Signaling Mechanisms of Mouse Sperm Capacitation

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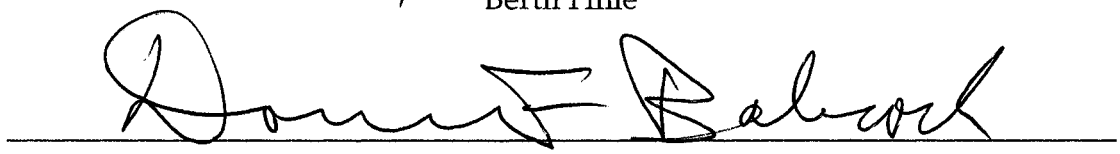

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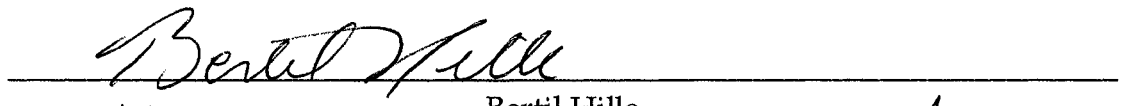

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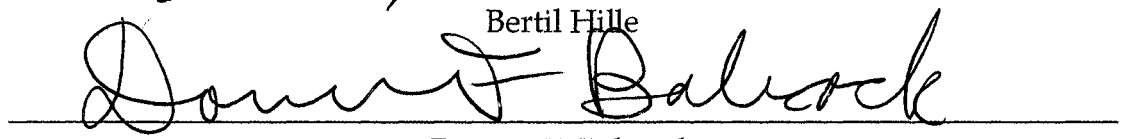

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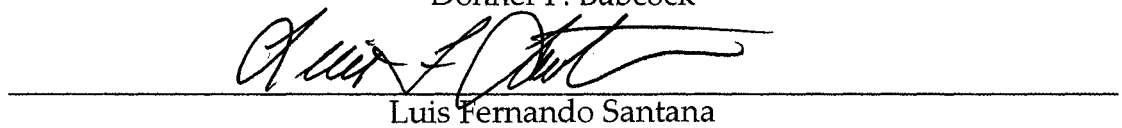

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Abstract

Signaling Mechanisms of Mouse Sperm Capacitation

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Although successful meeting of sperm and egg is vital for continuation of life, critical gaps exist in our understanding of fertilization and events that prepare sperm for it. Understanding how sperm prepare for fertilization after mating may facilitate the design of new forms of contraception or assist in infertility treatment. The overall goal of the experiments described in this proposal is to elucidate the signaling pathways involved in sperm capacitation, the process by which sperm gain the ability to undergo the acrosome reaction and to fertilize. I am interested in studying signaling events in mammalian sperm that occur within the first seconds after ejaculation when relatively immotile sperm initiate rapid swimming – a process termed activation. I am also interested in another change in motility that occurs late in capacitation, hyperactivation, that may be required for sperm to complete fertilization.

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LIST OF ABBREVIATIONS

ATP – Adenosine triphosphate

BSA – Bovine serum albumin

cAMP - cyclic adenosine monophosphate

PDE – phosphodiesterase

PKA – cAMP dependent protein kinase

sAC – soluble adenylyl cyclase

LIST OF FIGURES

GLOSSARY

Acrosome: A membrane-bounded vesicle covering the anterior surfaces of the sperm nucleus, which contains hydrolytic enzymes required for digestion and subsequent penetration of the protective vestments of the egg. Etymology: From the Greek words *akros* meaning 'elevated', and *soma* meaning 'body'.

Acrosome reaction: The exocytotic process involving fusion of the outer acrosomal membrane with the sperm plasma membrane to form a composite membrane with openings through which the acrosomal enzymes pass.

Activation: The earliest stage of capacitation that occurs at mating when the relatively immotile sperm of the vas deferens initiate rapid swimming upon exposure to the male and female reproductive fluids.

Axoneme: The central portion of the sperm flagellum (and of cilia and flagella in general) containing a microtubular array with associated dynein motor proteins; directly responsible for sperm motility. Sperm axonemes are comprised of a '9+2' array of microtubule doublets occupying the central position of the flagellum throughout its length, and is directly responsible for the motile properties of the flagella.

Capacitation: Collectively, the physiological processes that occur in the female reproductive tract to render sperm competent to fertilize.

Epididymis (plural *epididymides*): A tubular organ joining the testis and vas deferens. Comprised of three main parts: caput, corpus, and cauda, they hold sperm during maturation,. Etymology: *epi* for 'on' which were once referred to as *didymi* from Greek 'twins' or 'paired'.

Flagellum (plural *flagella*): The motile apparatus of the sperm with associated accessory structures. The function of the flagellum is to provide motility to the sperm. Within the flagellum, the major structural features are the axoneme, the mitochondrial sheath, the outer dense fibers, and the fibrous sheath and plasma membrane. Etymology: Latin for 'whip'.

