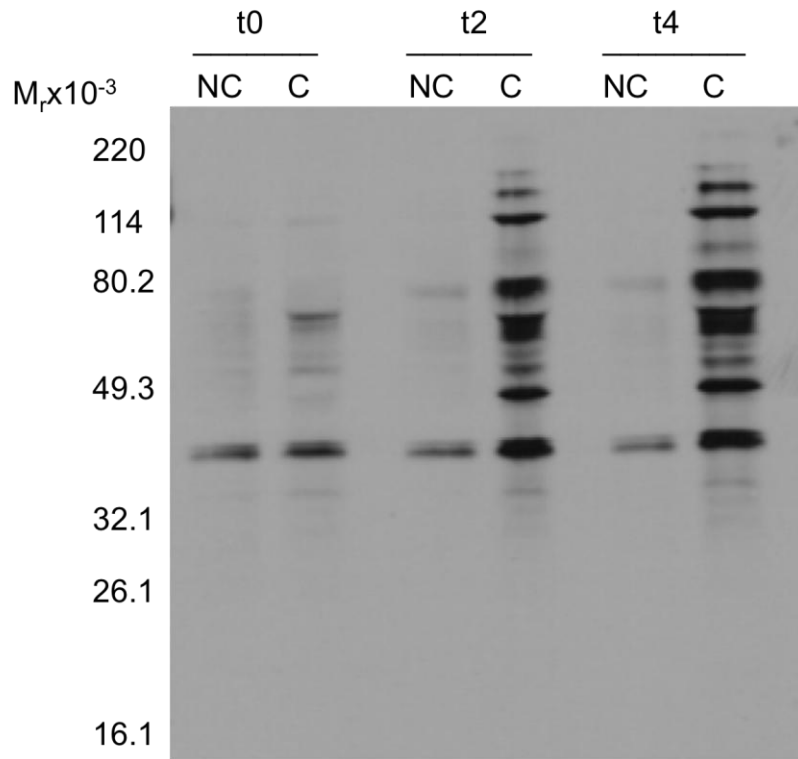
We then compared the individual and combined effects of BSA and HCO<sub>3</sub><sup>-</sup> for their ability to support tyrosine phosphorylation. The concentration of 25 mM NaHCO<sub>3</sub> was chosen based on sperm capacitation reports from other large animal species [5,40]. When sperm were incubated in an air atmosphere, medium containing BSA but not NaHCO<sub>3</sub> was consistently better at supporting both sperm motility and tyrosine phosphorylation of protein residues than the non-capacitating base medium (Figure 2.3A; P≤0.02) for both protein bands analyzed. Conversely, levels of tyrosine phosphorylation did not differ (P>0.05) between sperm incubated in non-capacitating medium and medium containing NaHCO<sub>3</sub> with no BSA. The combination of both BSA and HCO<sub>3</sub><sup>-</sup> (capacitating

**Table 2.1.** Total and progressive motility data for stallion sperm incubated in three different capacitating media.

Medium	t0 h		t2 h		t4 h		t6 h	
	Total *	Progressive	Total	Progressive	Total	Progressive	Total	Progressive
spTalp	78 (3.9)	60 (8.0)	66 (4.2)	44 (6.0)	58 <sup>a</sup> (6.7)	40 (7.8)	41a (6.7)	26 <sup>a</sup> (6.2)
BWW	79 (2.9)	66 (4.3)	72 (5.1)	56 (5.2)	72 <sup>a,b</sup> (4.0)	56 (3.8)	68b (4.5)	54 <sup>b</sup> (4.0)
MW	75 (2.6)	56 (5.8)	80 (3.1)	61 (5.5)	76 <sup>b</sup> (3.3)	58 (5.2)	72b (4.0)	55 <sup>b</sup> (3.9)

a,b Within a column, means without a common superscript differed ( $P < 0.05$ ). Results are means from 13 different experiments.

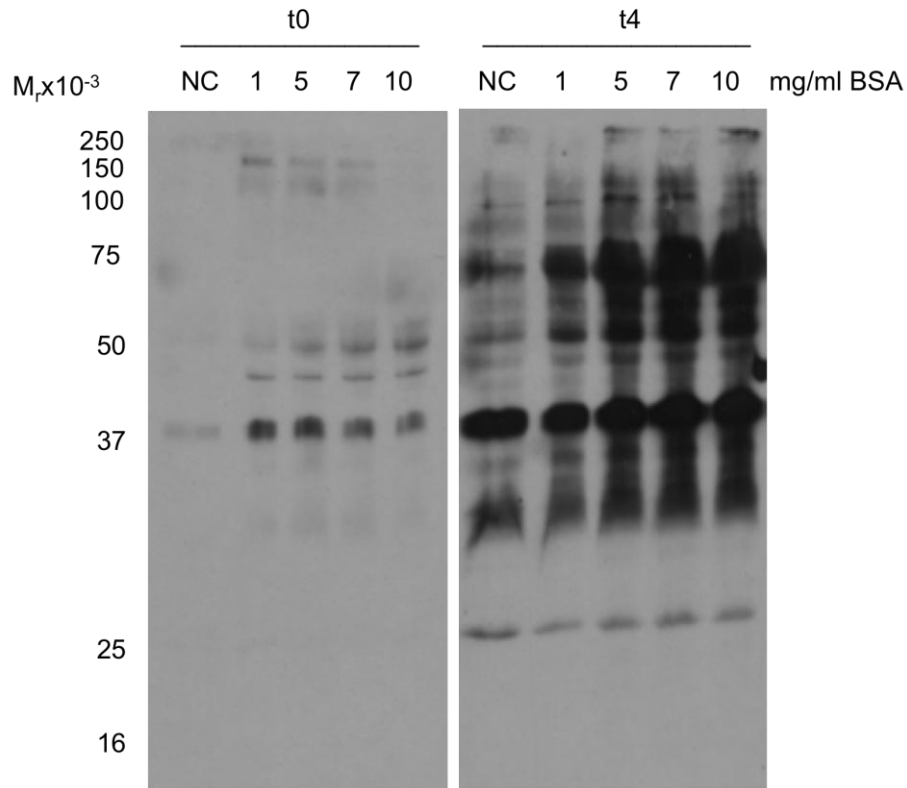
\* Data are expressed as % (S.E.M.).



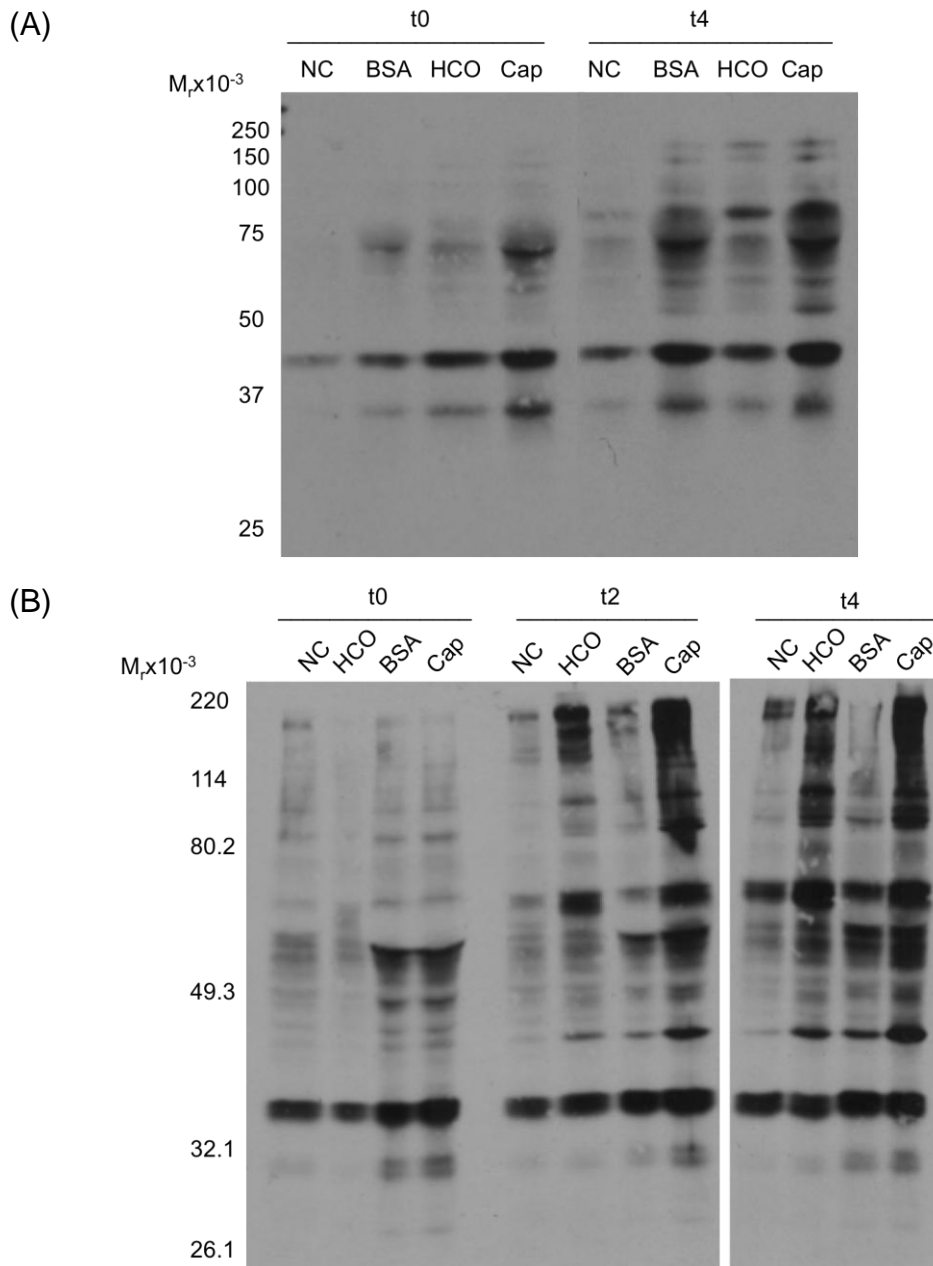
**Figure 2.1.** Representative immunoblot for protein tyrosine phosphorylation in stallion sperm incubated in an air atmosphere at 37°C in modified Whitten's for 0, 2 and 4 h. Capacitation medium (C) contained 25 mM NaHCO<sub>3</sub> and 7 mg/ml BSA; non-capacitating medium (NC) was devoid of NaHCO<sub>3</sub> and BSA. Protein bands at ~150 and ~114 kDa were used for statistical analysis. Densitometric analysis of these bands yielded differences ( $P < 0.05$ ) between NC and C at both 2 and 4 h.

condition) yielded the highest levels of protein tyrosine phosphorylation when compared to all other conditions ( $P < 0.05$ ). However, in an air atmosphere,  $\text{CO}_2$  mixing with water in the medium generates carbonic acid, and therefore bicarbonate. Consequently we suspected that the effectiveness of BSA alone might have been due to the presence of small amounts of  $\text{HCO}_3^-$  forming from exchange with the surrounding air. To investigate this possibility, the same experiment was repeated under nitrogen saturation. When incubated under a nitrogen atmosphere, BSA alone did not provide an advantage over  $\text{HCO}_3^-$  alone when compared to sperm incubated in the non-capacitating condition ( $P > 0.05$ ). As in the previous experiment, our capacitating conditions (BSA +  $\text{HCO}_3^-$ ) provided the most intense pattern of tyrosine phosphorylation ( $P < 0.01$ ).

The above findings suggested that a very small concentration of  $\text{HCO}_3^-$  in BSA-containing medium should be sufficient to support tyrosine phosphorylation events in stallion sperm. Therefore we compared medium containing 1, 5, 10 and 25 mM  $\text{NaHCO}_3$  in the presence of 7 mg/ml BSA under a  $\text{N}_2$  atmosphere. Although not significant ( $P = 0.08$ ), as little as 1 mM  $\text{HCO}_3^-$  was sufficient to consistently induce an increase in protein tyrosine phosphorylation (Figure 2.4). Over time, both 10 and 25 mM  $\text{NaHCO}_3$  supported the highest levels of protein tyrosine phosphorylation ( $P < 0.05$ ) when compared to the non-capacitating condition for both bands analyzed. Furthermore, these higher concentrations of  $\text{HCO}_3^-$  were required to promote tyrosine phosphorylation in the higher molecular weight proteins (75-250 kD;  $P < 0.05$ ). For these reasons and because 25 mM  $\text{HCO}_3^-$  approximates the concentration present in body fluids [41], presumably what sperm encounter when traveling through the female's reproductive tract, the higher concentration was chosen for subsequent experiments.



**Figure 2.2.** Representative immunoblot for protein tyrosine phosphorylation in stallion sperm incubated in an air atmosphere at 37°C in modified Whitten's for 0 and 4 h. Capacitation medium (C) contained 25 mM NaHCO<sub>3</sub> and various concentrations (1, 5, 7 and 10 mg/ml) of BSA; non-capacitating medium (NC) was devoid of NaHCO<sub>3</sub> and BSA. Protein bands at ~150 and ~114 kDa were used for statistical analysis. Medium containing BSA supported higher levels ( $P < 0.05$ ) of protein tyrosine phosphorylation when compared to the NC condition.



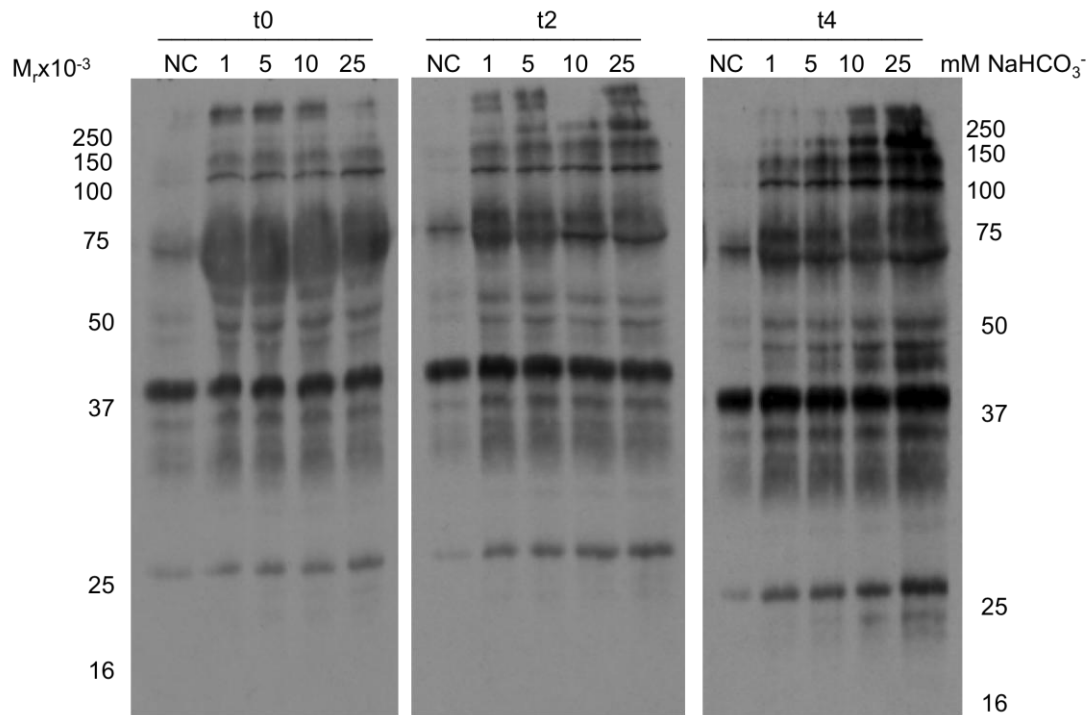
**Figure 2.3.** Representative immunoblots for protein tyrosine phosphorylation in stallion sperm. Non-capacitating medium (NC) was devoid of  $\text{NaHCO}_3$  and BSA; the BSA lane contained 7 mg/ml BSA and no  $\text{NaHCO}_3$ ; the HCO lane contained 25 mM  $\text{NaHCO}_3$  and no BSA; capacitation medium (Cap) contained 25 mM  $\text{NaHCO}_3$  and 7 mg/ml BSA. Protein bands at ~150 and ~114 kDa were used for statistical analysis. (A) Sperm were incubated in an air atmosphere at 37°C in modified Whitten's for 0 and 4 h. BSA supported higher levels of protein tyrosine phosphorylation than NC ( $P < 0.02$ ). (B) Sperm were incubated in a  $\text{N}_2$  saturated atmosphere at 37°C in modified Whitten's for 0 and 4 h. For both immunoblots (A and B), Cap yielded higher levels of protein tyrosine phosphorylation than all other conditions ( $P < 0.05$ ).

### *Evaluation of Exogenous cAMP Pathway Modulators*

Protein tyrosine phosphorylation of sperm undergoing capacitation is believed to occur via a cAMP-PKA-dependent pathway. To test for a role of cAMP as a key mediator of tyrosine phosphorylation in stallion sperm, we compared our non-capacitating and capacitating conditions, both with and without the addition of 1 mM dbcAMP and 0.1 mM 3-Isobutyl-1-methylxanthine (IBMX) [4,5]. As expected, these reagents initially accelerated the rate of protein tyrosine phosphorylation in medium containing BSA and  $\text{HCO}_3^-$  as compared to sperm incubated under our capacitating conditions (Figure 2.5;  $P \leq 0.002$  at 2 h); however, at 4 and 6 h of incubation there were no differences ( $P > 0.05$ ) between the two conditions for both protein bands analyzed. Notably, sperm capacitated in the presence of dbcAMP and IBMX had lower total motility at 6 h than sperm incubated in capacitating conditions without these reagents (56% vs. 73%, respectively;  $P < 0.05$ ). Unexpectedly, sperm incubated in medium devoid of BSA and  $\text{HCO}_3^-$  but with the addition of the cAMP agonist and phosphodiesterase inhibitor (lane NC<sup>++</sup>) did not display a time-dependent increase in tyrosine phosphorylation when compared to our non-capacitating condition ( $P > 0.05$ ), as it has been reported to occur in other species.

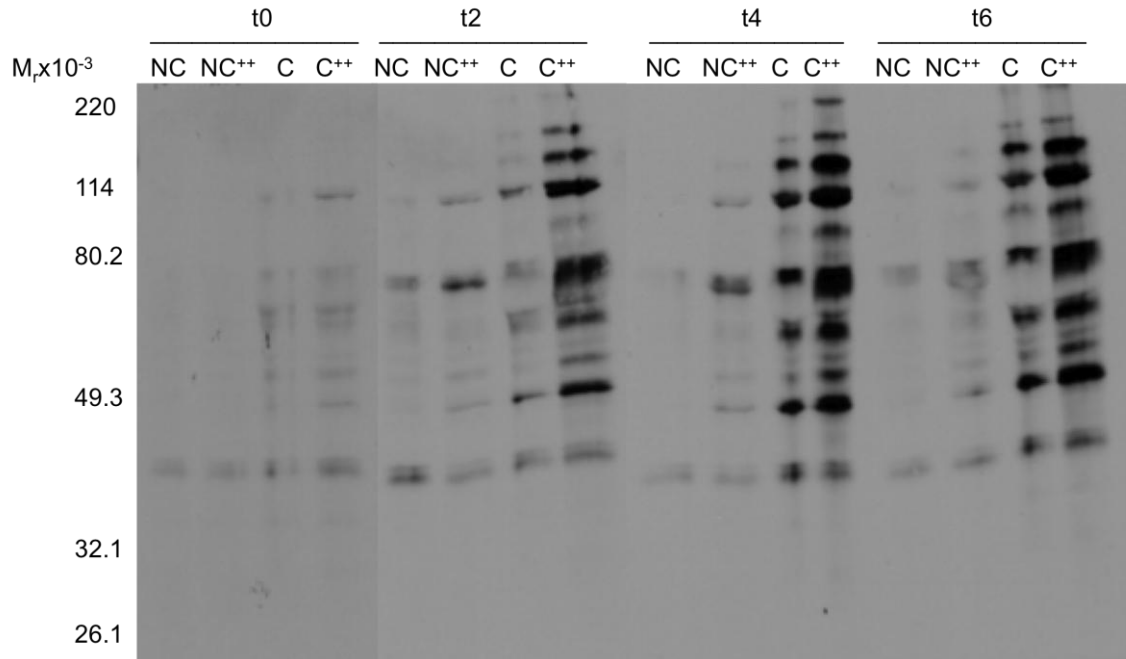
Because the addition of dbcAMP and IBMX to non-capacitating conditions did not support the intensity of tyrosine phosphorylation observed under our capacitating conditions, we predicted that the presence of either BSA or  $\text{HCO}_3^-$ , or both, was essential to facilitate these events. To test these possibilities we incubated sperm in medium containing dbcAMP/IBMX in the presence of BSA and/or  $\text{HCO}_3^-$  under a nitrogen atmosphere (Figure 2.6). Interestingly, when either BSA or  $\text{HCO}_3^-$  was present in conjunction with dbcAMP and IBMX, increases in tyrosine phosphorylation levels were

comparable to those seen in capacitating medium with and without these reagents ( $P > 0.05$ ).