Hyperactivation: The flagellar beating pattern of sperm characterized by a vigorous whiplash-like flagellar motion. Hyperactivated sperm swim with an average circular path. The development of the hyperactivated waveform is associated with capacitation and is thought to be necessary for sperm to penetrate the zona pellucida.

Meiosis: A type of cell division found in the gonads, in which the daughter cells are haploid.

Mitochondrial sheath: A helical wrapping of mitochondria around the outer dense fibers of the sperms midpiece, the mitochondria a usually situated end-to-end in parallel helices (2 parallel arrays in the mouse, 3 in the bull).

Mitosis: Process of cell division in which a diploid cell divides to produce two diploid progeny.

Outer dense fibers: Fibers that extend down the length of the flagellum. Outer dense fibers are not believed to play an active role in axonemal or flagellar movements, cross-lined by extensive disulfide bridges. Rather, these fibers may serve to stiffen or provide elastic recoil properties.

Oviduct: The tubal structure that is the passageway for the egg between the ovary and the internal uterus, and is the site of internal fertilization; called the fallopian tube in the human.

Signal transduction: Biochemical pathways using which intracellular proteins send signals from the cell surface to the inside of the cell.

Spermatid: The haploid germinal cells arising from meiotic divisions of spermatocytes, which differentiates within the seminiferous epithelium into a sperm.

Spermatogenesis: The gradual development of male germ cells into sperm, occurring over an extended period of time within the seminiferous tubules of the testes. This process involves cellular proliferation by repeated mitotic divisions, duplications of chromosomes, genetic recombination and reduction by meiotic division to produce haploid spermatids, and terminal differentiation of spermatids into spermatozoa or sperm.

Spermiogenesis: Maturation of a spermatid to a mature sperm, the last phase of spermatogenesis. This process involves cytoplasmic reduction and differentiation of the tail pieces. Sperm are essentially immotile on completion of spermiogenesis.

Testis (plural *testes*): The male gonad and the site of sperm formation. Etymology: derived from the Latin word for 'witness' or 'testify' – so named from the Roman ritual of placing one hand over their genitals while taking an oath.

Vas deferens (plural *vasa deferentia*): The tube that carries mature sperm from the epipdiymis to the urethra. Etymology: Latin for *vas* meaning 'vessel' + *deferre* meaning 'to carry away from'.

PREFACE

Some of the work presented in this dissertation has been published as separate manuscripts:

Wennemuth, GA, AE Carlson, AJ Harper, DF Babcock. 2003. Bicarbonate actions on flagellar and Ca^{2+} -channel responses: Initial events in sperm activation. Development 130: 1317-1326.

Carlson AE, RE Westenbroek, T Quill, D Ren, DE Clapham, B Hille, DL Garbers, DF Babcock. 2003. CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. Proc Natl Acad Sci USA 100: 14864-14868.

Carlson AE, TA Quill, RE Westenbroek, SM Schuh, B Hille, DF Babcock. 2005. Identical phenotypes of CatSper1 and CatSper2 null sperm. J Biol Chem 280: 32238-32244.

Schuh, SM, AE Carlson, GS McKnight, M Conti, B Hille, DF Babcock. 2006. Signaling pathways for modulation of mouse sperm motility by adenosine and catecholamine agonists. Biol Reprod 74: 492-500.

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Finally, I thank my family. My parents Phil and Cheryl Carlson, and my brother Marc Carlson. Your love and support got me here. You have always told me that I can do anything, and I have believed you. Thank you.

DEDICATION

To my grandmothers Violet and Fern.

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 General background on sperm

1.1.1 The discovery of sperm.

The first report of sperm came from the Dutch scientist Anton van Leeuwenhoek (1632-1723), famous for his homemade microscopes capable of magnification of up to 300 times (Pinto-Correia 1997). Leeuwenhoek left school at age 16, and was likely self-taught in natural history. In his own semen, Leeuwenhoek reported hundreds of vigorously swimming 'animalcules'; the name 'spermatozoa' refers to these 'animals of semen'. Leeuwenhoek hypothesized that the microscopic creatures swimming in the semen entered the egg and resulted in fertilization (Pinto-Correia 1997). During that time, Leeuwenhoek's findings were considered controversial and were not widely accepted (Birkhead 2000). Another hundred years would pass before another scientist would provide evidence supporting Leeuwenhoek's hypothesis. The Italian priest and scientist Lazzaro Spallanzani studied reproduction in frogs, and his research, surprisingly, was financially supported by the Catholic Church (Birkhead 2000). Spallanzani constructed pairs of oilskin pants for male frogs which prevented their semen from contacting eggs. Despite the encumbrance of the pants, the male frogs still grasped the females, yet the eggs were not fertilized. In a well-controlled experiment, Spallanzani then recovered drops of semen from inside the pants, applied the sample to eggs and subsequently documented fertilization and tadpole development (Pinto-Correia 1997). We have learned much since the discovery of sperm and documentation of its role in fertilization.