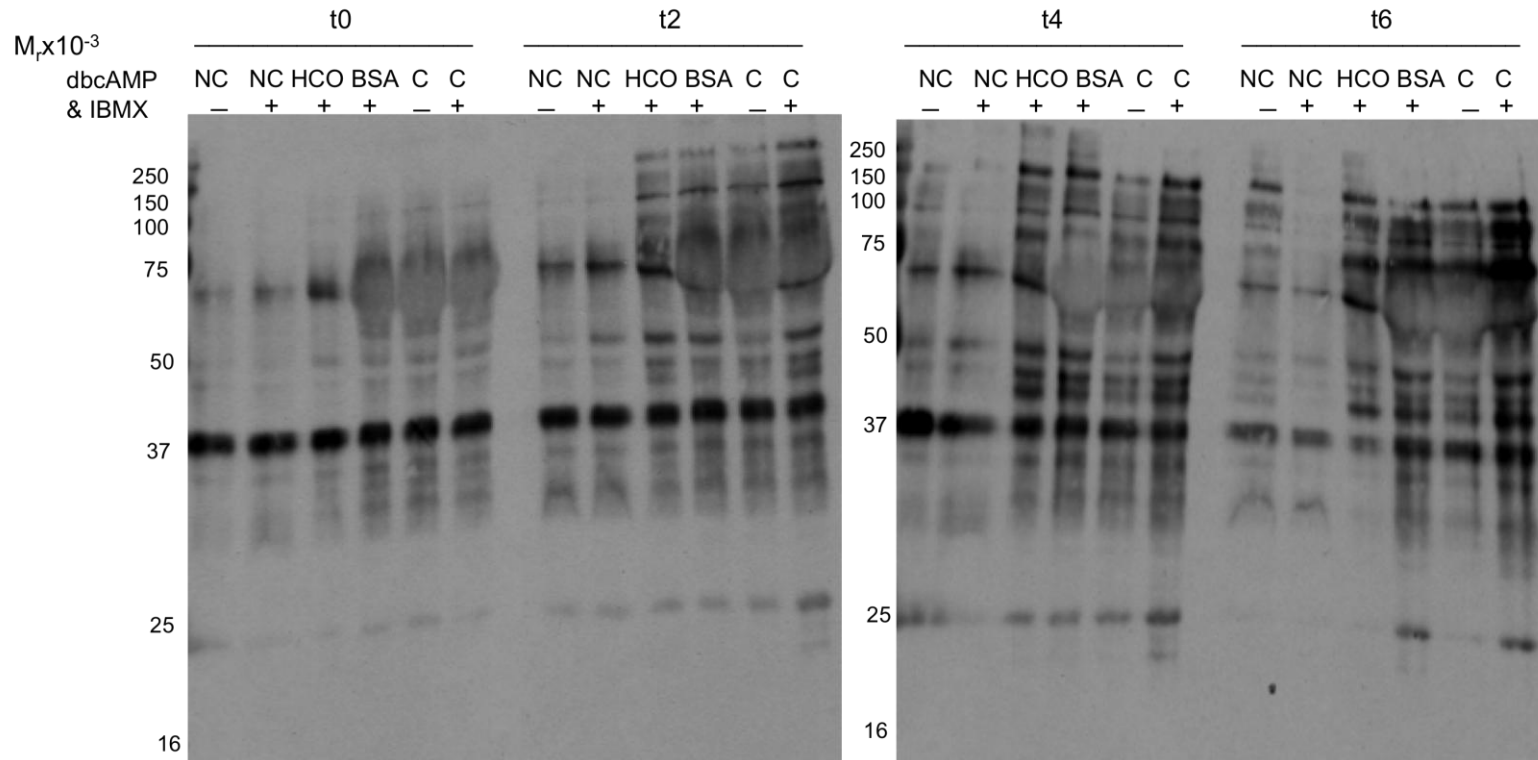


**Figure 2.4.** Representative immunoblot for protein tyrosine phosphorylation in stallion sperm incubated in a  $N_2$  atmosphere at  $37^\circ C$  in modified Whitten's for 0, 2 and 4 h. Non-capacitating medium (NC) was devoid of  $NaHCO_3$  and BSA; remaining lanes contain 7 mg/ml BSA plus various concentrations of  $NaHCO_3$  (1, 5, 10 and 25 mM). Protein bands at  $\sim 150$  kD and  $\sim 114$  kD were used for statistical analysis. Over time, both 10 and 25 mM  $NaHCO_3$  supported the highest levels of protein tyrosine phosphorylation ( $P < 0.05$ ) when compared to NC for both bands analyzed.





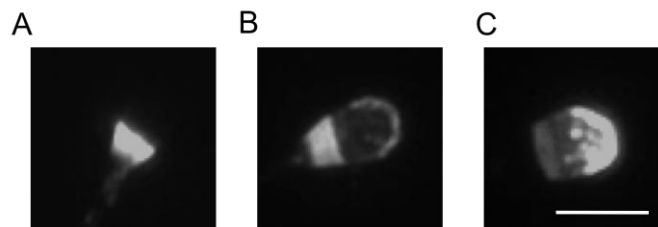
**Figure 2.5** Representative immunoblot for protein tyrosine phosphorylation in stallion sperm incubated in an air atmosphere at 37°C in modified Whitten's for 0, 2, 4 and 6 h with and without the addition of cAMP modulators. Non-capacitating medium is devoid of both NaHCO<sub>3</sub> and BSA (NC); non-capacitating medium plus the addition of 1 mM dbcAMP and 0.1 mM IBMX (NC++); capacitation medium contains 25 mM NaHCO<sub>3</sub> and 7 mg/ml BSA (C); capacitation medium plus the addition of 1 mM dbcAMP and 0.1 mM IBMX (C++). Protein bands at ~150 kD and ~114 kD were used for statistical analysis. C and C++ were different ( $P \leq 0.002$ ) at 2 h, but not at 0, 4 or 6 h ( $P > 0.05$ ). Over time, both C++ and C yielded higher levels of protein tyrosine phosphorylation than NC and NC++ ( $P < 0.01$ ).



**Figure 2.6.** Representative immunoblot for protein tyrosine phosphorylation in stallion sperm incubated in a  $N_2$  atmosphere at  $37^\circ C$  in modified Whitten's for 0, 2, 4 and 6 h with (+) and without (-) addition of the cAMP modulators dbcAMP (1 mM) and IBMX (0.1 mM). Non-capacitating medium (NC) is devoid of both  $NaHCO_3$  and BSA; HCO lane contains 25 mM  $NaHCO_3$  and no BSA; BSA lane contains 7 mg/ml BSA and no  $NaHCO_3$ ; capacitation medium (C) contains 25 mM  $NaHCO_3$  and 7 mg/ml BSA. Protein bands at  $\sim 150$  kD and  $\sim 114$  kD were used for statistical analysis. Levels of tyrosine phosphorylation were comparable between the capacitating (C), BSA and HCO conditions both with and without the addition of cAMP modulators ( $P > 0.05$ ).

### *Acrosome Reaction Assay*

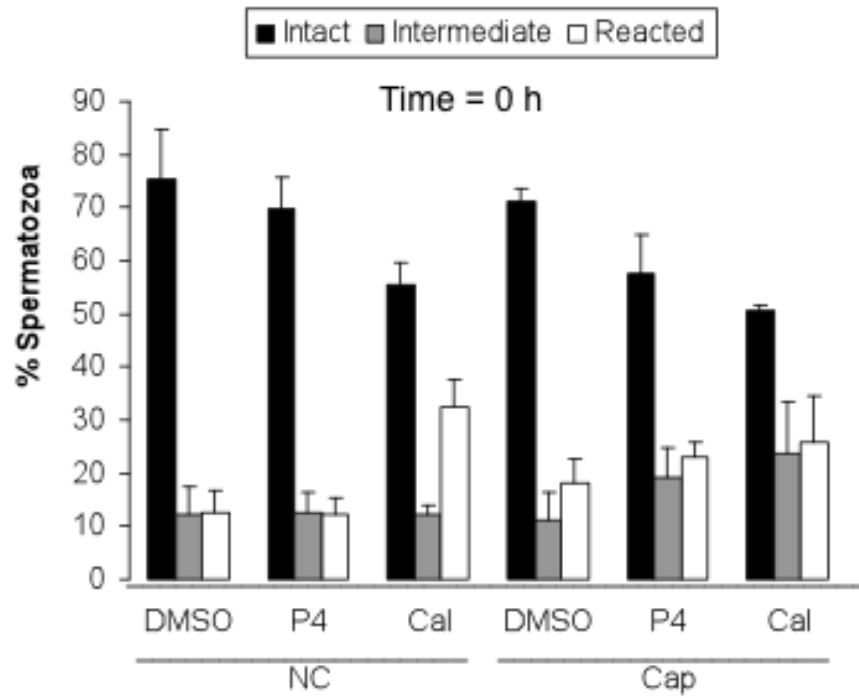
We next tested the ability of sperm incubated in capacitation conditions to undergo agonist-induced acrosomal exocytosis. To accomplish this, we exposed sperm previously incubated for 0 or 6 h in non-capacitating and capacitating conditions to a physiological concentration of progesterone, 3.2  $\mu\text{M}$  [42], 5  $\mu\text{M}$  calcium ionophore A23187, or DMSO (vehicle control) for 30 min. Sperm were scored by assessing the pattern of PNA-Alexa 488 fluorescence [22] and classified as acrosome intact (post acrosomal fluorescence only; Figure 2.7A), intermediate (fluorescence on the apical ridge of acrosome; Figure 2.7B) or acrosome reacted (bright acrosomal fluorescence; Figure 2.7C). At 0 h, there were no differences observed between media conditions or between treatments for any of the above classifications ( $P > 0.05$ ; Figure 2.8A). After 6 h of incubation in capacitating medium, both progesterone and calcium ionophore were able to induce higher percentages ( $P \leq 0.001$ ) of acrosome reacted sperm (44.6 % and 51.6%, respectively) when compared with exposure to DMSO (15.0%; Figure 2.8B). Conversely, there were no differences ( $P > 0.05$ ) in the percent of acrosome reacted sperm for any of the treatments when incubation (6 h) was performed in non-capacitation medium (Figure 2.8B). Furthermore, there were no significant interactions between treatment and stallion or ejaculate in any of the time points analyzed.



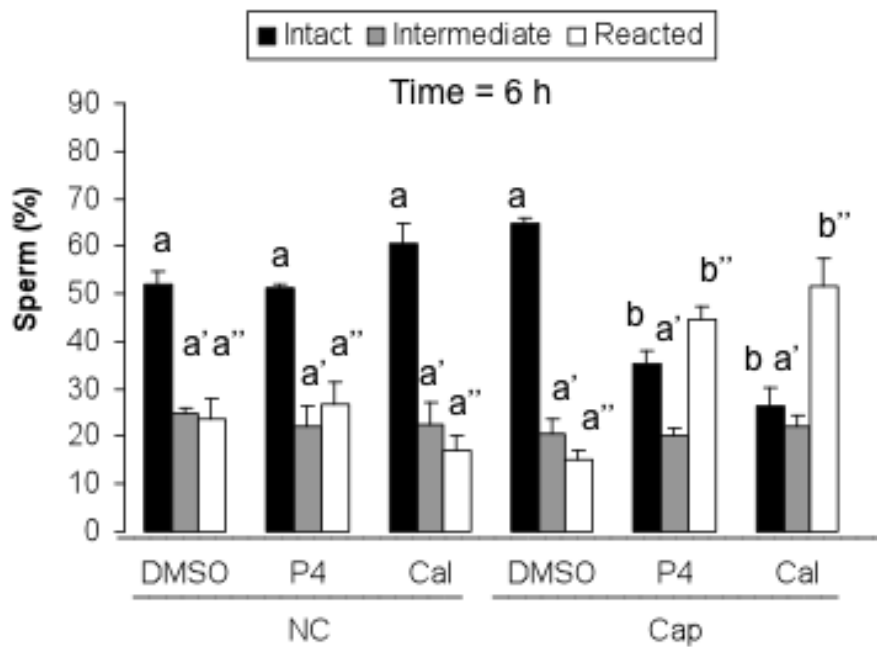
**Figure 2.7** PNA-Alexa 488 fluorescence pattern of: (A) an acrosome intact sperm lacking signal over the acrosomal cap; (B) an intermediately reacted sperm with signal restricted to the apical portion of the acrosomal cap; and (C) an acrosome reacted sperm with strong fluorescence over the entire acrosome. Scale bar represents 5  $\mu\text{m}$ .

**Figure 2.8.** Percent of three different acrosomal patterns detected by PNA-Alexa 488 under a fluorescent microscope at 600x. Sperm were incubated in an air atmosphere at 37°C in non-capacitating (NC) or capacitating medium (Cap) for 0 hours (A) and 6 hours (B). For acrosome reaction induction sperm were incubated for 30 min in vehicle control (DMSO; 2.5  $\mu$ l), progesterone (P4; 3.2  $\mu$ M) or calcium ionophore A23187 (Cal; 5  $\mu$ M). One-hundred sperm were scored per treatment at each time point and the percentages of each category (acrosome intact, intermediate or reacted) are reported. At 0 hr (A) there were no significant differences in the percent of acrosome intact, intermediate or reacted sperm for any of the conditions (NC or Cap) or inducing agents (DMSO, P4 or Cal). At 6 hr (B) each of the sets of superscripts (a,b; a',b' or a'',b'') represent differences ( $P \leq 0.001$ ) between treatments (NC and Cap) or acrosome reaction inducing agent (DMSO, P4 and Cal) for each of the three categories of sperm (intact, intermediate and reacted, respectively). Within incubation medium (NC or C) different superscripts for each of the treatments denote significant differences ( $P \leq 0.001$ ). Comparison of sperm incubated for 6 h in NC versus Cap yielded significant differences in the rates of acrosome intact (a,b) and reacted (a'',b'') sperm for both the progesterone and calcium ionophore treatments but not for DMSO ( $P < 0.001$ ). Within Cap, there were no differences ( $P > 0.05$ ) in the percent of acrosome intact, intermediate or reacted sperm between P4 and Cal.

**A**



**B**



## DISCUSSION

We show for the first time that significant levels of protein tyrosine phosphorylation can be achieved in stallion sperm incubated under defined conditions, in the absence of exogenous signaling intermediates that in other reports have bypassed upstream signaling events [17]. In other species, the molecular changes we show have been correlated with capacitation as defined by the ability of sperm to undergo an induced acrosomal exocytosis and/or to fertilize oocytes *in vitro* [43]. The observed levels of protein tyrosine phosphorylation coupled with the significant rates of acrosomal exocytosis reported herein suggest that sperm incubated in the conditions described underwent molecular and membrane changes that are consistent with capacitation.

In our laboratory, the medium that was best at supporting sperm motility coupled with the most consistent rates of protein tyrosine phosphorylation was modified Whitten's, which has been used to capacitate mouse sperm [12] and to support *in vitro* fertilization of mouse oocytes [11]. While optimizing the media requirements that support protein tyrosine phosphorylation, we demonstrated that BSA and  $\text{HCO}_3^-$  are critical components for tyrosine phosphorylation in stallion sperm as has been shown in other species. When the effect of each of these components was studied separately, we found that BSA was required to maintain sperm motility as well as to support an increase in protein tyrosine phosphorylation over time. Additionally, when another protein source, casein, was substituted for BSA, motility was also maintained; however, there was no increase in protein tyrosine phosphorylation (data not shown). It is worth noting that a very low concentration of  $\text{HCO}_3^-$  (1 mM) in the presence of BSA was sufficient to induce an increase in protein tyrosine phosphorylation levels when

compared to the non-capacitating condition (Figure 2.4;  $P=0.08$ ). In fact, low levels of  $\text{HCO}_3^-$  produced in the medium from exchange with the surrounding air were sufficient to induce marked changes in protein tyrosine phosphorylation (Figure 2.3A), suggesting that stallion sperm are more sensitive to  $\text{HCO}_3^-$ -induced tyrosine phosphorylation than sperm from other species studied to date [2-4].

In order to investigate the upstream signaling pathway leading to tyrosine phosphorylation of stallion sperm proteins, we added dbcAMP and IBMX to our non-capacitating medium. Because these chemicals are membrane permeable and have been shown to support increases in protein tyrosine phosphorylation in the absence of BSA and bicarbonate in other species [2-5,44], we anticipated that their addition to our non-capacitating medium would induce phosphorylation levels comparable to those observed in our capacitating conditions.

Unexpectedly, they were unable to produce these changes in the absence of BSA and/or  $\text{HCO}_3^-$ . Although Pommer et al. [17] showed increases in protein tyrosine phosphorylation when stallion sperm were incubated in the presence of dbcAMP and caffeine, the incubation medium used in this study always contained BSA. That study did not investigate the role of the cAMP modulators in the absence of a sterol acceptor, which could be critical in elucidating the specific requirements and pathways involved in stallion sperm capacitation.

Conceivably, changes in membrane fluidity and/or phospholipid scrambling induced by BSA and/or  $\text{HCO}_3^-$  might be playing key roles that can not be overcome by the use of the cAMP modulators alone. For example, bicarbonate enters the cell via a  $\text{Na}/\text{HCO}_3^-$  co-transporter [45] causing a rapid increase in plasma membrane disorder [33,46], most likely by inducing phospholipid scrambling [6,47]. This re-organization of the sperm plasma

membrane might have downstream effects similar to BSA, perhaps by activating the sAC-cAMP-PKA pathway directly or potentially through a positive feedback loop. A second possibility is that these reagents may not be as membrane permeable in stallion sperm in the absence of BSA and/or  $\text{HCO}_3^-$  as they are in other species [4,5,14], or perhaps that BSA and/or  $\text{HCO}_3^-$  might be exerting effects on tyrosine phosphorylation other than through the generation of cAMP. These possibilities are currently under investigation. In any event, these experiments demonstrate that either BSA or  $\text{HCO}_3^-$  needs to be present in order for these specific exogenous chemicals to exert their effects (Figure 2.6). These results are different than what has been previously shown in other species and further research to investigate possible causes for these findings is underway.

Using the lectin PNA to assess the percentage of acrosome reacted sperm, we were able to correlate our tyrosine phosphorylation results to another marker of capacitation. Indeed, after a 6 h incubation period, both progesterone and calcium ionophore were able to induce significantly higher percentages of acrosome reacted sperm in our capacitating condition as compared to sperm incubated in either non-capacitating medium or exposed to DMSO. Furthermore, the rates of acrosomal exocytosis in sperm incubated under capacitating conditions and exposed to either progesterone or calcium ionophore were higher than previously reported [20,24,48]. Since progesterone is considered a more physiological inducer of the acrosome reaction than calcium ionophore, these data strongly support the notion that sperm in this study had undergone the necessary molecular changes for acrosomal exocytosis.