1.1.2 Sperm development.

Mammalian sperm are produced in the male gonad, the testes. The testes are comprised of numerous long seminiferous tubules which converge and join at one end. Sperm develop from special stem cells called spermatogonia that line the outer walls of the seminiferous tubule. Beginning at puberty and continuing throughout life, these spermatogonia divide mitotically to give rise to diploid germ-line cells (Oko and Clermont 1998). Some spermatogonia enter the first stage of meiotic division and become primary spermatocytes and begin spermatogenesis. These cells move progressively inwards from the outer tubular lining as they advance though several developmental stages.

When sperm leave the testes they are incapable of progressive motility and unable to fertilize (Yanagimachi 1994). From the testes, sperm enter the epididymis. The cells lining the epididymal lumen secrete important substances that help sperm mature and survive, and within the epididymis, sperm undergo maturational processes that endow them with the capabilities of progressive motility and fertilization (Yanagimachi 1994). The epididymis empties into the vas deferens, which is then responsible for storing mature sperm until mating.

1.1.3 Sperm anatomy.

Evolution has selected for mammalian sperm cells that efficiently transport paternal DNA from the site of sperm storage in the adult male to the egg deep within the female. Mature sperm cells are terminally differentiated and highly polarized. A sperm cell has three main anatomical regions: a head, a midpiece, and principal piece (together the midpiece and principal piece form the flagellum). The exact details of cellular morphology, for example, the size and shape of the head, and the length of the flagellum vary greatly even between closely related species (Bedford and Cross 1998). The sperm head contains its nucleus and therefore its haploid set of DNA. Whereas the DNA of somatic cells is packaged around histone, sperm DNA is packaged in a more compactly bound form to protamine. This compact packaging leads to the

unique arrest of transcription in sperm. At the tip of a sperm head is the acrosome, an exocytotic organelle that contains enzymes that may allow for sperm to penetrate the protective coating of the egg. The sperm midpiece contains mitochondria that provide some of the ATP to fuel the sperm's journey to the egg. A prominent cellular feature of sperm is the tail-like flagellum. It propels sperm from one place to another.

1.2 Capacitation background

Historically, early fertilization research used primarily invertebrate model systems because of ease in collecting and manipulating their gametes. Mammalian model systems presented several obstacles including difficulty in obtaining eggs, and more interestingly, it was observed that sperm did not fertilize when placed near eggs in the oviduct or *in vitro*. The failure of transplanted sperm to fertilize was explained in 1951 by two scientists working independently. In experiments that were both pioneering and elegant, Chang reported that in the rabbit, fertilization success varied with time of tubal insemination relative to time of ovulation (Chang 1951), and Austin found that rat fertilization varied in relation to the time sperm spent in a female (Austin 1951). Both groups found that sperm recovered after longer residence in the female-donor fertilized recipient females more quickly. These findings suggested that sperm undergo physiological changes while residing in the female reproductive tract, and that these changes confer upon them the capacity to fertilize. Austin later coined the term *capacitation* to denote these physiologic changes (Austin 1967). We now understand capacitation to represent a set of biochemical changes in sperm, with the ability to undergo the acrosome reaction being one consequence (Bedford and Cross 1998). The exact details of the requirements and processes involved in capacitation vary between species (Bedford and Cross 1998).

Since the pioneering work of Austin and Chang, scientists have used incubations that are intended to approximate conditions of the female reproductive tract to study capacitation *in vitro*. Incubations used for *in vitro* capacitation are species specific and largely empirical, but they typically use media that contain Ca^{2+}, HCO_3^-/CO_2 and a protein source such as bovine serum albumin (BSA), and are pH

controlled. These incubations are typically last at least one hour for most sperm to complete capacitation. *In vitro* capacitation is assessed traditionally by the ability of sperm to fertilize and has allowed for dissection of the various stages and cellular changes associated with capacitation. Such capacitation-associated changes include increases in metabolic rate (e.g. glycolytic activity and oxygen consumption), serine/threonine phosphorylation by the cyclic AMP-dependent protein kinase (PKA) and tyrosine phosphorylation by unknown tyrosine kinases signaling pathways, alteration of lipid and protein composition of the plasma membrane, and changes in swimming patterns (Yanagimachi 1994).

1.2.1 Cellular changes associated with capacitation.

Membrane events – During fertilization, sperm undergo two membrane fusion events, exocytosis of the acrosome secretory granule known as the acrosome reaction, and sperm-egg fusion. The existence of these two fusion events suggests that at the time of fertilization, the sperm plasma membrane must be in a metastable fusible state (Gadella and Van Gestel 2004). In contrast, during spermiogenesis and storage in the male, the sperm membrane needs to be stable. Hence, membrane destabilization must take place following the sperm entry into the female reproductive tract and before encountering the egg (Gadella and Van Gestel 2004). HCO_3^- appears to be particularly important for membrane destabilization (Harrison et al. 1996).