Our results are an important step forward in efforts to capacitate stallion spermatozoa as evidenced both by the high rates of protein tyrosine phosphorylation as well as percentages of acrosome reacted sperm. Furthermore, our findings may lead to understanding the molecular mechanisms supporting sperm capacitation and the improvement of IVF success rates in the horse.

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## CHAPTER 3

### CYCLIC AMP ACTS VIA PROTEIN KINASE A OR GUANINE-NUCLEOTIDE EXCHANGE FACTORS TO MODULATE DIFFERENT CAPACITATION-ASSOCIATED EVENTS IN STALLION SPERM

#### **ABSTRACT**

Capacitation is a complex and poorly understood process that encompasses the molecular changes sperm must undergo to successfully fertilize an oocyte. Our laboratory has recently defined *in vitro* incubation conditions that yield time-dependent increases in protein tyrosine phosphorylation coupled with significant rates of progesterone-induced acrosomal exocytosis in equine sperm. Moreover, these incubation conditions support high rates of homologous *in vitro* fertilization, further demonstrating that sperm are capacitated. Recent experiments in our lab corroborate the notion that cAMP production is required for sperm capacitation. Due to the recent discovery of cAMP-dependent guanine nucleotide exchange factors EPAC1 and 2 in somatic cells and mammalian sperm, the objectives of this study were to investigate their role in capacitation-associated events. Indirect immunofluorescence localized EPAC1 to the acrosome and sub-acrosomal ring and EPAC2 to the midpiece in equine sperm. In murine sperm, EPAC1 and EPAC2 were also localized to the acrosome and midpiece, respectively. In somatic cells, activation of EPAC has been shown to activate the downstream MAPK pathway. Therefore, the EPAC-specific cAMP analogue 8pCPT was added to sperm incubated under both non-capacitating and capacitating conditions and the effects on protein tyrosine phosphorylation were evaluated. Based on one-dimensional electrophoresis, there were no changes in the pattern or levels of protein tyrosine phosphorylation seen at any time point in



either species. Activation of EPAC with 8pCPT did, however, induce acrosomal exocytosis in capacitated equine (34%) and murine sperm (37.3%) as compared to DMSO-vehicle controls (24.2% and 17%, respectively;  $P < 0.03$ ). These rates were similar to those obtained with progesterone and calcium ionophore-treated sperm ( $P > 0.05$ ). Reportedly, EPAC plays a role in the regulation of ion channels in somatic cells. Moreover, capacitation dependent hyperpolarization of the sperm plasma membrane has been shown to recruit low voltage activated (LVA) T-type  $\text{Ca}^{2+}$  channels, which later open in response to zona pellucida-induced membrane depolarization. We hypothesized that EPAC activation may be inducing acrosomal exocytosis via depolarization-dependent  $\text{Ca}^{2+}$  influx. To investigate this we first determined the resting membrane potential ( $E_m$ ) of non-capacitated equine sperm to be  $-37.11$  mV and the  $E_m$  of capacitated sperm to be hyperpolarized to  $-53.74$  mV ( $P = 0.002$ ). Interestingly, when equine sperm were incubated under capacitating conditions in the presence of 8pCPT,  $E_m$  remained in the depolarized state at  $-32.06$  mV, even after 6 hr of incubation. This result was similar to sperm in the non-capacitated state ( $P > 0.05$ ) but different ( $P < 0.001$ ) from capacitated sperm incubated in the absence of the EPAC activator. Taken together, these experiments demonstrate the presence of EPAC1 and 2 in equine and murine sperm, support the hypothesis that EPAC activation regulates acrosomal exocytosis via its modulation of membrane potential and demonstrate a novel role for EPAC in the regulation of sperm capacitation.

## INTRODUCTION

Ejaculated mammalian spermatozoa are not immediately able to fertilize an oocyte but acquire this ability during transit within the female reproductive tract through a process termed capacitation [1]. In several mammalian species this process can be mimicked in the laboratory by incubating sperm in a defined medium [2-5]. Although the molecular basis of capacitation is still poorly understood, the ability of sperm to fertilize an oocyte has been correlated with time-dependent increases in protein tyrosine phosphorylation, the acquisition of acrosomal responsiveness and the induction of hyperactivated motility in all species studied thus far (for review see [6] and [7]). Previous attempts to capacitate equine sperm *in vitro* have remained elusive as evidenced by low levels of protein tyrosine phosphorylation [8, 9], low rates of acrosomal exocytosis [10-13] and low sperm penetration rates when *in vitro* fertilization (IVF) has been attempted [14-20]. Recently, our laboratory has reported defined incubation conditions that yield significant time-dependent increases in protein tyrosine phosphorylation correlated with significant rates of progesterone-induced acrosomal exocytosis in equine sperm [21; Chapter 2]. Moreover, by pairing these *in vitro* capacitation conditions with the induction of sperm hyperactivation we have achieved the highest rates of homologous IVF ever reported for this species [22; Chapter 4].

Although the molecular and biochemical changes leading to sperm capacitation are still under investigation, reports in species other than the equine show that this process is associated with bicarbonate-dependent increases in intracellular cAMP levels generated by soluble adenylyl cyclase (SACY; formally known as sAC). Cyclic AMP then activates protein kinase A (PKA), a serine/threonine kinase, which is then transduced into downstream events

including protein tyrosine phosphorylation [23]. The inhibition of SACY and/or PKA abolishes protein tyrosine phosphorylation signaling and ultimately inhibits capacitation [24, 25]. Previously, we demonstrated that the addition of the cAMP analog dbcAMP and phosphodiesterase inhibitor IBMX to capacitation medium increases the rate of protein tyrosine phosphorylation in stallion sperm. However, in non-capacitating medium devoid of BSA and/or bicarbonate, these cAMP analogues are unable to induce tyrosine phosphorylation of stallion sperm proteins [21]. This is in contrast to what has been reported in other species [2, 24], and raises the question of whether unique molecular pathways are involved in supporting capacitation-dependent events in stallion sperm.

In this regard, a family of cAMP-activated guanine-nucleotide exchange factors (EPAC) has been recently discovered [26]; as a result, a comprehensive re-evaluation of pathways previously attributed to cAMP-PKA activation is warranted. There are two known isoforms of EPAC (EPAC1 and EPAC2) that in somatic cells have been shown to play roles in ion channel function, intracellular calcium signaling, ion transport activity and exocytosis [26]. Because calcium is required for sperm capacitation, of special interest is the fact that EPAC1/2 can mediate calcium mobilization in three hypothesized ways: 1) by interacting directly with inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R) to release calcium from intracellular stores; 2) by acting through Rap GTPases to activate protein kinases that phosphorylate and regulate the function of calcium release; and/or 3) by stimulating Rap GTPases that activate phospholipase C (PLC), thus generating IP<sub>3</sub> [26]. Interestingly, EPAC1 has recently been identified in mature human and epididymal mouse sperm [27, 28] and EPAC2 has been identified in mouse spermatogenic cells [29]. Given the importance of the aforementioned signal transduction pathways in sperm capacitation, it is important to revisit the

regulation of these pathways, which were previously attributed to PKA-mediated events. Therefore, in this study, we investigated the roles of SACY, PKA and EPAC in capacitation-associated events in stallion sperm.

## **MATERIALS AND METHODS**

*Chemicals and Reagents.* Four-Bromo-Calcium Ionophore A23187 was obtained from Calbiochem (San Diego, CA). Tween 20 was purchased from BioRad (Hercules, CA). Anti-phosphotyrosine monoclonal antibody (mAb), 4G10, was purchased from Upstate Biotechnology (Lake Placid, NY). PNA-Alexa 488, valinomycin and 3,3'-dipropylthiadicarbocyanine iodide (DiSC<sub>3</sub>(5)) were purchased from Invitrogen Corp. (Carlsbad, CA). 8-(*p*-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8pCPT) was purchased from BioLog Life Science Institute (San Diego, CA). Sp-5,6-DCI-cBIMPS and myristolated protein kinase A inhibitor (PKI) were purchased from EMD Biosciences, Inc. (Gibbstown, NJ). The acquisition of the SACY inhibitor KH7 was facilitated by Drs. Levin and Buck (Department of Pharmacology, Weill Cornell Medical College, New York, NY [25]) and purchased from the Abby and Howard P. Milistein Synthetic Chemistry Core Facility also at Weill Cornell Medical College. EPAC1 (C-17) and EPAC2 (H220) antibodies, and EPAC1 peptide (C-17P) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Carbonyl cyanide *m*-chlorophenylhydrazone (cccp) and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

*Sperm Culture Media.* Sperm were incubated as previously described [21] in modified Whittens (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 5.5

mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt and 1.0 mM pyruvic acid) [30]. Negative control, non-capacitating medium was always devoid of BSA and NaHCO<sub>3</sub> and also served as transport medium. Capacitating conditions for stallion sperm were achieved by adding 25 mM NaHCO<sub>3</sub> and 7 mg/ml BSA to the non-capacitating base medium and the final pH was adjusted to 7.25 with HCl for both conditions. Capacitating conditions for mouse sperm were achieved by adding 15 mM NaHCO<sub>3</sub> and 3 mg/ml BSA to the non-capacitating base medium and the final pH was adjusted to 7.35 with HCl for both conditions.

*Semen Collection and Preparation.* Semen was collected with an artificial vagina from seven adult stallions of proven fertility in compliance with IACUC guidelines. After visual evaluation of sperm motility under light microscopy on a heated stage and assessment of sperm concentration using a 534B MOD1 Densimeter (Animal Reproduction Systems, Chino, CA), the sperm rich fraction was diluted 2:1 (vol:vol) in pre-warmed non-capacitating MW and transported to the laboratory at 37°C for immediate processing. Samples were centrifuged in 15-ml conical tubes at 100 x g for 1 min at 37°C to remove particulate matter and dead sperm. The supernatant was then transferred to a 14-ml round- bottom centrifuge tube and centrifuged at 600 x g for 5 min (37°C), and resuspended in non-capacitating or capacitating MW to a final concentration of 10 x 10<sup>6</sup> sperm/ml. Samples were incubated in 500 µl aliquots in polyvinyl alcohol coated 5-ml round-bottom tubes [31] at 37°C in a humidified air atmosphere.

Mouse epididymal sperm were isolated using a swim-out method. Briefly, epididymides were placed into 500 µl of pre-warmed non-capacitating MW in a 1.5-ml round bottom tube and allowed to swim out for 10 min in a 37°C water

bath. An additional 1 ml of medium was added before centrifugation at 150 x g at room temperature. The top 1 ml of supernatant was removed and sperm were resuspended at  $6.6 \times 10^6$  sperm/ml. Samples were incubated in non-capacitating and capacitating MW in 300  $\mu$ l aliquots in polyvinyl alcohol coated 5-ml round bottom tubes at 37°C.

*SDS-PAGE and Immunoblotting.* Stallion or mouse sperm were incubated under non-capacitating and capacitating conditions for 0, 2, 4 and 6 h or 0, 30 and 60 min, respectively, in the presence and absence of various pharmacological reagents. Following incubation, samples were processed for SDS-PAGE and immunoblotting as previously described [21]. Briefly, samples were washed by centrifugation and the final pellet was resuspended in sample buffer [32] containing 40 mM DTT. Samples were then boiled for 5 minutes. The total volume of extract corresponding to equal numbers of sperm in each sample ( $5 \times 10^6$  stallion or  $2 \times 10^6$  mouse sperm) was loaded on 10% SDS gels. Separated proteins were blotted onto Immobilon P membranes (Millipore, Inc., Billerica, MA), and blocked for 1 h with 1:10 (vol:vol) dilution of cold water fish skin gelatin in TBS-Tween 20. TBS-Tween 20 was used for all subsequent antibody incubations and washes. Immunodetection of tyrosine phosphorylated proteins was performed using a monoclonal antibody against phosphotyrosine at a 1:10,000 dilution for 1 h, and incubated for 30 min with goat anti-mouse horseradish peroxidase-coupled IgG. Immunodetection of EPAC proteins was performed using polyclonal antibodies against EPAC1 or EPAC2 at a 1:200 dilution for 3 h, and incubated for 2 h with either donkey-anti-goat (EPAC1) or goat-anti-rabbit (EPAC2) horseradish peroxidase-coupled IgG. Immunoreactivity was visualized using enhanced chemiluminescence detection with an ECL kit (Amersham Corp., Piscataway, NJ) according to the

manufacturer's directions. Immunoblots shown are representative of three replicates with three different stallions and/or mice.

*Immunofluorescence.* Sperm suspensions ( $0.5 \times 10^6$ ) were washed with PBS by centrifugation (2000 x g for 2 min), resuspended in 125  $\mu$ l of PBS and allowed to settle on glass slides for 5 min at room temperature. Excess fluid was then aspirated off, and slides were allowed to air dry. Slides were fixed in 2% paraformaldehyde for  $\geq 10$  min, permeabilized with 0.1% Triton X-100 in PBS and blocked with 5% BSA in PBS for 2 h at room temperature. Primary antibodies for EPAC1 or EPAC2 (1:100 in blocking solution) were added and slides were incubated at 4°C overnight in a humidified chamber. Controls for EPAC1 were incubated in blocking peptide (1:100 in blocking solution) and controls for EPAC2 were incubated in pre-immune rabbit IgG (5 mg/ml in normal saline) at 4°C overnight in a humidified chamber. Slides were washed with PBS and incubated with either donkey-anti-goat FITC conjugated IgG (EPAC1) or goat-anti-rabbit FITC conjugated IgG (EPAC2) secondary antibodies (1:200 in blocking solution) for  $\geq 2$  h at room temp in the dark. Slides were washed again with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Slides were evaluated using a fluorescent Zeiss Imager ZI microscope (Thornwood, NY) with Green Fluorescent Protein (GFP) filters the following day.

*Acrosome Reaction Assay.* Stallion sperm were resuspended in capacitating media for 0 or 6 h. Induction of acrosomal exocytosis was performed by incubating sperm for an additional 30 min in the presence of 1 mM 8pCPT, 3  $\mu$ M progesterone, 5  $\mu$ M calcium ionophore A23187 [21] or an equal volume of vehicle control (DMSO; 0.5% vol:vol). Sperm were washed with PBS by centrifugation and resuspended in 125  $\mu$ l of PBS. Acrosomal status of sperm was evaluated with PNA-Alexa 488 (final concentration 0.024 mg/ml) as

previously described [21]. Briefly, sperm were incubated for 10 min in the presence of PNA-Alexa 488, washed, fixed with 2% paraformaldehyde for 10 min, washed again and mounted with Vectashield. Slides were scored (630x) for acrosomal status using an upright fluorescent Zeiss Imager ZI microscope (Thornwood, NY) with Green Fluorescent Protein (GFP) filters. Two hundred morphologically normal sperm were counted per slide and evaluation was performed blindly of treatment status. Sperm were categorized as acrosome intact, intermediate or acrosome reacted as previously described.

Mouse sperm were incubated in capacitating medium for 0 or 1 h. Induction of acrosomal exocytosis was performed by incubating sperm for an additional 10 min in the presence of 1 mM 8pCPT, 20  $\mu$ M progesterone [33] or an equal volume of vehicle control (DMSO; 0.5% vol:vol). Sperm were washed with PBS by centrifugation and fixed with 1% paraformaldehyde (400  $\mu$ l total volume) for 10 min. Sperm were pelleted by centrifugation to remove fixative and washed twice with 500  $\mu$ l of 100 mM sodium acetate. After the second wash, the supernatant was removed down to ~ 35  $\mu$ l, then sperm were smeared onto slides and allowed to air dry. Slides were stained with 0.22% Coomassie blue G250 (wt:vol) in 50% methanol and 10% in acetic acid in a Coplin jar for 10 min. Slides were rinsed with PBS, air dried and mounted with Mount-Quick (Diado Sangyo Co., Ltd., Japan). Acrosomal status was immediately evaluated using an Olympus BH2 microscope (100X; Center Valley, PA); staining over the acrosome indicated acrosome intact sperm [34]. Two hundred morphologically normal sperm were counted per slide and evaluation was performed blindly of treatment status.

*Membrane Potential Assay in Stallion Sperm Populations.* Membrane potential ( $E_m$ ) was measured using freshly ejaculated stallion semen or semen



that was diluted in INRA 96 Extender (Breeder's Choice, LLC, Aubrey, TX) and cooled to 4°C for shipping in an Equitainer (Hamilton Research, Inc., South Hamilton, MA) following standard recommendations for commercial cooled-shipment. Fresh semen was washed and processed as described above; cooled semen was washed from extender by dilution in room temperature PBS and centrifugation. Sperm were then resuspended at  $10 \times 10^6$  sperm/ml (500  $\mu$ l total volume) in non-capacitating, capacitating, or capacitating medium plus 1 mM 8pCPT and incubated in a 37°C water bath. Membrane potential measurements for non-capacitated sperm were taken at time points throughout the experiment, whereas sperm incubated under capacitating conditions with and without 1 mM 8pCPT were evaluated between 3-6 h of incubation.

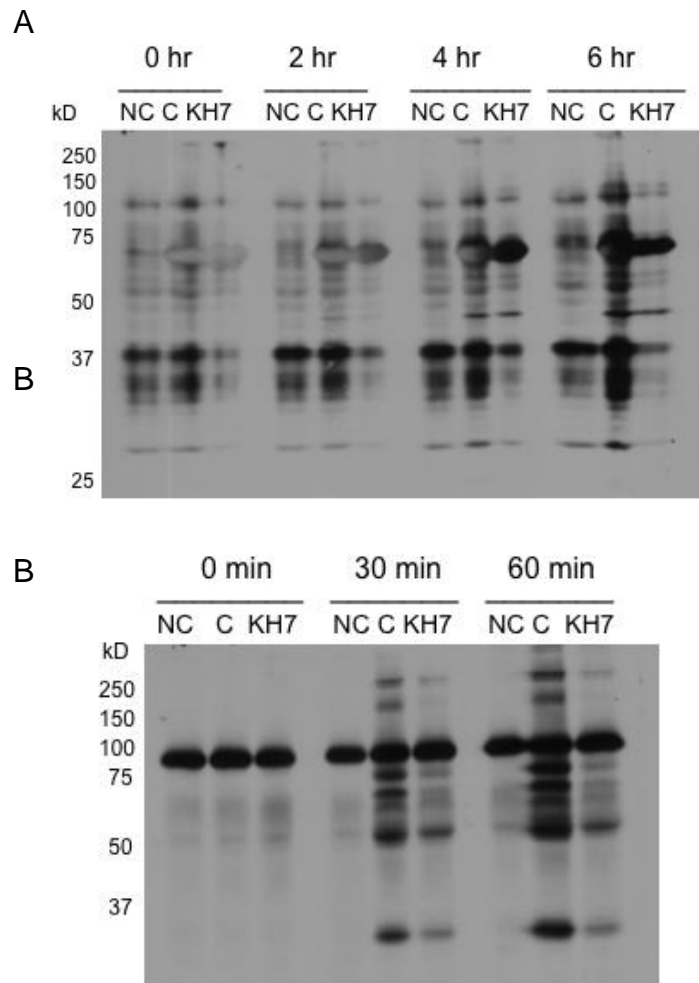
Membrane potential was measured as previously described [35]. Prior to  $E_m$  measurements, sperm were diluted in 1.5 ml total volume of either non-capacitating or capacitating medium. The fluorescent probe DiSC<sub>3</sub>(5) (1  $\mu$ M final concentration) was added to the sperm suspension followed by the addition of 1  $\mu$ M cccp (final concentration) was also added, to collapse mitochondrial membrane potential. After a 10-min incubation with these reagents (37°C), sperm were transferred to a gently stirred cuvette (37°C) and the fluorescence was continuously recorded at 620/670 nm (excitation/emission). Calibration was performed as described by Demarco et al. [35] with the addition of 1  $\mu$ M valinomycin and sequential additions of KCl; membrane potential was then calculated using the Nernst equation and expressed as mV, as previously described [36].

*Statistical Analysis.* Raw data for percent acrosome reacted sperm were transformed using arcsin square root and then analyzed by two-way repeated measures ANOVA using SigmaStat software (San Jose, CA). Raw

data for membrane potential was analyzed using a one-way ANOVA; when significant differences were detected ( $P < 0.05$ ) the Student-Newman-Keuls method was applied to assess all pairwise multiple comparisons.

## RESULTS

*SACY-Generated cAMP Supports Capacitation-Induced Increases in Protein Tyrosine Phosphorylation.* Based on studies performed in mammalian spermatozoa, it is accepted that the activation of SACY by bicarbonate and the subsequent generation of cAMP plays a crucial role in the downstream accumulation of tyrosine-phosphorylated proteins [6]. In this regard, the SACY-specific inhibitor KH7 can abrogate the time-dependent increases in protein tyrosine phosphorylation in mouse sperm incubated in capacitating conditions [25]. Because capacitation-dependent protein tyrosine phosphorylation in stallion sperm requires the presence of bicarbonate in the incubation medium [21], we hypothesized that KH7 would inhibit tyrosine phosphorylation in this species as well. We performed parallel experiments in mouse sperm as an internal laboratory control. Notably, twice the concentration of KH7 was required in stallion sperm (60  $\mu\text{M}$ ; Figure 3.1A) to achieve the same level of inhibition obtained in mouse sperm (30  $\mu\text{M}$ ; Figure 3.1B; [25]). Lower concentrations of KH7 (15 and 30  $\mu\text{M}$ ) were not sufficient to inhibit tyrosine phosphorylation in stallion sperm (data not shown). This may suggest a more robust control of these pathways in equine sperm or perhaps inherent differences related to the origin of the sperm samples; that is, ejaculated (equine) vs. epididymal, thus possibly affecting membrane permeability to KH7.



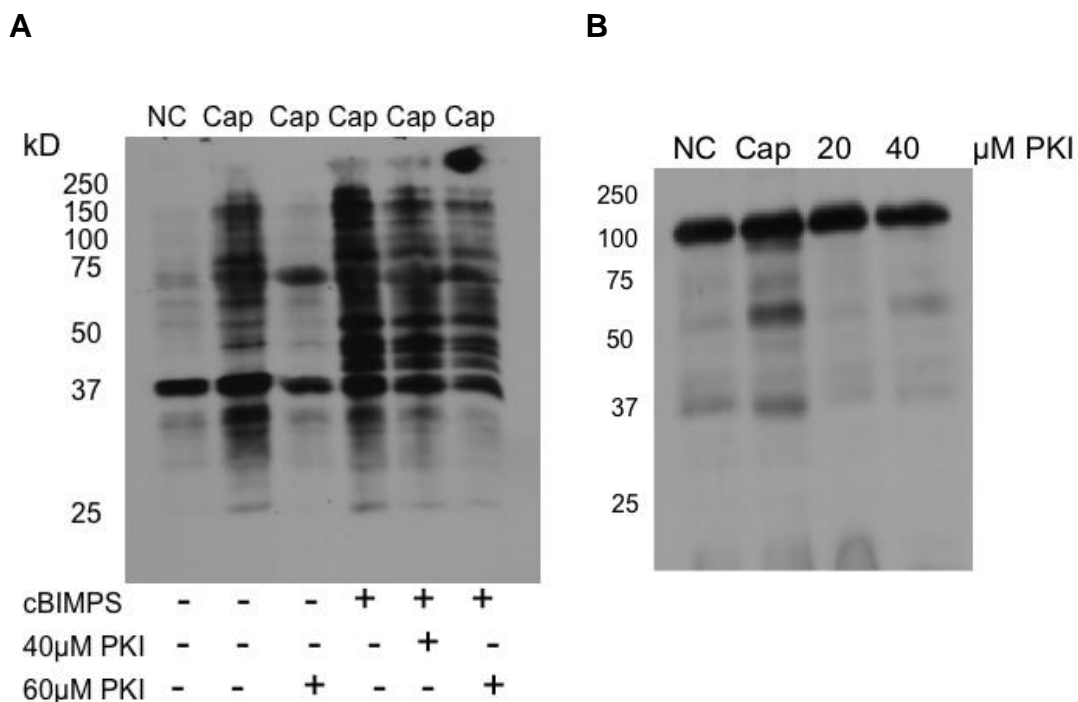
**Figure 3.1** Representative immunoblot for protein tyrosine phosphorylation of stallion (A) and mouse sperm (B) incubated in non-capacitating (NC), capacitating (C) or capacitating conditions plus KH7 (60  $\mu$ M in stallion sperm; 30  $\mu$ M in mouse sperm).

Although this experiment supports previous studies demonstrating that SACY activation is responsible for protein tyrosine phosphorylation in stallion sperm, we had previously reported that the combination of dbcAMP and IBMX was unable to support time-dependent increases in protein tyrosine phosphorylation in stallion sperm incubated under non-capacitating conditions [21]. Therefore, we tested the more membrane permeable and

phosphodiesterase-resistant cAMP analog cBIMPS. Sperm incubated in capacitating MW plus 1 mM cBIMPS demonstrated increases in protein tyrosine phosphorylation higher than those observed in sperm incubated under capacitating conditions alone (Figure 3.2A, lane 4). Altogether, we concluded that SACY-dependent cAMP production is required for the downstream capacitation-dependent protein tyrosine phosphorylation cascade in stallion sperm.

*Inhibition of PKA Blocks the Capacitation-Dependent Increase in Protein Tyrosine Phosphorylation.* Sperm capacitation research in several mammalian species points at PKA as being the immediate effector of SACY-generated cAMP leading to downstream protein tyrosine phosphorylation events [2, 6, 24]. To investigate this, we incubated stallion sperm under capacitating conditions in the presence of the PKA-specific inhibitor PKI [37]. As shown in Figure 3.2A (lane 3), 60  $\mu$ M PKI was sufficient at inhibiting the capacitation-dependent increases in tyrosine phosphorylation; lower concentrations of PKI (10, 20, and 30  $\mu$ M) did not have the same inhibitory effect (data not shown). As a control for this pharmacological experiment, mouse sperm were also incubated under capacitating conditions in the presence of PKI. Figure 3.2B shows that as low as 20  $\mu$ M PKI completely inhibited capacitation-dependent tyrosine phosphorylation in mouse sperm.

These experiments supported the hypothesis that PKA promotes downstream protein tyrosine phosphorylation in stallion sperm, however, we wanted to test the possibility that other cAMP targets contribute to these effects. For this purpose, sperm were incubated in capacitating MW, with or without the PKA inhibitor PKI (60  $\mu$ M final concentration) and 1 mM cBIMPS. Interestingly,



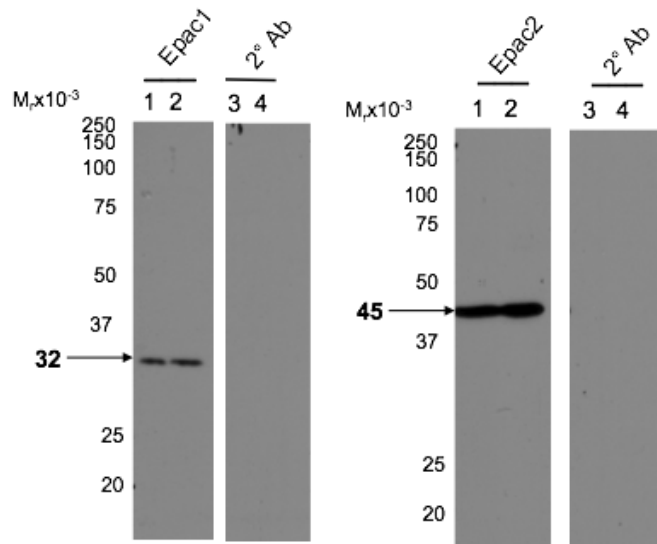
**Figure 3.2** Representative immunoblots for protein tyrosine phosphorylation in (A) stallion and (B) mouse sperm. Sperm were incubated under capacitating (Cap) and non-capacitating (NC) conditions in the presence of the cAMP analog cBIMPS (1mM) and/or increasing concentrations of the PKA inhibitor PKI.

in the presence of PKI, sperm incubated with the addition of cBIMPS still underwent tyrosine phosphorylation to levels markedly higher than those seen in the non-capacitating condition or capacitating condition with the addition of PKI alone (Figure 3.2A, lanes 5, 6). That is, the addition of PKI was not sufficient at completely inhibiting the effects of cBIMPS, which suggests that there are cAMP targets other than PKA that stimulate tyrosine phosphorylation in stallion sperm.

*EPAC1 and EPAC2 are Present in Stallion and Mouse Sperm.* The cAMP-dependent guanine nucleotide exchange factors EPAC1 and EPAC2 have been recently identified in somatic cells [26] and mammalian sperm [27, 38]. EPAC1/2 has been shown to play a role in activating the MAPK pathway in

somatic cells [39, 40]; paired with the results reported above (Figure 3.2), we hypothesized that their activation contributes to PKA-independent increases in protein tyrosine phosphorylation.

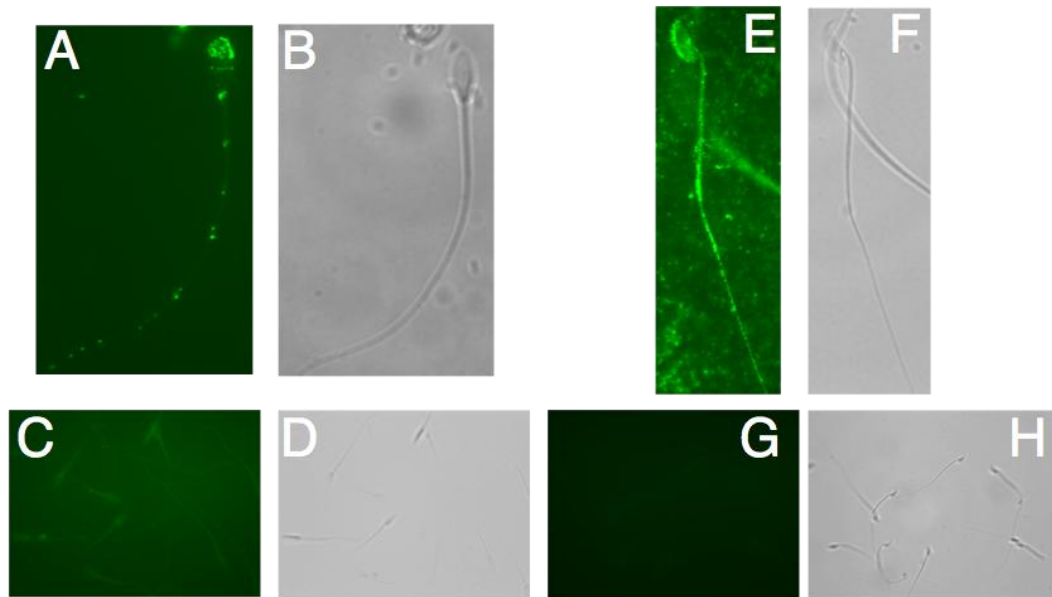
We first investigated the expression of these proteins in stallion sperm. Immunoblotting of sperm extracts was performed using polyclonal antibodies against EPAC1 or EPAC2. A band at ~ 32 kD (Figure 3.3) and at ~ 45 kD (Figure 3.3), corresponding to EPAC1 and EPAC2 proteins, respectively, was observed. These bands did not appear when the primary antibodies were omitted (Figure 3.3). Additionally, while the full length EPAC proteins are 120 – 126 kD, the size of the observed protein bands correspond to the predicted sizes of the N-term regulatory subunits for EPAC1 and EPAC2, as determined by BLAST and BLAT searches of the mouse and horse genomes, respectively



**Figure 3.3** Representative immunoblot of EPAC1 and EPAC2 in stallion (lanes 1 and 3) and mouse (lanes 2 and 4) sperm.

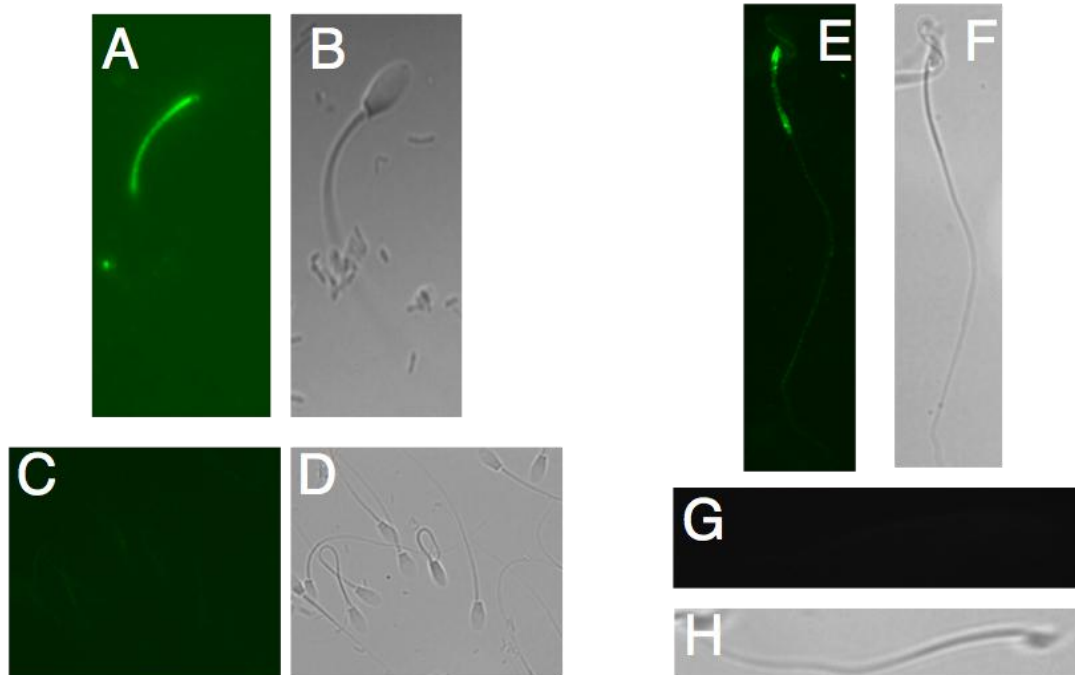
[26] (NCBI accession numbers NP\_659099 and CAM24393; University of California, Santa Cruz horse genome browser [www.genome.ucsc.edu]). Therefore, these protein bands are most likely degradation products of the full-length EPAC proteins. As detected via indirect immunofluorescence, EPAC1 localized to the acrosome and sub-acrosomal ring of stallion sperm (Figure 3.4A); in agreement with previous reports, EPAC1 localized to the acrosome of mouse sperm (Figure 3.4E) [27]. EPAC 2 localized to the midpiece of both stallion (Figure 3.5A) and mouse sperm (Figure 3.5 E).

Because there are no membrane permeable antagonists for EPAC available [41], we used the EPAC-specific cAMP analogue 8pCPT [26] to investigate the potential involvement of these proteins in capacitation-dependent protein tyrosine phosphorylation events. For this purpose, stallion and mouse sperm were incubated under non-capacitating and capacitating conditions with and without 1 mM 8pCPT for 0, 2, and 4 h (stallion sperm) or 0, 30 and 60 min (mouse sperm). As shown in Figure 3.6, there was no discernable effect of 8pCPT on tyrosine phosphorylation in either incubation condition or species. These results support a primary role for a cAMP-PKA pathway in driving these events, as reported in other species.



**Figure 3.4** Immunofluorescence localization of EPAC1 in stallion and mouse sperm. (A) EPAC1 localizes to the acrosome and sub-acrosomal ring in stallion sperm; (B) corresponding bright field image for A; (C) peptide control demonstrating background levels of fluorescence with corresponding bright field image (D); (E) EPAC1 localizes to the acrosome in mouse sperm; (B) corresponding bright field image for E; (G) pre-immune rabbit IgG control demonstrating background levels of fluorescence with corresponding bright field image (H).

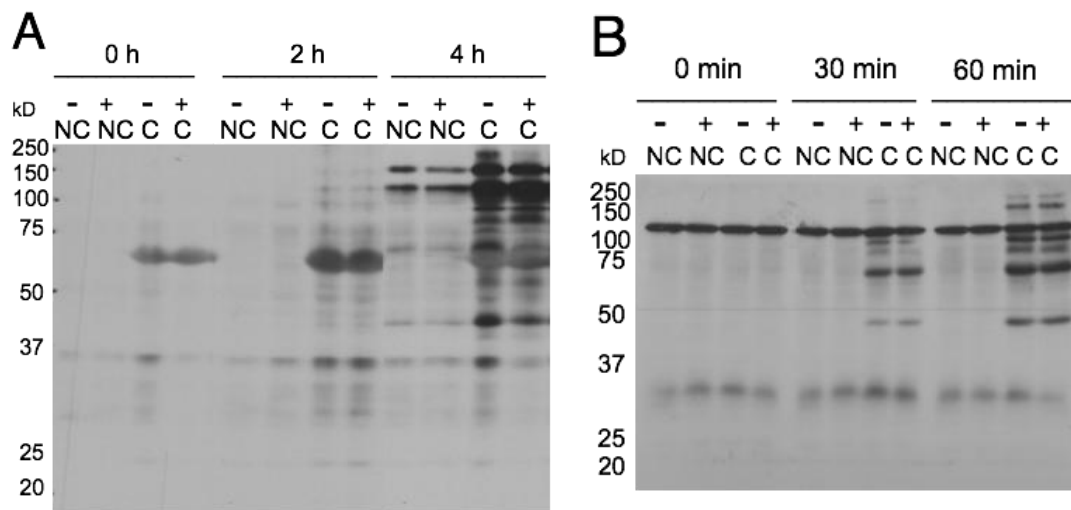




**Figure 3.5** Immunofluorescence localization of EPAC2 in stallion and mouse sperm. EPAC2 localizes to the midpiece in stallion (A) and mouse (E) sperm; (B and F) corresponding bright field images for A and E, respectively; (C and G) secondary antibody alone controls demonstrating background levels of fluorescence with corresponding bright field images (D and H).

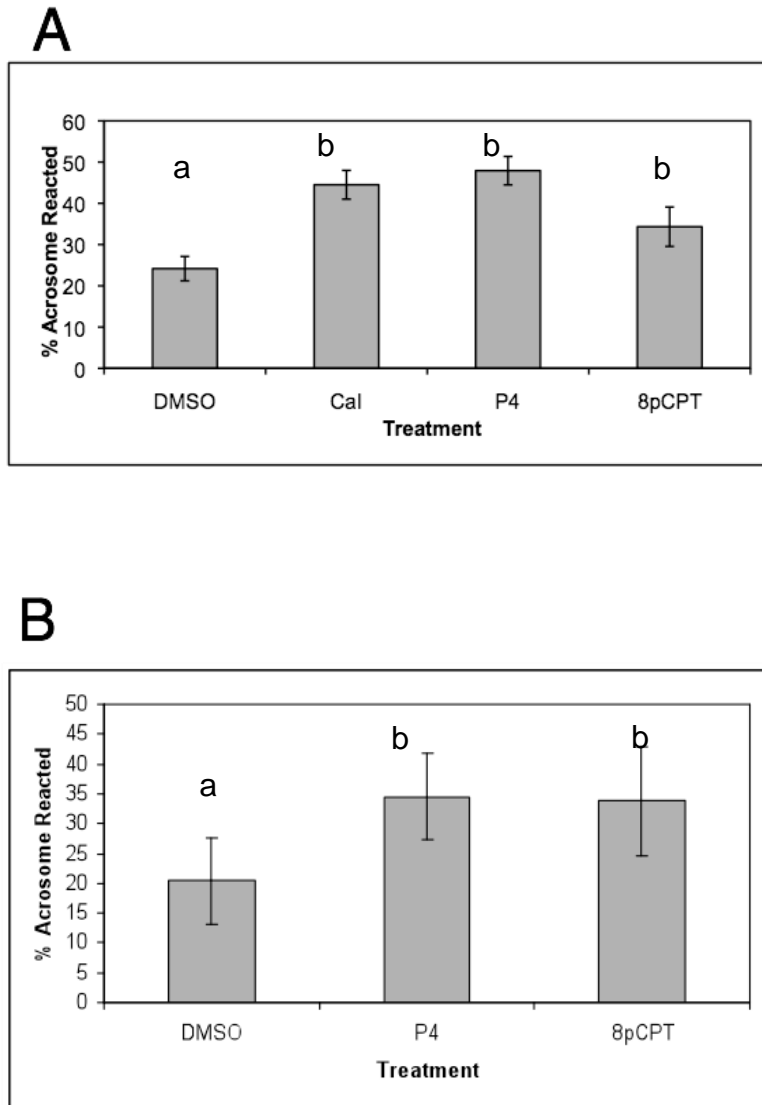
*The EPAC-specific cAMP Analogue 8pCPT Induces Acrosomal Exocytosis in Capacitated Stallion and Mouse Sperm.* Because EPAC1 localized to the acrosome, we next hypothesized that EPAC activation might play a role in the induction of acrosomal exocytosis in stallion and/or mouse sperm. In support of this, Branham et al. have shown that activation of EPAC via 8pCPT in permeabilized human spermatozoa contributes to the calcium-dependent phase of the acrosome reaction [28].