Cyclic AMP-dependent phosphorylation – Activation of PKA has long been recognized as an important event in sperm capacitation (Garbers and Kopf 1980; Tash and Means 1983). Activation of this kinase may then produce phosphorylation of motor proteins. Further, it is possible that PKA-mediated phosphorylation is an important regulator in the membrane events of capacitation (Harrison and Miller 2000). The roles of cAMP and PKA in capacitation are discussed in more detail later in this chapter.

Tyrosine phosphorylation – A long-term effect of sperm capacitation is the activation of tyrosine kinases and the subsequent tyrosine phosphorylation of diverse sperm proteins. This tyrosine phosphorylation in mouse sperm appears slowly, with some phosphorylation appearing at 60 min, and maximal at 90 min, a time that coincides with the incubation time required for sperm to be competent for undergoing the acrosome reaction and *in vitro* fertilization (Yanagimachi 1994). This tyrosine phosphorylation pathway seems to require prior elevation of cAMP and activation of PKA (Nolan et al. 2004; Visconti et al. 1995a; Visconti et al. 1995b). Proteins known to be tyrosine phosphorylated during capacitation include scaffolding proteins of the AKAP family of A-kinase anchoring proteins (Carr et al. 2001; Carrera et al. 1996; Ficarro et al. 2003). AKAPs are found in the sperm flagellum (Ficarro et al. 2003), and have been implicated in cross-talk between the cAMP/PKA signaling pathway and the tyrosine phosphorylation cascade.

1.3 How sperm swim

1.3.1 General overview of sperm flagella.

The flagellum allows sperm to swim and its axonemal core contains nine pairs of microtubules that surround a central pair. The '9+2' axoneme is found in virtually all eukaryotic cilia and flagella. This characteristic '9+2' arrangement can be seen when the axoneme is viewed in cross section with an electron microscope. The two central singlet and nine outer doublet microtubules are continuous for the entire length of the axoneme.

The basic mechanochemical mechanism which defines flagellar motility is the ATP-induced sliding of microtubules. Flagella beat back and forth when the microtubule pairs in the core slide past each driven by the motor protein dynein (Summers and Gibbons 1971). The energy for microtubule sliding is derived from the ATPase activity associated with the dynein arms on the inner and outer doublet microtubules (Summers and Gibbons 1971). While this basic axonemal mechanism is

fairly well agreed upon, the variety of waveforms that produce diverse types swimming patterns, suggests additional regulatory mechanisms.

1.3.2 Flagellar control.

Mammalian sperm cells have a limited repertoire of swimming behaviors and intracellular signaling paths to mediate swimming changes. The cellular changes that mediate these swimming patterns are not yet completely understood, but must be tightly regulated and harmonious with the location of the sperm as it travels through the female reproductive tract.

The signaling pathways that control swimming behaviors of broadcast-fertilizing sea urchin sperm are better characterized than those for internal-fertilizing mammalian sperm. For instance, a well-understood pathway is the sea urchin swimming response to chemoattractants released by eggs. The peptide resact is released by eggs of the sea urchin *Arbacia punctulata* (Suzuki and Garbers 1984). Resact binds to guanylyl cyclase receptors on the sperm flagellum, and this activates a signal transduction pathway that elevates intracellular pH and Ca^{2+} and causes the sperm to turn unless they continuously move up the peptide concentration gradient toward the egg (Cook et al. 1994; Kaupp et al. 2003; Kirkman-Brown et al. 2003). Specifically, resact triggers a rapid elevation of cGMP content which is followed by a delayed rise in Ca^{2+} levels (Kaupp et al. 2003). How do these cellular signals change swimming patterns?

Ca^{2+} likely changes swimming patterns by binding directly to proteins of the axoneme. This hypothesis is supported by the finding that in reactivated, demembranated sea urchin sperm, the flagellar beat asymmetry is dependent on the Ca^{2+} concentration of the bathing solution (Brokaw et al. 1974). Specifically, as the Ca^{2+} content was increased, the flagellar beat pattern became more asymmetric, and sperm swam in circular paths of decreasing radius (Brokaw et al. 1974). Therefore, resact-evoked elevation of intracellular Ca^{2+} causes sperm to swim towards the resact source, the egg. As the axoneme of demembranated, reactivated sperm is open to the bathing

solution, Ca^{2+} must signal by binding either directly to the axoneme or to a complex anchored near the axoneme.