In stallion sperm incubated under capacitating conditions (6 h), activation of EPAC with 8pCPT induced 34.3% acrosomal exocytosis rates, which was



**Figure 3.6** Representative immunoblots of protein tyrosine phosphorylation in stallion (A) and mouse sperm (B) incubated under non-capacitating (NC) and capacitating (C) conditions in the presence (+) and absence (-) of 8pCPT.

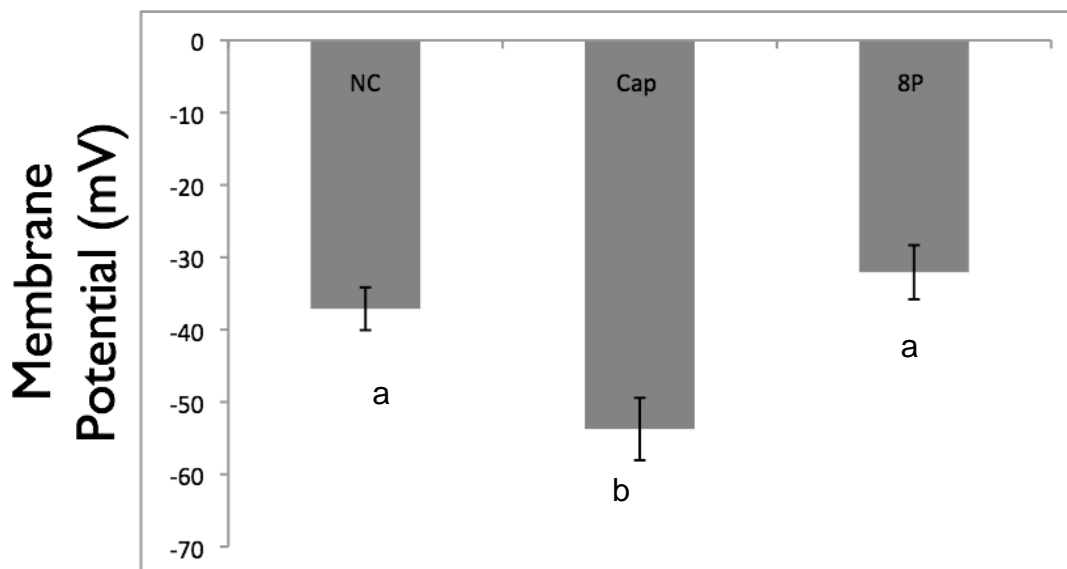
higher ( $P=0.03$ ) than DMSO-vehicle controls (24.2%; Figure 3.7A). Moreover, this percentage was similar ( $P>0.05$ ) to rates achieved with both progesterone and calcium ionophore (47.9% and 44.5%, respectively). Conversely, 8pCPT did not induce exocytosis in non-capacitated (0 h) stallion sperm (data not shown;  $P>0.05$  for all conditions). Similarly, in mouse sperm, 8pCPT induced 45% exocytosis rates (Figure 3.7B), which was higher ( $P=0.004$ ) than DMSO controls (27.5%) but comparable ( $P>0.05$ ) to progesterone (47.9% acrosomal exocytosis). From these studies, we concluded that activation of EPAC via 8pCPT induces acrosomal exocytosis in capacitated stallion and mouse sperm.



**Figure 3.7** Percentages of acrosomal exocytosis in stallion (A) and mouse sperm (B) incubated under capacitating conditions and exposed to DMSO (solvent control), calcium ionophore A23187 (Cal), progesterone (P4), or the EPAC-specific cAMP analogue 8pCPT. (Mean  $\pm$  SEM).

### *8pCPT Maintains Sperm Plasma Membrane in the Depolarized State.*

Hyperpolarization of the sperm plasma membrane, which has been demonstrated to occur during capacitation, is required for fertility [36, 42, 43]. In the mouse, this represents a change in membrane potential ( $E_m$ ) from  $\sim -30$  mV to  $\sim -60$  mV, is dependent on the activation of various ion channels and prepares the sperm for acrosomal exocytosis [36, 42, 44]. Currently, cAMP is believed to play a role in hyperpolarization of the sperm plasma membrane through a PKA-mediated pathway [42]; however, in various somatic cell lines, activation of EPAC via 8pCPT has been shown to have effects on ion channels known to play a role in the modulation of  $E_m$  [40, 45, 46]. The potential role of EPAC in this event has yet to be investigated in mammalian sperm. For this purpose, stallion sperm were incubated in non-capacitating, capacitating, and capacitating medium with the addition of 1 mM 8pCPT for up to 6 h. Membrane potential of non-capacitated sperm was measured throughout the experiment;  $E_m$  for sperm incubated under capacitating conditions with and without 8pCPT was measured between 3-6 h of incubation. Non-capacitated stallion sperm displayed a resting mean  $E_m$  of  $-37.11$  mV versus capacitated sperm, which hyperpolarized to  $-53.74$  mV; this capacitation-dependent hyperpolarization was significant ( $P=0.002$ ). Interestingly, when sperm were incubated under capacitating conditions in the presence of 8pCPT, sperm  $E_m$  was maintained in the depolarized state at  $-32.06$  mV; this result was comparable to non-capacitated sperm ( $P>0.05$ ) but different from capacitated sperm ( $P<0.001$ ; Figure 3.8). This experiment not only determines the resting and capacitation-associated membrane potentials for stallion sperm, it also demonstrates that EPAC activation results in membrane depolarization.



**Figure 3.8** Average membrane potential ( $E_m$ ) of non-capacitated stallion sperm (NC), capacitated stallion sperm (Cap) and stallion incubated under capacitating conditions in the presence of 8pCPT (8P). (Mean  $\pm$  SEM).

## DISCUSSION

Our previous work suggested inherent differences in the physiological response of stallion sperm to pharmacological agents used to ascertain the pathways linked to sperm capacitation [21]. Moreover, the recent discovery of cAMP-dependent guanine nucleotide exchange factors EPAC1 and EPAC2 in somatic and germ cells prompted the re-evaluation of pathways previously attributed to cAMP-PKA activation. Therefore, in this study, we sought to separate the roles of PKA and EPAC activation in capacitation-associated events in stallion sperm.

While it is widely reported that capacitation-dependent protein tyrosine phosphorylation follows a cAMP-PKA driven pathway, we had previously shown that dbcAMP-IBMX were unable to enhance tyrosine phosphorylation in stallion sperm incubated in non-capacitating conditions [21], as shown in all other

species studied. In the present study, we report that PKI can inhibit, albeit not completely (band at ~ 75 kD), time-dependent increases in protein tyrosine phosphorylation in stallion sperm incubated under capacitating conditions (Figure 3.2, lane 3). Moreover, PKI did not completely inhibit the effects of cBIMPS in our protein tyrosine phosphorylation studies (Figure 3.2, lanes 5,6). Altogether, these results made us reconsider the analysis of these pathways in stallion sperm.

Additionally, in somatic cells and in mouse sperm, activation of EPAC with 8pCPT results in the direct activation of Rap 1 [27, 47], and in some cell types, this is followed by ERK1/2 activation [39]. Therefore, we hypothesized that EPAC might be playing a role in capacitation-associated protein tyrosine phosphorylation in stallion sperm. In one-dimensional gel electrophoresis, however, we were unable to detect any changes in phosphotyrosine with the addition of 8pCPT in either horse or mouse sperm. Under capacitating conditions, marked levels of tyrosine phosphorylation are normally seen; therefore it is possible that an effect of 8pCPT could be masked. Under non-capacitating conditions, the absence of BSA and bicarbonate may preclude appropriate membrane permeability of the cAMP analogue (particularly in stallion sperm which are ejaculated and therefore exposed to coating by components of seminal plasma). Alternatively, 8pCPT may have been rapidly hydrolyzed to an inactive form by phosphodiesterases (PDEs). To be certain that our negative results were not due to membrane permeability issues or PDE hydrolyzation, we incubated stallion sperm under non-capacitating conditions plus 8pCPT in the presence of BSA, IBMX or both. In these studies, we observed no effect on tyrosine phosphorylation and levels of tyrosine phosphorylation remained at non-capacitated control levels (data not shown).

Our results, therefore, support only cAMP-PKA driven induction of protein tyrosine phosphorylation during stallion sperm capacitation.

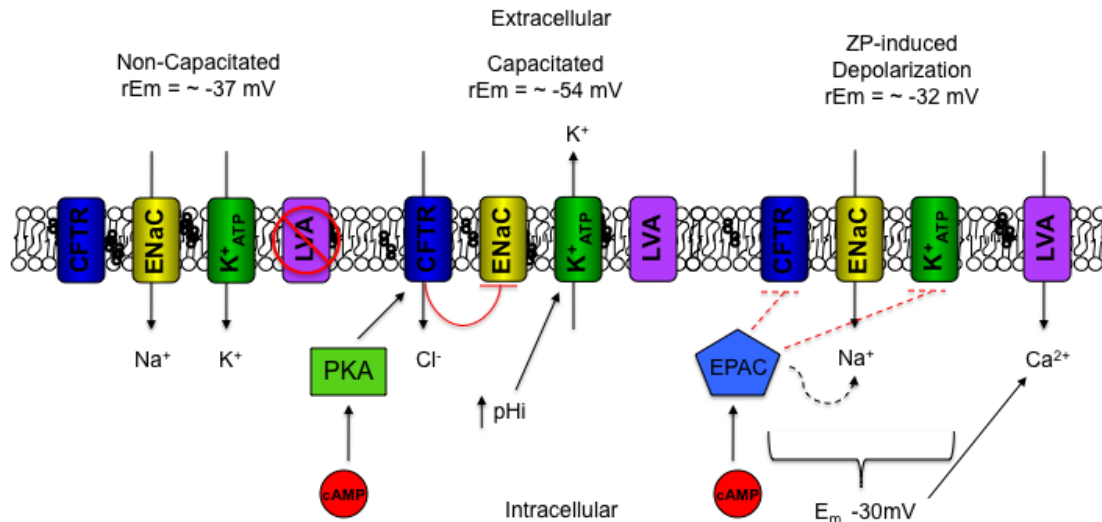
Most notably, however, activation of EPAC with 8pCPT induced rates of acrosomal exocytosis in capacitated stallion (34%) and mouse sperm (45%) comparable to those obtained with progesterone and/or calcium ionophore-treated sperm ( $P>0.05$ ). We hypothesized a potential link between EPAC activation, changes in membrane potential and acrosomal exocytosis. In support of this, we demonstrated that incubation of stallion sperm under capacitating conditions in the presence of the EPAC-selective agonist 8pCPT maintained sperm  $E_m$  in the depolarized state, thus precluding membrane hyperpolarization as observed under the capacitating conditions.

Indeed, capacitation-dependent hyperpolarization of the sperm plasma membrane is required for fertility [36]. In the mouse, this represents a change in membrane potential from  $\sim -30$  mV to  $\sim -60$  mV [36]. We report herein the resting membrane potential of stallion sperm to be  $-37.11$  mV, and show that capacitated stallion sperm hyperpolarize to  $-53.74$  mV ( $P=0.002$ ). Currently, cAMP is believed to play a role in hyperpolarization of the sperm plasma membrane through a PKA-mediated pathway [42]. The current model of capacitation-induced plasma membrane hyperpolarization involves increases in intracellular cAMP, the activation of PKA, followed by phosphorylation of the cystic fibrosis transmembrane conductor (CFTR). Activation of CFTR allows the influx of negatively charged  $Cl^-$  ions as well as the inhibition of the constitutively active epithelial sodium channels (ENaC), thus preventing the influx of  $Na^+$  [36, 42]. Moreover, capacitation-associated increases in intracellular pH activate  $K^+_{ATP}$  channels, also contributing to membrane hyperpolarization [48].

It has been shown that the activation of EPAC with 8pCPT will induces hyperpolarization in cerebellar neurons [40]. Conversely, in various other excitable and non-excitable somatic cells, EPAC activation has been observed to have effects on the above mentioned ion channels such that a depolarization of the plasma membrane would be expected. For instance, in pancreatic beta cells, Kang et al. observed an EPAC-mediated inhibition of  $K^+_{ATP}$  channels [46]; in rat pulmonary epithelial cells 8pCPT increased the channel open probability of ENaC [45]; and, in rat hepatocytes, 8pCPT stimulated an outwardly rectifying  $Cl^-$  current [49].

Additionally, the observations in the present study that: 1) EPAC1 localized to the acrosomal region of stallion and mouse sperm; 2) 8pCPT induced acrosomal exocytosis similarly in both species; and, 3) 8pPCT maintained stallion sperm in the depolarized state, are all in accordance with work demonstrating ZP3-induced exocytosis as a result of plasma membrane depolarization [43, 44]. In this model, capacitation-dependent hyperpolarization results in the recruitment of low voltage-activated (LVA) T-type ion channels, which are then available for activation. Contact with ZP3 stimulates membrane depolarization and opening of LVA channels, thus resulting in calcium influx and the initiation of acrosomal exocytosis [43]. Since 8pCPT interfered with fluorescent recordings during membrane potential determination, we were unable to ascertain its immediate effect when added to capacitated stallion sperm. However, because 8pCPT did maintain sperm in the depolarized state, we propose a model (Figure 3.9) in which ZP-directed EPAC activation may be involved in inducing acrosomal exocytosis by triggering membrane depolarization, through yet to be determined ion channel(s). We hypothesize that this, in turn, results in the activation of LVA channels previously recruited as





**Figure 3.9** In the non-capacitated state, the cystic fibrosis transmembrane regulator (CFTR) is in the closed state, while the epithelial sodium ion channel (ENaC) is constitutively open allowing for the influx of  $Na^+$  ions. Additionally, the potassium ATP ion channel ( $K^+_{ATP}$ ) is inwardly rectifying. Collectively, this results in a depolarized resting membrane potential of  $\sim -37$  mV. At this membrane potential, the low voltage activated T-Type calcium channels (LVA) are not able to open. During capacitation, cAMP-dependent PKA phosphorylates CFTR, thus opening this channel allowing for the influx of  $Cl^-$  ions; additionally, activation of CFTR inhibits ENaC channels. The increase of intracellular pH that also accompanies capacitation switches the  $K^+_{ATP}$  channel from an inwardly rectifying channel to an outwardly rectifying channel. As a result, capacitated sperm have a hyperpolarized membrane potential of  $\sim -54$  mV. This hyperpolarization allows for the recruitment of LVA channels, which can now be opened upon proper stimulation. We hypothesize that when a capacitated sperm contacts the zona pellucida of an oocyte, cAMP-dependent EPAC is activated, which in turn inhibits CFTR, allowing ENaC channels to become constitutively active again. Evidence also suggests that activation of EPAC inhibits  $K^+_{ATP}$  channels. Collectively, this results in a rapid depolarization of the plasma membrane ( $\sim -32$  mV), the opening of LVA channels, and the resulting  $Ca^{2+}$  influx triggers acrosomal exocytosis.

a result of capacitation-dependent hyperpolarization, a sudden calcium influx, and acrosomal exocytosis. Future studies should aim at identifying the particular ion channels through which EPAC may facilitate sperm membrane depolarization.

Taken together, these experiments demonstrate a novel role for EPAC in stallion sperm capacitation, as it pertains to acrosomal exocytosis and plasma membrane potential. Moreover, this study highlights the importance of investigating EPAC in a comparative manner with PKA, as both are cAMP-dependent proteins potentially playing diverging roles in sperm physiology.

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CHAPTER 4  
HYPERACTIVATION OF STALLION SPERM IS REQUIRED FOR  
SUCCESSFUL IN VITRO FERTILIZATION OF EQUINE OOCYTES<sup>2</sup>

**ABSTRACT**

Capacitation is a complex and not well-understood process that encompasses all the molecular changes sperm must undergo to successfully fertilize an oocyte. *In vitro* fertilization has remained elusive in the horse as evidenced by low *in vitro* fertilization (IVF) rates (0-33%); moreover, only two foals have ever been produced using IVF. Incubation of stallion sperm in modified Whittens supplemented with BSA and sodium bicarbonate yielded significant rates of time-dependent protein tyrosine phosphorylation and induced acrosomal exocytosis, consistent with capacitation. The objective of this study was to characterize stallion sperm hyperactivation and to test whether hyperactivation of capacitated sperm supported equine IVF. Treatment of sperm with procaine, an anesthetic shown to induce hyperactivation in other mammalian species, resulted in the decrease of three motility variables indicative of hyperactivation: Straight line velocity (VSL;  $P = 0.029$ ), straightness (STR;  $P = 0.001$ ), and linearity (LIN;  $P = 0.002$ ). We demonstrated that procaine-induced hyperactivation was not regulated by changes in protein tyrosine phosphorylation, and that it did not induce acrosomal exocytosis in capacitated sperm as compared to calcium ionophore ( $P > 0.05$ ), similar to findings in the bovine. Most notably, by coupling our capacitating conditions with the induction of hyperactivation using procaine,

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<sup>2</sup> McPartlin, LA, Suarez, SS, Czaya, CA, Hinrichs, K, Bedford-Guaus, SJ. Biol Reprod. 2009 February 4;81(1):199-206. Reprint with permission from the Society for the Study of Reproduction.

we have achieved the novel result of substantial and reproducible percentages of fertilized mare oocytes (60.7%) in our IVF experiments. Conversely, sperm incubated in capacitating conditions but not treated with procaine did not fertilize (0%). These results support the hypothesis that capacitation and hyperactivation are required for successful IVF in the equine.

## INTRODUCTION

Only two foals have ever been produced using *in vitro* fertilization (IVF) technology [1] despite decades of work addressing this topic. Reported fertilization rates have ranged from 0% to 33% [2-8] and these results were inconsistent even between studies performed by the same laboratories [4-6]. Since equine IVF is equally unsuccessful with *in vivo*-matured and *in vitro*-matured oocytes [1], and oviductal transfer of *in vitro*-matured equine oocytes to inseminated mares results in fertilization rates comparable to those obtained by natural mating [8], it is likely that the low sperm penetration rates observed in IVF studies have stemmed from the inability to appropriately capacitate and/or hyperactivate stallion sperm in the laboratory.