Transduction events in mammalian sperm are similarly likely to involve intracellular second messengers such as Ca^{2+}, and cyclic nucleotides and to use ion channels. However, elucidating these signals has been difficult. Studying mature sperm presents a variety of obstacles. Mammalian sperm are very small, differentiated cells that do not synthesize new proteins and are difficult to study by the patch clamp method. Thus, studying their ion channels and intracellular ionic events has required alternative methods, such as using transgenic animals, fluorescent indicators, and pharmacologic approaches. Mature sperm cells are motile, adding an additional difficulty in studying them at the single-cell level. In this dissertation, my aim was to elucidate the signaling pathways underlying changes in mouse sperm swimming patterns during capacitation. It is not yet understood whether or how these other capacitation-related cellular changes, such as membrane events and tyrosine phosphorylation, fit into signaling changes in swimming patterns.

1.3.3 Sperm motility and fertility.

Sperm can be moved towards the site of fertilization mechanically by the contractile forces of the female musculature and by the ciliated oviductal epithelium (Bedford and Cross 1998) raising the possibility that sperm motility might not be required for internal fertilizers. Several studies, however, document that mammalian sperm motility is important for male fertility. A recent report evaluating human semen characteristics found that motility and sperm counts were the best predictors of male fertility and that men with motile sperm were likely to be fertile whereas men with immotile sperm were likely to be infertile (Nallella et al. 2006). Other human-based studies document that sperm motility predicts success of intrauterine sperm injection (Branigan et al. 1999; Shulman et al. 1998). Therefore, sperm motility is important for moving sperm from the site of deposition at mating, to the site of fertilization.

We can look elsewhere in the animal kingdom for more examples of the importance of sperm motility for fertility. For instance, in the opossum and many

other species of American marsupials, approximately 80-90% of sperm pair-up during epididymal maturation (Moore and Taggart 1995). Two sperm unite at their heads and together traverse the female reproductive tract, only to separate near the vicinity of the egg. The two flagella beat with the same frequency which is off beat in phase (Moore and Taggart 1995). The end result is less lateral movement and more forward progression. One consequence of this locomotion pattern is an increase in the proportion of sperm arriving at the vicinity of the eggs (Moore and Taggart 1995). These results indicate that as a result of better motility, opossum sperm from locomotive-pairs are more likely to fertilize than are single sperm, and further highlight the importance of sperm motility for fertilization.

1.4 Activation

The earliest stage of capacitation involves activation of sperm motility. While residing in the epididymis and vas deferens, sperm are densely packed ($>10^9$ sperm/ml) and are nearly or completely immotile. Sperm initiate a low basal level of 'resting' motility when they are diluted in isotonic media. Activation specifically refers to the initiation of vigorous movement that occurs when sperm come into contact with the HCO_3^- contained in male and/or female reproductive fluids or in physiological salt solutions (Bedford and Yanagimachi 1992; Yanagimachi 1994). A low HCO_3^- concentration and an acidic pH in the luminal fluid of the epididymis and vas deferens help to maintain sperm in a quiescent state during maturation and storage in these organs (Breton 2001). Freshly ejaculated sperm need to travel a large distance from the site of sperm deposition (typically the vagina or uterus) to the site of fertilization (oviducts). Vigorous swimming in relatively linear trajectories may aid sperm in achieving this goal. *In vitro*, HCO_3^- is sufficient to initiate this vigorous motility; it is believed to be the physiologic initiator of activation as it is present in both male and female reproductive fluids (Boatman and Robbins 1991; David et al. 1969; David et al. 1973; Hamner and Williams 1965; Okamura et al. 1985).

Mammalian male germ cells contain an atypical adenylyl cyclase (Braun et al. 1977). Unique characteristics of sperm adenylyl cyclase have long been recognized and

include: 1) Presence in the cytosolic fraction (Braun et al. 1977); 2) A requirement for HCO_3^- to increase the intracellular cAMP concentration (Garbers et al. 1982); 3) Resistance to known activators of other adenylyl cyclase (Braun et al. 1977). However, the link between HCO_3^- and cAMP was not clear until the atypical adenylyl cyclase of sperm, named "soluble adenylyl cyclase" (sAC), was cloned (Buck et al. 1999). Demonstration that sAC is present in mature sperm and that recombinant sAC is stimulated by HCO_3^- suggests a cAMP-hypothesis for sperm activation (Chen et al. 2000; Jaiswal and Conti 2001; Sinclair et al. 2000). The sAC lacks identifiable transmembrane domains and differs from conventional transmembrane adenylyl cyclases in that it is neither regulated by heterotrimeric G-proteins nor activated by forskolin (Buck et al. 1999; Chen et al. 2000). *In vitro*, recombinant sAC was stimulated by $NaHCO_3$. It was also stimulated by the bisulfite ion derived from Na_2SO_3 or $NaHSO_3$, which structurally resemble the HCO_3^- anion, but not by dissimilar ions such as chloride (NaCl), sulfate (Na_2SO_4) or phosphate (Na_2HPO_4) (Chen et al. 2000). These findings indicated that HCO_3^- and not CO_2 binds to and activates sAC (Chen et al. 2000). Identification of sAC provided a link between HCO_3^- and a signaling pathway that may be involved in sperm activation.