Capacitation comprises the changes that ejaculated sperm must undergo to become fertilization-competent [9]. Although the molecular basis of capacitation is still poorly understood, the ability of sperm to fertilize an oocyte has been correlated with time-dependent increases in protein tyrosine phosphorylation and the acquisition of acrosomal responsiveness in all species studied thus far (for review see [10]). Previous attempts to capacitate sperm *in vitro* have remained elusive in the horse as evidenced by low levels of protein tyrosine phosphorylation [11, 12], low rates of acrosomal exocytosis [13-16] and low sperm penetration rates when IVF has been attempted [2-8]. Recently, our

laboratory has reported defined incubation conditions that yield significant time-dependent increases in protein tyrosine phosphorylation correlated with significant rates of progesterone induced acrosomal exocytosis, suggesting that sperm are undergoing changes consistent with capacitation [17].

In addition, penetration of the outer vestments of the oocyte at fertilization requires a change in the pattern of sperm motility that has been termed hyperactivation [18-20]. The acquisition of hyperactivated sperm motility has been observed within the oviducts of mammals at the time of fertilization [21-23] and is required for zona pellucida penetration [19, 24]. It is also believed to play a role in the ability of sperm to detach from the oviductal wall [21], navigate the labyrinthine environment of the oviduct [25], and penetrate the viscous mucoid environment encountered in the female reproductive tract [22, 26; for review see 27]. The precise motility pattern that defines hyperactivation is species-specific; however, it is generally characterized by increases in lateral head displacement, flagellar bend amplitude and beat asymmetry [20, 24, 28].

Capacitation and hyperactivation are often linked together in the continuum of changes that a sperm must undergo to fertilize an oocyte; moreover, both processes require calcium, bicarbonate and the activation of cAMP synthesis [29-32]. However, the pathways that regulate these events are considered separable and independent [27, 33, 34]. This has been demonstrated in *Catsper* null mutant mice whereby disruption of any CATSPER proteins (which form alkaline activated  $\text{Ca}^{2+}$  channels) resulted in normal sperm production, protein tyrosine phosphorylation patterns and rates of acrosomal exocytosis; however, the *Catsper* null sperm failed to hyperactivate, could not penetrate zona-intact oocytes and as a consequence these mice were infertile [18, 19]. *In vitro*-incubated bovine sperm that exhibit markers of capacitation do

not hyperactivate; however, hyperactivation can be exogenously induced in capacitated as well as non-capacitated sperm by exposure to procaine [33], further supporting the hypothesis that capacitation and hyperactivation are independent processes. Although the exact mechanism by which procaine induces hyperactivation is not known, it is believed to stimulate calcium influx by increasing the permeability of the plasma membrane to calcium [34]. Although Rathi et al. [35] designated certain motility parameters, as measured by computer assisted semen analysis (CASA), as indicative of hyperactivated motility in equine sperm, none of the treatments tested in that study significantly affected the proportions of sperm showing these patterns. Thus, the pattern of hyperactivation and its associated measures of motility have not been defined in stallion sperm.

Altogether, the information above suggests that successful IVF in the horse might require defined and specific conditions that support sperm capacitation *in vitro*, but also the acquisition of hyperactivated motility. Therefore, the first objective of this study was to characterize the pattern of motility and motion measures associated with stallion sperm hyperactivation. The second objective of this study was to test whether the induction of hyperactivation in sperm incubated under capacitating conditions supports fertilization of *in vitro* matured mare oocytes.

## **MATERIALS AND METHODS**

### *General Methods*

*Chemicals and reagents.* Four-Bromo-Calcium Ionophore A23187 was obtained from Calbiochem (San Diego, CA). Tween 20 was purchased from BioRad (Hercules, CA). Anti-phosphotyrosine monoclonal antibody (mAB),

4G10, was purchased from Upstate Biotechnology (Lake Placid, NY). PNA-Alexa 488 was purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS), TCM 199 with Hanks salts, TCM 199 with Earles salts, DMEM/F-12 and gentamicin were purchased from Gibco (Carlsbad, CA). Follicle stimulating hormone (FSH) was purchased from Sioux Biochemical (Sioux Center, IA). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

*Sperm culture media.* Sperm were incubated as previously described [17] in modified Whittens (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt and 1.0 mM pyruvic acid) [36]. Negative control, non-capacitating medium lacked BSA and NaHCO<sub>3</sub> and also served as transport medium. Capacitating conditions were prepared by adding 25 mM NaHCO<sub>3</sub> and 7 mg/ml BSA to the non-capacitating MW base medium. For both media, the final pH was brought to 7.25 with HCl.

*Semen collection and preparation.* Semen was collected with an artificial vagina from six adult stallions of proven fertility in compliance with Institutional Animal Care and Use Committee guidelines. After visual evaluation of sperm motility under light microscopy on a heated stage and assessment of sperm concentration using a 534B MOD1 Densimeter (Animal Reproduction Systems, Chino, CA), the sperm-rich fraction was diluted 2:1 (vol:vol) in pre-warmed non-capacitating MW and transported to the laboratory (10 min) at 37°C for immediate processing. Samples were centrifuged in 15-ml conical tubes at 100 x g for 1 min (37°C) to remove particulate matter and dead sperm. The supernatant was then transferred to a 14-ml round-bottom centrifuge tube and centrifuged at 600 x g for 5 min (37°C), and resuspended in non-capacitating or

capacitating MW to a final concentration of  $10 \times 10^6$  sperm/ml. Samples were incubated in 500  $\mu$ l aliquots in polyvinyl alcohol coated 5-ml round-bottom tubes [37] at 37°C in a humidified air atmosphere.

*Statistical analysis.* Data for motility measures (detailed below) were analyzed by two-way ANOVA using SigmaStat software (San Jose, CA). Raw data for percent acrosome reacted sperm were transformed using arcsin square root and then analyzed by two-way ANOVA; raw data for percent hyperactivated sperm sorts were also transformed using arcsin square root and then analyzed by two-way repeated measures ANOVA. When significant differences were detected ( $P < 0.05$ ) the Student-Newman-Keuls method was applied to assess all pairwise multiple comparisons.

#### *Characterization of Hyperactivation in Stallion Sperm: Effects of Procaine on Motility Measures Pre- and Post-Capacitation*

*Induction of hyperactivation and motility analysis.* Hyperactivation of spermatozoa resuspended in capacitating MW was induced with the addition of procaine (5 mM final concentration [33]; dissolved in capacitating MW). Sperm motility was analyzed at 0 h and after 6 h of incubation under capacitating conditions using computer assisted semen analysis (CASA) with an HTM-IVOS equipped with a heated stage (version 12, V32.01L; Hamilton Thorne Biosciences, Beverly, MA) immediately following the addition of procaine. Eight motility parameters were evaluated: % motile, average path velocity (VAP,  $\mu$ m/sec, is the average velocity of sperm along the smoothed cell path), straight line velocity (VSL,  $\mu$ m/sec, is the average velocity measured in a straight line from beginning to end), curvilinear velocity (VCL,  $\mu$ m/sec, is the average rate of travel of the sperm head), amplitude of lateral head displacement (ALH, in  $\mu$ m,

is the mean width of head oscillations), beat cross frequency (BCF, in Hz, is frequency that the head crosses the sperm track), straightness (STR, is  $VSL/VAP \times 100$ ) and linearity (LIN is  $VSL/VCL \times 100$ ). Two ejaculates each from three stallions were used for data analysis and >200 sperm were analyzed per treatment sample.

After the identification of motility variables for which significant measures were indicative of hyperactivation in procaine-treated sperm, the 0 and 6 h control conditions were re-analyzed to determine the percentage of spontaneously hyperactivated sperm. For this purpose, two sets of parameters were compared using the CASA sorting system: (1)  $VSL \leq 50.9 \mu\text{m}/\text{sec}$  and  $LIN \leq 20.9\%$  vs (2)  $VCL \geq 246.8 \mu\text{m}/\text{sec}$  and  $LIN \leq 20.2\%$ . Each field was analyzed individually and overlapping and/or agglutinated sperm tracks were not counted. Videos of activated and hyperactivated sperm were obtained with a Zeiss Axiovert microscope on a 37°C heated stage. Sperm were videotaped using Hoffman Modulation Contrast optics at 300x with stroboscopic illumination at 30 Hz (Strobex, Chadwick-Helmuth Co., El Monte, CA). Images were obtained with a black and white video camera (Model CCD72, Dage-MTI, Inc., Michigan City, IN) and recorded with an HDD/DVD digital recorder (Lite-On, Fremont, CA).

#### *Effect of Procaine on Tyrosine Phosphorylation and Acrosomal Exocytosis*

*SDS-PAGE and immunoblotting.* Sperm were incubated in non-capacitating and capacitating MW for 0, 2, 4 and 6 h in the presence and absence of 5 mM procaine. Following incubation, samples were processed for SDS-PAGE and immunoblotting as previously described [17]. Briefly, samples were washed by centrifugation and the final pellet was resuspended in sample buffer [38] containing DTT (40 mM). Samples were then boiled for 5 minutes.



The total volume of extract corresponding to equal numbers of sperm in each sample ( $5 \times 10^6$  sperm) was loaded on 10% SDS gels. Separated proteins were blotted onto Immobilon P membranes (Millipore, Inc., Billerica, MA), and blocked for 1 h with 1:10 (vol:vol) dilution of cold water fish skin gelatin in TBS-Tween 20. TBS-Tween 20 was used for all subsequent antibody incubations and washes. Immunodetection of tyrosine-phosphorylated proteins was performed using a monoclonal antibody against phosphotyrosine at a 1:10,000 dilution for 1 h, followed by incubation for 30 min with goat anti-mouse horseradish peroxidase-coupled IgG. Immunoreactivity was visualized using enhanced chemiluminescence detection with an ECL kit (Amersham Corp., Piscataway, NJ) according to the manufacturer's directions. Experiment shown is representative of three replicates with three different stallions.

*Acrosome reaction assay.* Sperm were resuspended in capacitating MW for 0 or 6 h. Induction of acrosomal exocytosis was performed by incubating sperm for an additional 30 min in the presence of 5  $\mu$ M calcium ionophore A23187 (Cal; [17]), an equal volume of vehicle control (DMSO) or 5 mM procaine. Sperm were washed and resuspended in 125  $\mu$ l of PBS. To evaluate acrosomal status of sperm, PNA-Alexa 488 (final concentration 0.024 mg/ml; [17]) was added to the sperm suspension and incubated for 5 min in a 37°C water bath. The entire suspension was then pipetted onto clean glass slides and allowed to settle for 5 min at room temp. The slides were gently washed with 1 ml PBS (x 2), fixed with 2% paraformaldehyde for 10 min, washed again with 1 ml PBS (x 3) and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Slides were scored (630x) for acrosomal status using an upright fluorescent Zeiss Imager ZI microscope (Thornwood, NY) with Green Fluorescent Protein (GFP) filters. Two hundred morphologically normal sperm

were counted per slide and evaluation was performed blindly of treatment status. Sperm were categorized as acrosome intact (post-acrosomal fluorescence only), intermediate (fluorescence on the apical ridge of acrosome) or acrosome reacted (bright acrosomal fluorescence) as previously described [17].

#### *Effect of Procaine on In Vitro Fertilization*

*Oocyte maturation.* Equine cumulus-oocyte complexes (COCs) were obtained by scraping the follicular wall of ovaries [8] obtained from two different sources: (1) abattoir derived (Equine Reproduction Laboratory, Texas A&M University); and (2) post-mortem ovaries from the necropsy service at Cornell University College of Veterinary Medicine. Abattoir-derived COCs were shipped overnight in 1:1 (vol:vol) TCM 199 with Hanks salts and TCM 199 with Earles salts, supplemented with 25 µg/ml gentamicin, and 20% fetal bovine serum (FBS) at room temperature [39]. Upon arrival to our laboratory or upon retrieval from post-mortem ovaries, COCs were transferred to culture dishes in 50 µl droplets of maturation medium (TCM 199 with Earles salts supplemented with 25 µg/ml gentamicin, 10% FBS and 5 mU/ml FSH) under mineral oil and placed in an incubator at 38.2°C, with 5% CO<sub>2</sub>, in a humidified air atmosphere for 24 h. After maturation, COCs were partially denuded by gentle mouth pipetting in 0.05% porcine hyaluronidase diluted in TCM 199 (Hanks salts, 25 µg/ml gentamicin, 20% FBS) and evaluated for quality (i.e. smooth plasma membrane and apparent perivitelline space). Polar body visualization could not always be used as a maturation criterion because the cumulus was not completely removed prior to IVF experiments.

*In vitro fertilization.* Sperm were incubated at  $10 \times 10^6$  sperm/ml in capacitating MW for 6 h and then diluted to  $1 \times 10^6$  sperm/ml in capacitating MW with or without 5 mM procaine. Droplets (100  $\mu$ l) of the sperm suspensions were pipetted onto culture dishes and covered with mineral oil. Five mature partially denuded mare oocytes were transferred to each droplet and culture dishes were incubated at 38.2°C in 5% CO<sub>2</sub> in humidified air. After 18 h of coincubation, oocytes were transferred to an embryo culture medium (DMEM/F-12 supplemented with 25  $\mu$ g/ml gentamicin and 10% FBS [40]), in 5% CO<sub>2</sub>/90% N<sub>2</sub> mixed gas atmosphere) for an additional 24 h. Oocytes were then fully denuded by mouth pipetting in 0.05% porcine hyaluronidase (as above), fixed in 10% buffered formaldehyde with 0.1% Triton X-100 (vol:vol) and mounted onto glass slides with 6.5  $\mu$ l of 9:1 glycerol:PBS containing 2.5  $\mu$ g/ml Hoechst 33258 to assess fertilization status and chromatin configuration. Coverslips were sealed and oocytes evaluated on a Nikon Eclipse TE200 inverted microscope (Melville, NY) with fluorescent filters. Oocytes were considered fertilized if one or more decondensing sperm heads or pronuclei were observed, or if they had cleaved to the two-cell stage. Degenerating oocytes containing no chromatin or fragmented chromatin, and oocytes that had failed to mature to metaphase II were not counted in the assessment of fertilization rates.

## **RESULTS**

### *Characterization of Hyperactivation in Stallion Sperm: Effects of Procaine on Motility Measures Pre- and Post-Capacitation*

Procaine is a local anesthetic that has been demonstrated to induce hyperactivation in sperm from other mammalian species, possibly by increasing the plasma membrane's permeability to calcium [34]. The addition of procaine (5

mM final concentration [33]) to stallion sperm at 0 h elicited an immediate change in motility in nearly all motile sperm, characterized by a star-shaped movement pattern (Figure 4.1B). Procaine-treated sperm completed more than one circle within the measuring period (30 frames at 60 Hz) as depicted by the overlap of the sperm track (Figure 4.1B). This change in motility was characterized by an increase in ALH ( $P = 0.013$ ) as well as decreases in STR ( $P = 0.003$ ) and LIN ( $P = 0.005$ ; Table 1). Because movement of the sperm head is dependent on the pattern of flagellum movement, increases in ALH with a concomitant decrease in LIN and/or STR are consistent with hyperactivation [28]. After 6 h of incubation under capacitating conditions, the motility of control sperm was visually similar to that observed at 0 h (Figure 4.1A, C); nonetheless, these sperm were slower as detected by decreases in VAP ( $148.7 \mu\text{m}/\text{sec}$  vs.  $122.6 \mu\text{m}/\text{sec}$ ;  $P = 0.008$ ) and VCL ( $276.7 \mu\text{m}/\text{sec}$  vs  $237.7 \mu\text{m}/\text{sec}$ ;  $P = 0.023$ ; Table 2). Treatment with procaine at 6 h of incubation resulted in tracks that were shortened and more curved when compared to the non-treated control (Figure 4.1C, D). These sperm did not typically complete a full circle, as observed in the 0 h procaine-treated condition; however, treatment with procaine did elicit decreases in VSL ( $P = 0.029$ ), STR ( $P < 0.001$ ) and LIN ( $P = 0.002$ ; Table 2), changes consistent with hyperactivation [28]. There were no significant interactions between treatment and stallion or ejaculate within the 0 or 6 h time points. Figure 4.2 depicts still images obtained from videos of control and procaine-treated sperm at 0 and 6 h of incubation. Supplemental Movies 1-4 are available online at [www.biolreprod.org](http://www.biolreprod.org).

Stallion sperm require prolonged incubation to demonstrate markers of capacitation such as tyrosine phosphorylation and acrosomal responsiveness [17]. In other species it has been demonstrated that without these hallmarks of

**Table 4.1.** CASA measurements before and after the addition of 5 mM procaine to stallion sperm at Time 0 hour.

Condition	VAP( $\mu\text{m}/\text{sec}$ )	VSL( $\mu\text{m}/\text{sec}$ )	VCL( $\mu\text{m}/\text{sec}$ )	ALH( $\mu\text{m}/\text{sec}$ ) <sup>a</sup>	BCF(Hz)	STR(%) <sup>b</sup>	LIN(%) <sup>b</sup>
Cap	148.7 $\pm$ 5.1	89.5 $\pm$ 15.4	276.7 $\pm$ 7.3	9.4 $\pm$ 0.2	41.5 $\pm$ 0.9	60.6 $\pm$ 3.7	34.5 $\pm$ 2.2
Cap + Procaine	170.0 $\pm$ 15.4	66.2 $\pm$ 7.7	334.8 $\pm$ 25.4	12.8 $\pm$ 0.9	40.2 $\pm$ 1.3	37.9 $\pm$ 2.7	19.8 $\pm$ 1.4

Data is expressed as  $\pm$  SEM.

<sup>a</sup>Values within columns are significantly different; P = 0.013

<sup>b</sup>Values within columns are significantly different; P  $\leq$  0.005

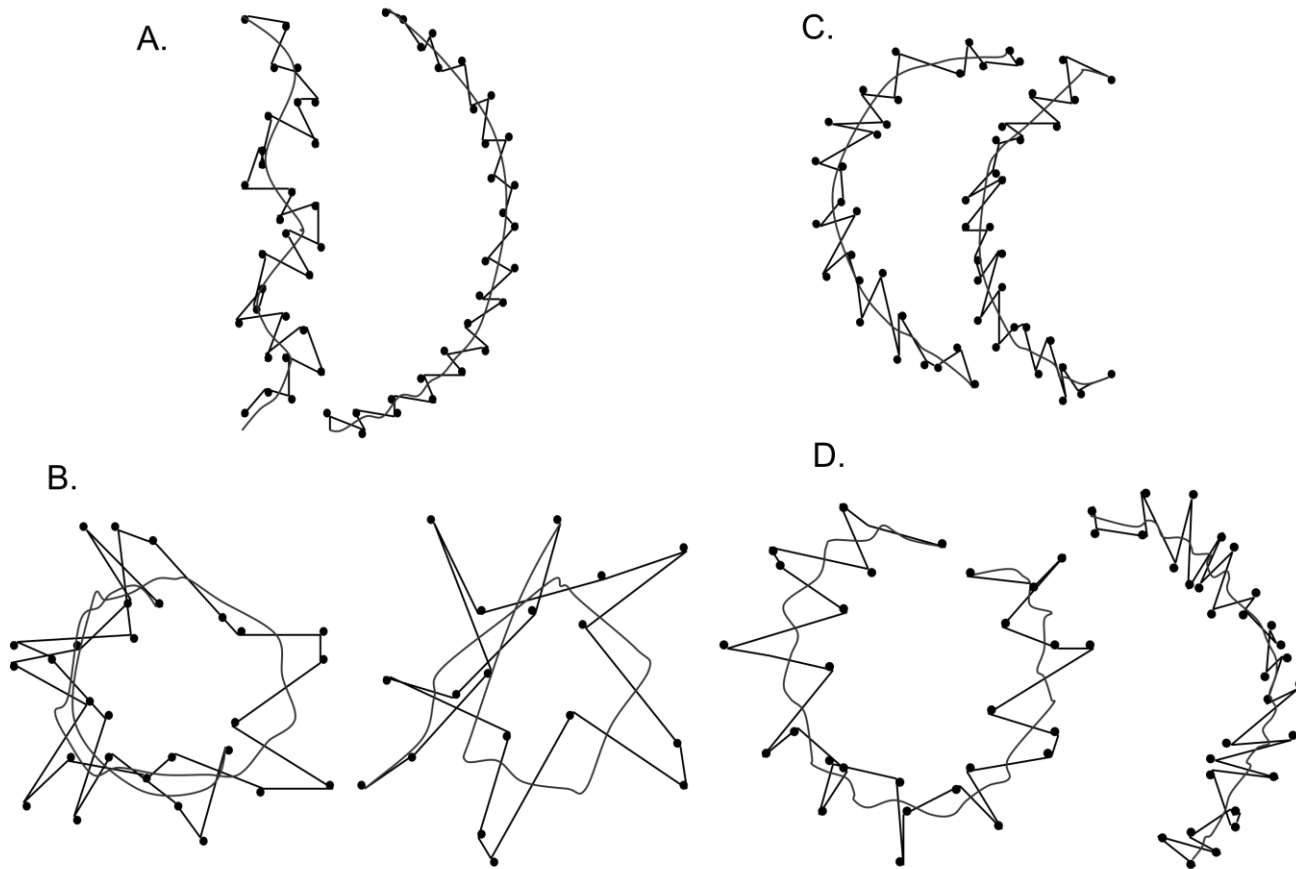
**Table 4.2.** CASA measurements before and after the addition of 5 mM procaine to stallion sperm at Time 6 hour.