The details of how HCO_3^- activates sAC and the pathways that putatively would link cAMP to the activation of sperm swimming at the time of mating are not known. In demembranated and reactivated mouse and rat sperm, cAMP increased microtubule sliding (Lindemann et al. 1987; Si and Okuno 1995), presumably via a pathway that involves the cAMP-dependent protein kinase, and phosphorylation of motility-related axonemal proteins.

1.5 Hyperactivation

Yanagimachi reported that hamster sperm incubated in follicular fluid or serum exhibited a movement different from that of an activated sperm (Yanagimachi 1969; Yanagimachi 1970). He called this behavior hyperactivated swimming. It is characterized by large amplitude, 'whiplash'-like beats of the sperm flagellum (Yanagimachi 1970). Whereas ejaculated sperm swim in linear trajectories with a

relatively symmetrical flagellar beat pattern, hyperactivated sperm swim a circular average path with an asymmetric beat pattern (Singh et al. 1983; Suarez et al. 1983). *In vivo*, mouse sperm removed from the female reproductive tract after capacitation time display hyperactivated motility (Suarez et al. 1992). Hyperactivated sperm can be seen through the wall of the oviductal ampulla near the time of fertilization (Katz and Yanagimachi 1980). Sperm that have undergone capacitation and display hyperactivated motility can respond to the physiologic initiators of the acrosome reaction. In fact, there is a close correlation between hyperactivated motility of the sperm and the ability to fertilize (Fraser 1982). What is the purpose of hyperactivated motility? Several possible functions have been proposed for hyperactivation: 1) It may aid sperm in penetrating mucoid oviductal secretions or the matrix of the cumulus oophorus. Indeed, hyperactivated hamster sperm are more capable of penetrating viscous media than activated sperm (Suarez 1996; Suarez et al. 1991); 2) It may help sperm escape from pockets or troughs created by mucosal folds in the oviduct (Smith and Yanagimachi 1989; Smith and Yanagimachi 1990). Only hyperactivated sperm have been observed detaching from the mucosa of the oviducts in mice (Demott and Suarez 1992); 3) Finally, it may assist in the penetration of the zona pellucida, the protective matrix surrounding the oocyte (Katz et al. 1989; Suarez et al. 1984). Hyperactivated sperm penetrate the zona pellucida more effectively than activated sperm (Stauss et al. 1995).

1.6 Aims

The aims of my dissertation research were to elucidate the signaling pathways mediating two swimming patterns associated with mouse sperm capacitation, activation and hyperactivation. Specifically I have characterized the role of HCO_3^- in signaling these two swimming behaviors. I have also described the role of a newly discovered, putative Ca^{2+} channel CatSper in mouse sperm capacitation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Fura-2 acetoxymethyl (AM) ester, sodium-binding benzofuran isophthalate (SBFI) AM, BCECF (2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein) AM, NP-EGTA-AM (o-nitrophenyl-EGTA AM), and Pluronic F127 were from Molecular Probes (Eugene, OR), and FFP18-AM from TEF Labs (Austin, TX). Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3',5'-monophosphorothioate (cBiMPS) from Axxora (San Diego, CA), cGMP-AM from BioMol (Hamburg, Germany) and H89 were from Calbiochem (San Diego, CA). The cAMP enzyme immunoassay kit was from Assay Designs (Ann Arbor, MI). All other chemicals were from Sigma (St. Louis, MO).

2.2 Animals

Wild-type sperm were obtained from male Swiss-Webster retired-breeder mice, unless noted otherwise. Mutant sperm were obtained from adult males from three lines of knockout mice bred on a C57BL/6 background. Mice null for sAC were generated by Esposito and co-workers (Esposito et al. 2004), and we obtained these from the colony maintained by Marco Conti's lab at Stanford University. Experiments characterizing the phenotype of sAC null sperm were done in comparison with sperm from the Swiss-Webster retired breeder mice as controls. CatSper1-null mice were developed by the David Clapham lab at Harvard University (Ren et al. 2001). We obtained these mice from the colony maintained by David Garbers' lab at University of Texas Southwestern Medical Center. Mice null for CatSper2 were developed by David Garbers and coworkers (Quill et al. 2001). In experiments sperm from CatSper1 and CatSper2 null sperm were compared to sperm from their wild-type littermates. All animal procedures were in accordance with accepted standards of humane animal care and were approved by the Animal Care and Use Committee at the University of Washington.