Condition	VAP( $\mu\text{m}/\text{sec}$ )	VSL( $\mu\text{m}/\text{sec}$ ) <sup>a</sup>	VCL( $\mu\text{m}/\text{sec}$ )	ALH( $\mu\text{m}/\text{sec}$ )	BCF(Hz)	STR(%) <sup>b</sup>	LIN(%) <sup>b</sup>
Cap	122.6 $\pm$ 10.1	69.9 $\pm$ 9.1	237.7 $\pm$ 15.5	8.8 $\pm$ 0.3	40.9 $\pm$ 0.7	59.2 $\pm$ 4.3	32.1 $\pm$ 2.4
Cap + Procaine	109.3 $\pm$ 14.3	46.5 $\pm$ 4.4	246.8 $\pm$ 23.2	9.8 $\pm$ 1.0	43.6 $\pm$ 1.0	46.6 $\pm$ 2.9	20.2 $\pm$ 0.7

Data is expressed as  $\pm$  SEM.

<sup>a</sup>Values within columns are significantly different; P < 0.03

<sup>b</sup>Values within columns are significantly different; P  $\leq$  0.002

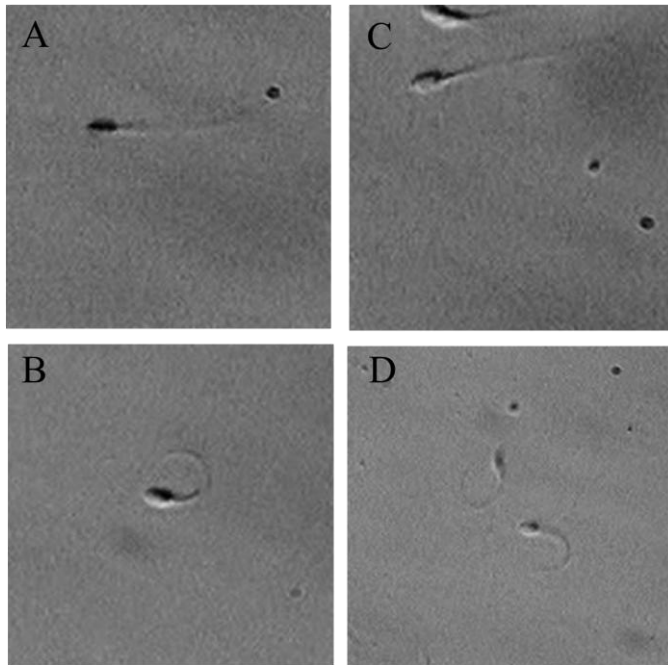


**Figure 4.1.** Hyperactivation induced by procaine. Representative motion tracks of control sperm at 0 h (A) and 6 h (C), and sperm treated with 5 mM procaine at 0 h (B) and 6 h (D). Points on the track represent positions of the head in 30 successive video frames in 0.5 sec.

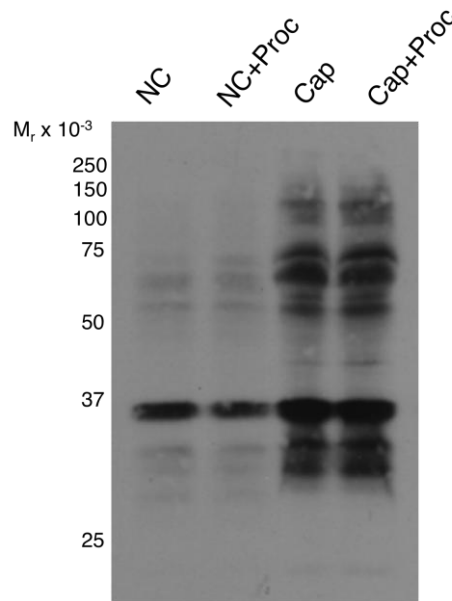
capacitation, sperm are fertilization incompetent [10, 41-43]. Therefore, when defining sorting thresholds to determine the percentage of sperm that underwent spontaneous hyperactivation (i.e. not treated with procaine), we used the measures obtained from the 6 h time point described above. For this purpose, sperm with VSL  $\leq 50.9$   $\mu\text{m}/\text{sec}$  and LIN  $\leq 20.9$   $\mu\text{m}/\text{sec}$  were considered hyperactivated. Based on these parameters, ~26% of sperm at 0 h and ~29% of sperm at 6 h were hyperactivated under capacitating conditions. In contrast, after treatment with procaine ~73% ( $P = 0.004$ ) and ~55% ( $P = 0.013$ ) of sperm were hyperactivated at 0 and 6 h, respectively. There were no significant differences between stallions or ejaculates at the 0 h time point ( $P > 0.05$ ); however, stallion to stallion variability ( $P < 0.05$ ) was observed after 6 h of incubation. Additionally, to rule out the possibility that changes in these parameters were associated with decreased velocities of sperm, a second sort was performed using a VCL  $\geq 246.8$   $\mu\text{m}/\text{sec}$  in conjunction with a LIN  $\leq 20.2\%$ . Differences between procaine and non-procaine treated sperm were again observed at 0 and 6 h ( $P = 0.004$  and  $P = 0.038$ , respectively). There were no significant differences between stallions or ejaculates at either time point with these parameters ( $P > 0.05$ ).

#### *Effect of Procaine on Tyrosine Phosphorylation and Acrosomal Exocytosis*

The signaling pathways that initiate and maintain hyperactivated motility are not well defined, but calcium, bicarbonate and cAMP have been demonstrated essential for this process [24]. Since similar requirements support capacitation, the purpose of this experiment was to rule out whether procaine was exerting its effects on sperm motility via the induction of protein tyrosine phosphorylation. Therefore, sperm were incubated in non-capacitating and



**Figure 4.2.** Representative images from Supplemental Movies 1-4 (available online at [www.biolreprod.org](http://www.biolreprod.org)) of control and hyperactivated stallion sperm. (A) Control sperm at 0 h. (B) Procaine-treated sperm at 0 h. (C) Capacitated sperm at 6 h. (D) Capacitated-procaine treated sperm at 6 h.



**Figure 4.3.** Representative immunoblot of sperm incubated for 4 h in non-capacitating (NC) or capacitating (Cap) modified Whittens medium with and without the addition of 5 mM procaine (+ Proc) and probed with anti-phosphotyrosine antibodies.



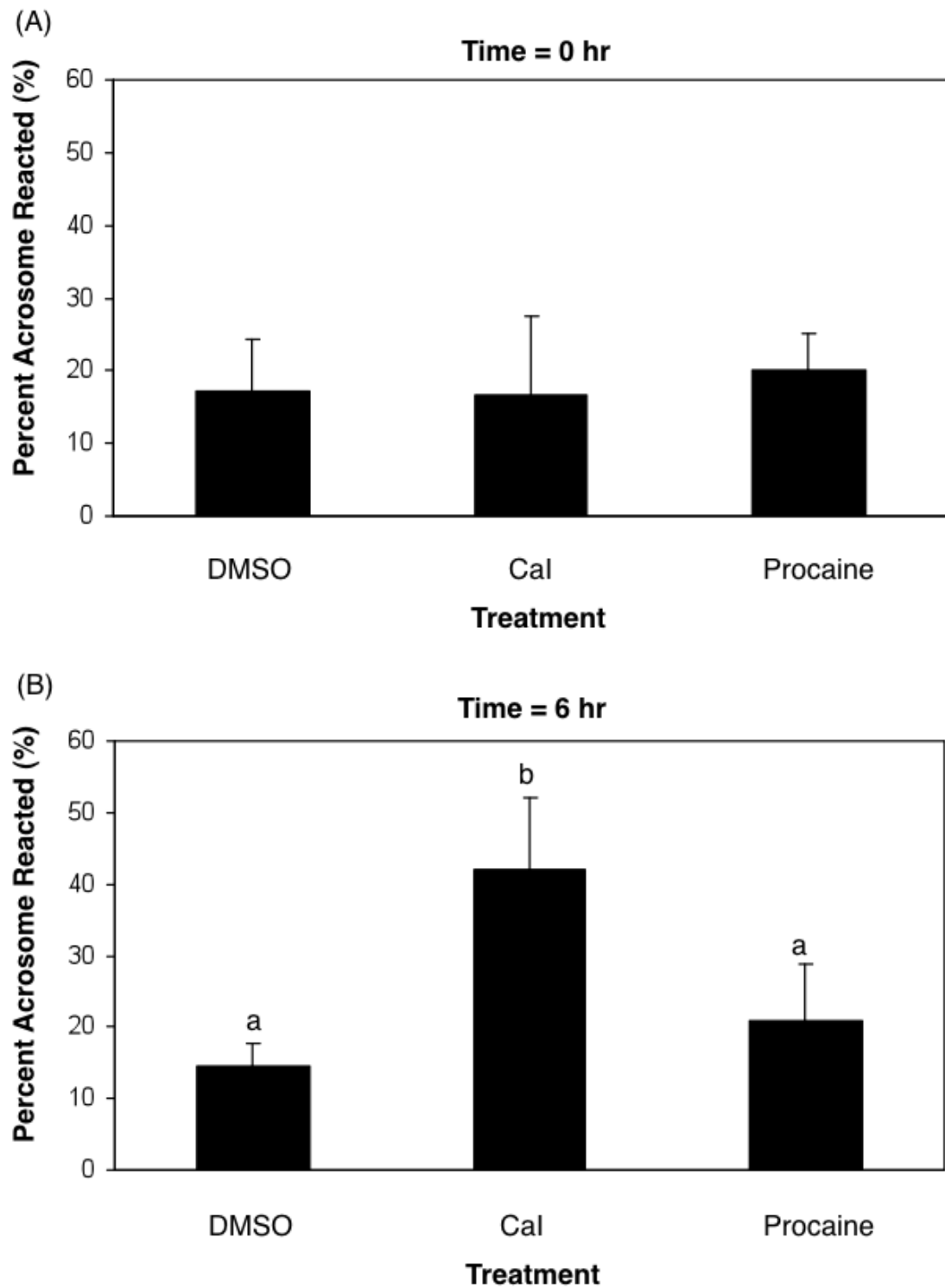
capacitating media in the presence and absence of 5 mM procaine for 0, 2, 4 and 6 h. As shown in Figure 4.3, incubation in the presence of procaine did not have an effect on protein tyrosine phosphorylation. As reported for bovine sperm, our results support the hypothesis that the pathways regulating capacitation (acrosomal responsiveness) and hyperactivation are separable and independent [20, 33, 34].

Because the acrosome reaction is a  $\text{Ca}^{2+}$ -dependent exocytotic event and procaine may induce hyperactivation by increasing plasma membrane permeability to calcium [34], we also wanted to investigate whether the induction of hyperactivation with procaine had any effect on acrosomal exocytosis. We have previously reported significant rates of calcium ionophore -induced acrosomal exocytosis in stallion sperm incubated under capacitating conditions. In this experiment, we exposed sperm previously incubated for 0 or 6 h in capacitating conditions to 5  $\mu\text{M}$  calcium ionophore A23187, 5 mM procaine, or 0.5% (vol:vol) DMSO (vehicle control; equal volume) for 30 min to test whether procaine can induce acrosomal exocytosis. At 0 h, there were no differences observed among any of the treatments ( $P > 0.05$ ; Figure 4.4A). After 6 h of incubation, calcium ionophore induced a significant percentage of acrosome reacted sperm as compared to procaine and DMSO-treated sperm (35% vs. 15.2% and 11.8%, respectively;  $P < 0.03$ ; Figure 4.4B). There was no difference between DMSO controls and procaine treated sperm at 6 h ( $P > 0.05$ ). There were also no significant interactions between treatment and stallion or ejaculate in either of the time points analyzed. Altogether, these data suggest that procaine-induced hyperactivation of stallion sperm is independent from protein tyrosine phosphorylation and acrosomal exocytosis events.

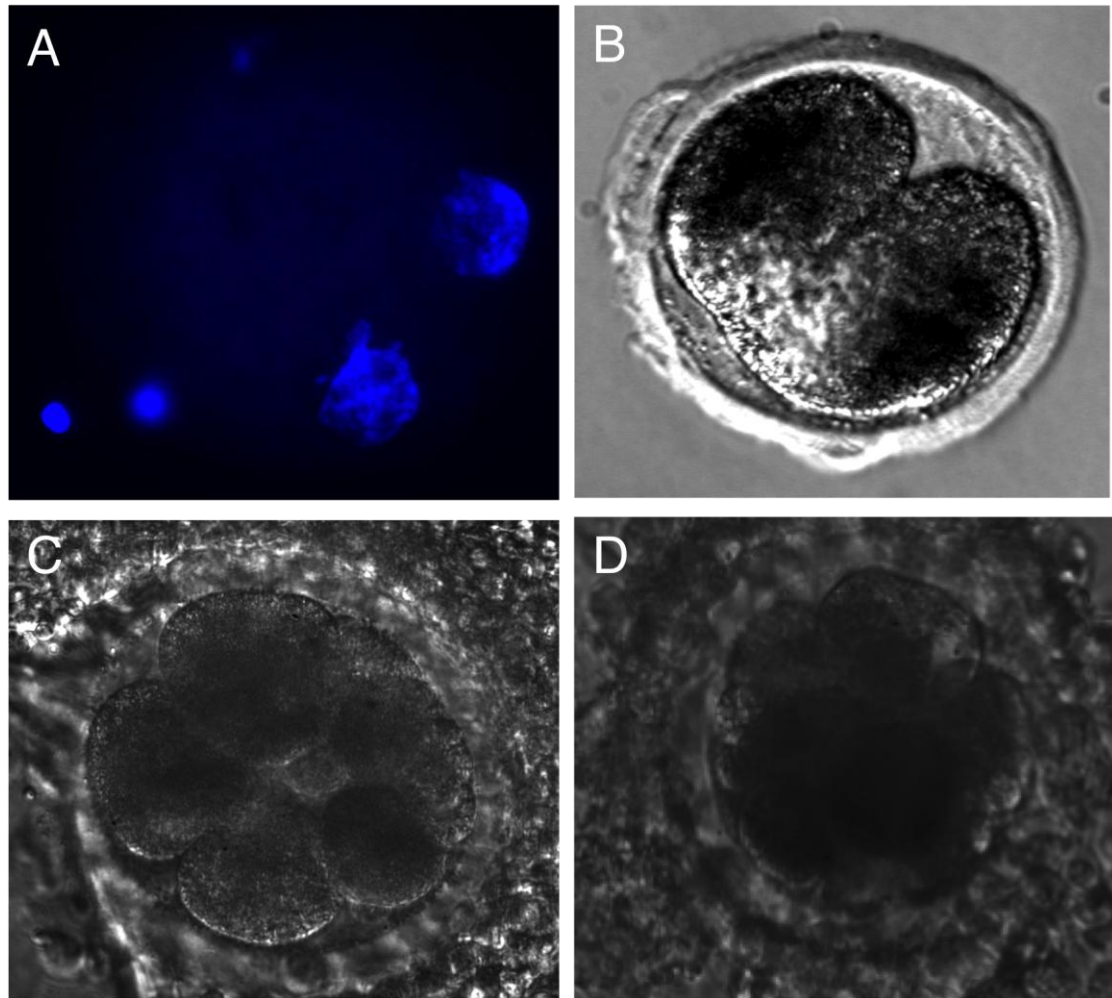
### *Effect of Procaine on In Vitro Fertilization*

The ability of a population of sperm to undergo protein tyrosine phosphorylation and induced acrosomal exocytosis is suggestive of capacitation [10, 31, 32, 41, 44]; however, the most definitive test of capacitation is the ability of sperm to fertilize an oocyte. Notably, *in vitro* fertilization (IVF) has been highly unsuccessful in the equine, as evidenced by low fertilization rates (0-33%; [2-8]); moreover, these results have not been replicable even within laboratories using the same protocols [4-6]. Therefore, we wanted to investigate whether our capacitation conditions, which conferred time-dependent increases in protein tyrosine phosphorylation and acrosomal responsiveness on sperm, would also support IVF of mare oocytes. Additionally, because procaine induced changes consistent with hyperactivated motility, we combined our capacitation conditions with the addition of procaine at the time of insemination. For these experiments, sperm were incubated for 6 h at  $10 \times 10^6$  sperm/ml in capacitation medium, then further diluted to  $1 \times 10^6$  sperm/ml in capacitation medium with or without the addition of procaine (5 mM final concentration) prior to co-incubation with mature equine oocytes. In independent replicates, using semen from two stallions, we achieved 54% (7/13), 40% (6/15), 66% (4/6), 84% (11/13), 50% (4/8), 66% (4/6) and 84% (11/13) fertilization rates, as evidenced by either pronuclear formation or cleavage to the two-cell stage (Figure 4.5A, B). In one experiment, potential embryos were further incubated until day 3 post-insemination in embryo culture medium and 4/6 (66%) had cleaved to the 8-16 cell stage (Figure 4.5C, D). When sperm were incubated in our capacitation conditions but procaine was not added at the time of insemination, no oocytes were fertilized (0/66 oocytes; total of five experiments). Following these results, we wanted to test whether hyperactivation alone in non-capacitated sperm was

sufficient to fertilize oocytes. For this purpose, semen from one of these stallions was incubated in non-capacitating or capacitating medium for 6 h and diluted to  $1 \times 10^6$  sperm/ml plus 5 mM procaine as described. Sperm incubated under capacitating conditions in the presence of procaine achieved 40% (2/5) fertilization, whereas 0% (0/5) of oocytes were fertilized in the non-capacitating MW plus procaine condition. These data support the concept that stallion sperm must undergo changes consistent with capacitation (protein tyrosine phosphorylation and acrosomal responsiveness) as well as acquire hyperactivated motility in order to become fertilization-competent.



**Figure 4.4.** Percentages of acrosomal exocytosis in sperm incubated under capacitating conditions and exposed to calcium ionophore A23187 (Cal), 5 mM procaine, or DMSO (vehicle control) at 0 h (A) and 6 h (B).



**Figure 4.5.** Representative *in vitro* fertilized mare oocytes. (A) Hoechst 33258 staining showing 2 pronuclei (18 h post IVF); (B) phase contrast microscopy of a 2 cell embryo (24 h post IVF); (C & D) light microscopy of 8 cell embryos (Day 3).

## DISCUSSION

In this study, we characterized the motility pattern and movement measurements associated with hyperactivation in stallion sperm and demonstrated that stallion sperm hyperactivation is required for successful IVF. Because treatment with procaine was not associated with changes in protein tyrosine phosphorylation or the induction of acrosomal exocytosis, it is

unlikely that procaine facilitated fertilization via a mechanism other than the induction of sperm hyperactivation. Moreover, neither incubation of sperm in capacitation conditions alone nor treatment of non-capacitated sperm with procaine supported IVF in our experiments. Altogether, our results indicate that both capacitation and hyperactivation are required for successful IVF in the horse.