2.3 Experimental methods

2.3.1 Sperm preparation and incubation

Caudae epididymides and vasa deferentia were excised from male mice that were euthanized by CO_2 asphyxiation and cervical dislocation. Tissue were then rinsed in ~3 ml medium Na7.4 (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 20 HEPES [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 5 glucose, 10 lactic acid, 1 pyruvic acid, adjusted to pH 7.4 with NaOH. The cleaned tissue, obtained after removal of adipose and connective tissue, was transferred to 1 ml of a 'swimout/capacitation' medium (medium Na7.4 with 5 mg BSA/ml and 15 mM $NaHCO_3$). Semen was allowed to exude (15 minutes at 37°C, 5% CO_2) from several small incisions made with iridectomy scissors under a dissection microscope. All subsequent operations were at room temperature (22-25°C) in medium Na7.4, unless noted otherwise. Sperm were washed twice, then dispersed and stored at 1-2 x 10^7 cells ml^{-1}. A modified medium Na7.4 with no added $CaCl_2$ was used for 0 mM Ca^{2+} treatments. Experiments with pH photometry were done with a modified medium Na7.4 prepared with no added lactic or pyruvic acids and with 10 mM glucose. Potassium-evoked responses were produced with medium K8.6 (in mM): 135 KCl, 5 NaCl, 2 $CaCl_2$, 1 $MgSO_4$, 30 TAPS [N-tris(hydroxymethyl)-methyl-3-aminopropane sulfonic acid], 10 glucose, 10 lactic acid, 1 pyruvic acid, adjusted to pH 8.6 with NaOH. Some experiments used a sodium-free medium that consisted of (in mM): 135 N-methyl-D-glucamine (NMDG), 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 20 HEPES, adjusted pH to 7.4 with KOH. For in vitro capacitation, sperm were transferred to the 'swimout/capacitation' medium and incubated 90 min at 37°C in a 95% air/5% CO_2 atmosphere. Sperm then were washed twice in medium Na7.4 and examined at room temperature.

2.3.2 Waveform analysis

The flagellar waveform was analyzed from stop-motion digital images in a system unique to our laboratory. A custom-built stroboscopic power supply provided 3-5 V pulses of 1-2 ms duration to create stroboscopic illumination (~1 ms duration) of a pulsed ultrabright red LED (AND190H9Pl Newark Electronics, Chicago, IL). Stop-motion images were captured from these ~1 ms illuminations at a rate of 30 Hz from a 128 x 128 pixel region of the cooled CCD camera chip (larger regions were used for asymmetry measurements), under the direction of Metamorph (Universal Imaging; West Chester PA). Images were stored in TIFF format for subsequent semi-automated tracing of the flagellum. Additional software routines written by Donner F. Babcock analyzed flagellar images to: (*i*) determine the flagellar beat frequency; (*ii*) tabulate the distance along the flagellum (arc length), the angular deviation (tangent angle) along the flagellum, and the time-averaged tangent angle; (*iii*) present the time-averaged tangent angle versus arc length data (shear curves) as a measure of flagellar beat asymmetry; (*iv*) determine the flagellar beat envelope and the beat amplitude at regular intervals along the beat axis; and (*v*) calculate the maximal curvature of the 20 μm midpiece in both the pro-hook and anti-hook directions (Ishijima et al. 2002).

2.3.3 Acetoxymethyl esters

Loading charged molecules into cells can be challenging. To load sperm with indicators or cyclic nucleotides, we expose them to the acetoxymethyl (AM) ester form of the molecules (Tsien 1981). The AM ester group masks charges on molecules and allows for their passage through the sperm membrane. Once inside of the cell, intracellular esterases cleave the AM group to free the charged impermeant parent molecule.

2.3.4 Dye loading and photometry

Calcium and sodium photometry – Fura-2-AM, FFP18-AM, or SBFI-AM was dispensed from 2 mM stocks in DMSO, dispersed in 10-15% Pluronic F127, diluted to 20 µM in 0.5 ml sperm suspension in medium Na7.4. After 30-40 min, medium Na7.4 (0.5 ml) was added and the cells were sedimented. After resuspension in 0.3 ml fresh medium, incubation continued for 45 min before use to ensure de-esterification of the dye. Excitation light of 340 and 380 nm was provided from a computer-controlled galvanometric monochromator (T.I.L.L., Gräfelfing Germany), and >450 nm emitted light was collected by a photodiode detector from an adjustable viewfinder that selected a rectangular region containing a small cluster (3-5 cells) of loosely-tethered sperm, each pivoting about a single point of attachment at the base of the head. The raw photometric signals were corrected for the cell-free background, collected prior to each series of measurements.

For both fura-2 and FFP18, the ratio of the corrected signals was calibrated with the following equation (Grynkiewicz et al. 1985):

$$Ca^{2+} = B \times ((R-R_{FREE})/(R_{SAT} - R))$$

$$[Ca^{2+}]\ (nM) = 1228 \times ((R - 0.3795)/(1.7952 - R))$$

Values for B, R_{FREE} and R_{SAT} were obtained from cells equilibrated in solutions fortified with ionomycin (10 µM) and containing (in mM): 20 EGTA, 15 $CaCl_2$, or 20 EGTA with 15 $CaCl_2$ (calculated free Ca^{2+} concentration of 226 nM). The calibrated signal reports spatially-averaged internal $[Ca^{2+}]$ from the head and proximal flagellum of several sperm. Sodium photometry results are presented as the ratio of the background-corrected emission from excitation at 340 and 380 nm. Further analyses were performed in Igor (Wavemetrics, Lake Oswego, OR). Statistical analyses were performed in Excel (Microsoft, Redmond, WA). All results are presented as mean ± SEM, except where noted.