The addition of procaine to stallion sperm (both at 0 and 6 h of incubation) induced significant changes in the motion parameters that have been used to define hyperactivated motility in other species [22, 28, 29, 33, 45-47]. However, treatment with procaine after 6 h of incubation did not elicit as robust a response when compared to non-incubated sperm (Supplemental Movies 2, 4); rather, the sperm tracks shortened and became more curved (Figure 4.1C,D). Nonetheless, in both cases, decreases in LIN and STR were consistent with the acquisition of hyperactivation. Therefore, because only sperm incubated for 6 h underwent changes consistent with capacitation, and were presumably fertilization-competent, we chose to define hyperactivation based on the motion measures observed after procaine treatment of sperm incubated for 6 h. Based on these experiments, hyperactivated stallion sperm can be defined by a  $VSL \leq 46.5 \pm 4.4 \mu\text{m}/\text{sec}$ ,  $STR \leq 46.6 \pm 2.9\%$  and  $LIN \leq 20.2 \pm 0.7\%$  (Table 2). Notably, the difference between procaine and non-procaine-treated sperm was not attributed to decreased velocities or sluggishness of sperm as these differences were still significant when track speed (VCL) was  $\geq 246.8 \mu\text{m}/\text{sec}$ . In fact, treatment with procaine increased VCL at both 0 and 6 h (Tables 1 & 2).

Sperm from mouse, human and non-human primates undergo hyperactivation when incubated under capacitating conditions [28, 45, 46, 48].

Conversely, bull sperm do not undergo spontaneous hyperactivation when incubated in conditions supporting capacitation and therefore procaine has been used to characterize hyperactivation in this species [33]. Moreover, treatment of wild type mouse sperm with procaine induces a pattern of hyperactivation similar to that observed under *in vitro* capacitating conditions [49]. In order to ascertain whether stallion sperm undergo spontaneous hyperactivation under capacitating conditions, we re-analyzed our data using the average + SEM of the variables that defined hyperactivation in procaine-treated samples (VSL  $\leq$  50.9  $\mu\text{m}/\text{sec}$  and LIN  $\leq$  20.9%). In this context, we determined that only 26% of sperm at 0 h and 29% of sperm at 6 h spontaneously hyperactivated under capacitating conditions, whereas 73% and 55% of procaine-treated sperm fell within the hyperactivated category, at 0 and 6 h, respectively ( $P < 0.05$ ). This suggests that some stallion sperm undergo spontaneous hyperactivation, albeit below the critical threshold to support fertilization as evidenced by our IVF experiments in which 0% of non-procaine treated sperm fertilized mare oocytes. Moreover, only ~30–50% of stallion sperm in an incubated population are capacitated as determined by acrosomal responsiveness [17]. Capacitation and hyperactivation are separable and independent events, that is, a particular sperm may be hyperactivated but not capacitated and vice versa; therefore, only a small percentage of incubated sperm displaying hyperactivated motility are capable of fertilizing. Furthermore, based on our visual observations of sperm incubated in capacitating conditions, hyperactivation in untreated samples appeared to be mostly biphasic: Sperm will circle, become progressive, and then circle again. We hypothesize that this on and off motility pattern may enable sperm to navigate through the uterine or oviductal milieu. In contrast,

treatment with procaine synchronizes hyperactivation ensuring that a larger percentage of capacitated sperm are hyperactivated during these IVF experiments. Additionally, procaine-treated sperm do not exhibit biphasic motility but rather they circle continuously (Supplemental Movies 2, 4), perhaps giving the continuous thrust or propulsion needed to penetrate the zona pellucida.

Procaine exerts its effects on sperm motility presumably by increasing the permeability of the plasma membrane to calcium [34]. This is supported by the failure of procaine to elicit motility changes in bovine sperm incubated in calcium-free medium [33]. We have also observed this in stallion sperm (data not shown). However, calcium is also a critical component of the pathways regulating protein tyrosine phosphorylation and acrosomal exocytosis [50-53]. One might argue that procaine facilitated IVF through the activation of these pathways. However, as found previously in bovine sperm [33], incubation of stallion sperm with procaine did not induce time-dependent increases in protein tyrosine phosphorylation (Figure 4.3) nor did it induce acrosomal exocytosis (Figure 4.4). These results suggest that capacitation and hyperactivation are separable and independent events, as demonstrated in bovine sperm [33]. Moreover, this leads us to conclude that procaine facilitated IVF via the induction of hyperactivated motility.

Protocols used for equine IVF have typically mimicked those published for the bovine species without investigation into the specific incubation conditions required for stallion sperm [2, 5, 6, 54]. Moreover, previous reports have not adequately defined incubation conditions that supported time-dependent increases in protein tyrosine phosphorylation in the absence of pharmacological reagents, nor have they correlated these changes with the



acquisition of acrosomal responsiveness and/or fertilization potential [12, 55]. We have recently reported incubation conditions that support stallion sperm capacitation [17]; moreover, such conditions were similar to the requirements of murine sperm, which may partially explain the inability to previously capacitate stallion sperm in the laboratory. Reported IVF protocols yielding the highest fertilization rates (33%) have incubated sperm in the presence of calcium ionophore for short periods before co-incubation, which increases intracellular calcium levels. While previous thinking about the effect of the calcium ionophore in these protocols was that it induced capacitation and/or the acrosome reaction, it may have been acting in a similar manner to the procaine used in the present study, albeit less efficiently, by inducing hyperactivated motility. Fertilization rates with untreated horse sperm are essentially 0% [2, 4-7], whereas partial or complete removal of the zona pellucida results in high fertilization rates, including polyspermic fertilization upwards of 65% [3]. However, the essential relationship of hyperactivated motility to fertilization is demonstrated by *Catsper*-null mice, whose sperm lack the ability to hyperactivate, can not penetrate zona-intact oocytes *in vitro* and are infertile as a result [18, 19]. Altogether, these studies and ours suggest that the major barrier for successful equine IVF is penetration of the zona pellucida; moreover, our results corroborate the findings of oocyte transfer studies [8] in that that the failure of sperm to penetrate the zona pellucida of *in vitro* matured oocytes under standard IVF conditions is probably not due to premature zona hardening (i.e. cortical granule release).

Due to the difficulties associated with obtaining mare ovaries in the United States (all horse slaughterhouses in this country closed in 2007) and of superovulating mares for the recovery of meaningful numbers of oocytes,

intracytoplasmic sperm injection (ICSI) may still be the clinical assisted reproductive technique of choice in subfertility cases linked to poor semen quality or when a valuable mare is euthanized due to medical reasons. However, studies comparing IVF with ICSI in other species have demonstrated differences in the epigenetic imprinting of genes during embryonic development with these techniques [56, 57], which highlights the physiological relevance of IVF over ICSI. Therefore, understanding the underpinnings of stallion sperm capacitation and hyperactivation as it pertains to successful IVF may provide insight into the molecular events surrounding fertilization in the equine that can be applied to the improvement of other assisted reproduction techniques.

In summary, in this study, we characterized procaine-induced hyperactivation of stallion sperm and demonstrated that induction of hyperactivation facilitates fertilization. Most notably, by coupling our capacitating conditions with the induction of hyperactivation using procaine, we have achieved the novel result of substantial and reproducible percentages of fertilized mare oocytes in our IVF experiments.

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## CHAPTER FIVE

### SUMMARY AND DISCUSSION OF RESULTS

In several domestic and laboratory species, as well as in humans, capacitation can be recapitulated in the laboratory by incubating sperm in a defined medium. Two *in vitro* markers of capacitation include time-dependent increases in protein tyrosine phosphorylation and high rates of agonist-induced acrosomal exocytosis. In contrast to other species, capacitation of stallion sperm has been extremely difficult to achieve with inconsistent results reported between and within laboratories. Moreover, despite numerous attempts, only two foals have ever been produced via *in vitro* fertilization (IVF) technology, both of which were born over fifteen years ago. Evidence suggests that this is ultimately due to the inability to appropriately capacitate stallion sperm in the laboratory.

Therefore, the objectives of this research were to take a molecular approach to solving the clinical problem of unsuccessful IVF in this species. By taking a basic-science approach to solving the problems associated with stallion sperm capacitation, something that had been previously lacking in the field, the goal of this research was to translate our findings into the successful fertilization of mare oocytes. For this purpose, we felt it was of importance to define and characterize: 1) the incubation conditions that supported capacitation of stallion sperm, as evidenced by increases in protein tyrosine phosphorylation and agonist-induced acrosomal exocytosis; 2) the molecular pathways involved in both of these capacitation-dependent events; 3) the motion parameters associated with hyperactivated motility; and 4) the

incubation conditions that supported successful *in vitro* fertilization of mare oocytes.

By first performing a systematic comparison of the incubation media reportedly used in stallion sperm capacitation experiments, as well as testing a mouse sperm capacitation medium, we determined that incubation of stallion sperm in the mouse sperm medium modified Whitten's was superior to the media currently being used in the literature (i.e. SpTALP and BWW). Additionally, while optimizing the media requirements, we evaluated the key individual components in modified Whitten's and concluded that the addition of 25 mM sodium bicarbonate and 7 mg/ml BSA supported significant time-dependent increases in protein tyrosine phosphorylation and significant percentages of progesterone-induced acrosomal exocytosis. This research is the first demonstration of incubation conditions that support longevity of motility (Table 2.1), significant increases in time-dependent protein tyrosine phosphorylation (Figure 2.1), and significant percentages of progesterone-induced acrosomal exocytosis (Figure 2.8). Another major breakthrough in this study was the ability to achieve these markers of capacitation in the absence of exogenous signaling intermediates (i.e. cAMP analogues) that in other reports have bypassed upstream signaling events. As a result, these incubation conditions are considered physiologically relevant for the understanding of sperm capacitation in the stallion.

We hypothesize that modified Whitten's supports stallion sperm capacitation more effectively than the other media tested because stallion sperm may be more similar in their metabolism to mouse sperm than to bovine sperm. For example, mouse sperm utilize glycolysis, and as such, modified Whitten's contains glucose as the primary energy source. Bovine sperm

utilize oxidative phosphorylation to generate ATP; in fact, glucose inhibits capacitation in this species, and as such, the capacitation media contain lactate and pyruvate as energy sources. This difference in sperm physiology could account for the previous lack of success in stallion sperm capacitation.

Through the systematic evaluation of the protein tyrosine phosphorylation pathway, we demonstrated that the generation of cAMP via SACY was required for this event (Figure 3.1) and that the downstream target in this pathway is the serine/threonine kinase protein kinase A (PKA) (Figure 3.2). We also investigated the possible role of an alternative cAMP target in sperm, the guanine nucleotide exchange factor EPAC. We were surprised to find that EPAC activation had no effect on tyrosine phosphorylation (Figure 3.6), as it has been demonstrated to activate the MAPK pathway in somatic cells. However, due to the fact the EPAC activation can mobilize calcium release, it is involved in exocytotic events in excitable somatic cell types and it is present in the acrosome of stallion and mouse sperm, we hypothesized that it might play a role in acrosomal exocytosis. Indeed, the activation of EPAC with the EPAC-specific cAMP analog 8pCPT induced significant percentages of acrosomal exocytosis in both capacitated stallion and mouse sperm as compared to DMSO controls (Figure 3.7).

Probing deeper into this phenomenon, we demonstrated that when sperm were incubated under capacitating conditions in the presence of 8pCPT, sperm plasma membrane potential was maintained in the depolarized state ( $-32$  mV) as compared to capacitated sperm, which hyperpolarized ( $-54$  mV). This EPAC-induced depolarization was comparable to non-capacitated sperm, which were also depolarized ( $-37$  mV) (Figure 3.8). Collectively, these data lead us to the following two-part model of stallion sperm capacitation as it

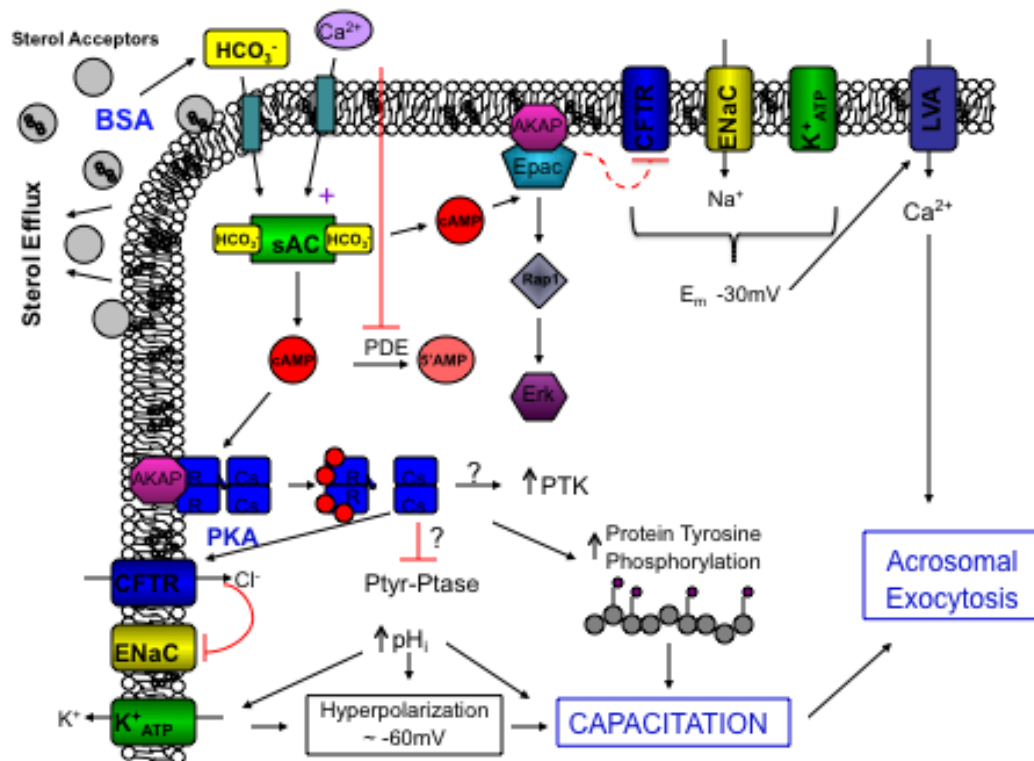
pertains to protein tyrosine phosphorylation and acrosomal exocytosis (Figure 5.1).

The first part of the capacitation model, as it pertains to protein tyrosine phosphorylation, is considered the 'early stage' of capacitation. This involves changes in plasma membrane fluidity, which are postulated to occur as a result of sterol efflux from the plasma membrane; in this regard BSA is used as a sterol acceptor during *in vitro* incubations. These changes then impact enzymatic function and/or ion transport. For example, calcium and bicarbonate enter the cell and activate soluble adenylyl cyclase (sAC or SACY), amongst other functions, with a resulting increase in intracellular cAMP levels. Altogether, these events lead to an increase in PKA activity, which is then transduced into a series of protein tyrosine phosphorylation events.

The second part of the capacitation model, or the 'late stage' of capacitation, involves both PKA and EPAC impacting ion channels in various ways resulting in agonist-induced acrosomal exocytosis. In the non-capacitated state, a depolarized resting membrane potential of  $\sim -37$  mV is observed. During capacitation, however, cAMP-dependent PKA phosphorylates the cystic fibrosis transmembrane conductor (CFTR), thus opening this channel and allowing for the influx of  $\text{Cl}^-$  ions; additionally, activation of CFTR inhibits epithelium sodium ion channels (ENaC). The increase of intracellular pH ( $\text{pH}_i$ ) that also accompanies capacitation, switches the potassium ATP ( $\text{K}^+_{\text{ATP}}$ ) channel from an inwardly rectifying channel to an outwardly rectifying channel. As a result, capacitated sperm have a hyperpolarized membrane potential of  $\sim -54$  mV. This hyperpolarization allows for the recruitment of low voltage activated T-Type calcium channels

(LVA), which can now be opened upon proper stimulation. We hypothesize that when a capacitated sperm contacts the zona pellucida of an oocyte, cAMP-dependent EPAC is activated, which in turn inhibits CFTR, allowing ENaC channels to become constitutively active again. Evidence also suggests that activation of EPAC inhibits  $K^+_{ATP}$  channels. Collectively, this results in a rapid depolarization of the plasma membrane ( $\sim -32$  mV), the opening of LVA channels, and the resulting  $Ca^{2+}$  influx triggers acrosomal exocytosis.

Capacitation and hyperactivation are separable and independent events; that is, a particular sperm may be hyperactivated but not capacitated and vice versa; therefore, only a small percentage of incubated sperm displaying hyperactivated motility are capable of fertilizing, as both are required for this event. Sperm from mouse, human and non-human primates undergo hyperactivation when incubated under capacitating conditions, whereas bull sperm do not. Therefore procaine has been used to characterize hyperactivation in this species. Additionally, treatment of wild type mouse sperm with procaine induces a pattern of hyperactivation similar to that observed under *in vitro* capacitating conditions. In order to ascertain whether stallion sperm undergo spontaneous hyperactivation under capacitating conditions, we re-analyzed our data using the average + SEM of the variables that defined hyperactivation in procaine-treated samples ( $VSL \leq 50.9$   $\mu\text{m}/\text{sec}$  and  $LIN \leq 20.9\%$ ). In this context, we determined that only 26% of sperm at 0 h and 29% of sperm at 6 h spontaneously hyperactivated under capacitating conditions, whereas 73% and 55% of procaine-treated sperm fell within the hyperactivated category, at 0 and 6 h, respectively ( $P < 0.05$ ). This suggests that some stallion sperm undergo spontaneous hyperactivation, albeit below



**Figure 5.1.** Postulated pathways leading to capacitation-dependent increases in protein tyrosine phosphorylation, plasma membrane hyperpolarization and acrosomal exocytosis. BSA: bovine serum albumin; HCO<sub>3</sub><sup>-</sup>: sodium bicarbonate; sAC: soluble adenylyl cyclase; PDE: phosphodiesterase; PKA: protein kinase A; PTK: protein tyrosine kinases; Ptkase: protein tyrosine phosphatases; AKAP: A-kinase anchoring protein; pHi: intracellular pH; CFTR: cystic fibrosis transmembrane conductor; ENaC: epithelium sodium ion channel; K<sup>+</sup> ATP: potassium ATP channel; EPAC: guanine-nucleotide exchange factor; LVA: low voltage activated T-Type calcium channel. See text for pathway details.

the critical threshold to support fertilization as evidenced by our IVF experiments in which 0% of non-procaine treated sperm fertilized mare oocytes. Moreover, only ~30–50% of stallion sperm in an incubated population are capacitated as determined by acrosomal responsiveness.

Furthermore, based on our visual observations of sperm incubated in capacitating conditions, hyperactivation in untreated samples appeared to be mostly biphasic: Sperm will circle, become progressive, and then circle again. We hypothesize that this on and off motility pattern may enable sperm to navigate through the uterine or oviductal milieu. In contrast, treatment with procaine synchronizes hyperactivation ensuring that a larger percentage of capacitated sperm are hyperactivated during our IVF experiments. Additionally, procaine-treated sperm do not exhibit biphasic motility but rather they circle continuously, perhaps giving the continuous thrust or propulsion needed to penetrate the zona pellucida.

By characterizing the motion parameters associated with procaine-induced hyperactivation in stallion sperm in juxtaposition with our capacitating conditions, we were able to achieve the highest rates of homologous *in vitro* fertilization reported in this species (60.7% vs. 0-30% previously reported). These findings were therefore critical in determining the incubation conditions that support successful IVF in the horse.

Collectively, these studies demonstrate newly defined incubation conditions for capacitation and *in vitro* fertilization in the horse, as well as contribute to our understanding of the pathways governing these events in mammalian sperm.