Intracellular pH photometry – Intracellular pH photometry was similar to Ca^{2+}/Na^{+} photometry with the following exceptions. BCECF-AM was dispensed from a 50 µM stock in DMSO, similarly dispersed in 10-15% Pluronic F127, and diluted to 0.5 µM in

0.5 ml of sperm suspended in medium Na 7.4. After 20-25 min, medium Na7.4 (0.5 ml) was added and the cells were sedimented. After resuspension in 0.3 ml fresh medium, incubation continued for 45 min before use. The T.I.L.L. monochromator provided excitation light of 460 and 490 nm, and >500 nm emitted light was collected from rectangular region that contained one single, loosely-tethered sperm. Neutral-density filters were used to reduce illumination intensity and thus decrease bleaching and photo-toxicity.

The ratio of the background-corrected signals was calibrated (Thomas et al. 1979) with the constants R_{min} (0.996), R_{max} (2.433), and K_{eff} (1.26×10^{-7} M) obtained from cells equilibrated in solutions fortified with 10 µM nigericin and containing (in mM): 10 MES (2[N-morpholino]ethanesulfonic Acid) and pH 5, 10 HEPES and pH 7, or 10 CHES (2-[N-cyclohexylamino] ethane-sulfonic acid) and pH 9, and 120 KCl, 10 NaCl, and 1 $MgSO_4$.

2.3.5 Ester loading of cyclic nucleotides.

The cAMP-AM or cGMP-AM was dispensed from a 20 mM stock in DMSO, dispersed in 10-15% Pluronic F127, diluted in 0.35 ml medium Na7.4, then immediately mixed with 0.15 ml of sperm suspension for a final concentration of 60 µM. After 30 min, an aliquot was added to the imaging chamber containing medium Na7.4. Data were collected within 5 min to preclude loss of signal due to declining cyclic nucleotide content that follows dilution of external cAMP-AM or cGMP-AM.

2.3.6 Local perfusion and bath exposure.

All waveform analyses and photometric experiments were performed in incubation chambers constructed from 35 mm tissue culture dishes whose bottoms were replaced with #0 glass coverslips. An uncoated, ~5 mm square #00 coverslip was placed in the dish and the chamber was gently flooded with medium Na7.4 for local perfusion experiments, or with treatment solution for bath-exposure experiments. A 5-10 µl aliquot of cell suspension was then placed directly onto the smaller coverslip and

allowed to settle. Many of the sperm spontaneously adhered to the glass coverslip at their head only. For local perfusion experiments, test solutions were applied by a solenoid-controlled, gravity-fed, multi-barreled local perfusion devise with an estimated exchange time of <0.5 s.

2.3.7 Cyclic-AMP measurements.

For each experimental trial, sperm from 3-6 animals were prepared as described above, and pooled in Na7.4. Samples destined for 0 mM Ca^{2+} treatment were washed in Na7.4 with no added $CaCl_2$. A 100 µl aliquot of sperm suspension was added to an equal volume of medium with 0 or 30 mM $NaHCO_3$. Treatments were continued for 30 s at room temperature. Reactions were stopped by lysis with 1 ml of ice-cold acidified ethanol (ethanol: 1M HCl; 100:1, v/v%). I had more predictable and repeatable results when the reaction was stopped with this method rather than stopping by placing the tube in boiling water. All samples were run in triplicate on samples of 1.5 – 4.3 x 10^6 sperm, and experiments were repeated on three separate occasions. The lysed cell suspensions were cooled on ice for at least 30 min. I found that the assay was sensitive to the ionic content of the samples, so $NaHCO_3$ and $CaCl_2$ were added as needed to ensure identical ionic composistions of the samples for assay. Samples were then lyophilized for 2-3 hrs to dryness, reconstituted in 250 µl of sodium acetate buffer, and transferred to microtiter plates. The cAMP content was measured using an enzyme immunoassay kit (Assay Designs, Ann Arbor MI). Acetylation was used according to the kit's instruction, to increase cAMP-antibody binding. The Assay Designs instructions were followed with the following changes. All samples and cAMP standards contained medium of the same ionic composition to control for ionic-induced alterations in cAMP-antibody binding and sensitivity. I also unsuccesfully tried using the cAMP elisa kit from Biomedical Technologies Inc. (Stoughton, MA).

CHAPTER 3

HCO_3^- SIGNALING PATHWAYS OF MOUSE SPERM CAPACITATION

3.1 Introduction

The available evidence strongly indicated that the HCO_3^- present in male and female reproductive fluids is required to signal capacitation. The goals of the experiments described here are to elucidate the HCO_3^- mediated signaling pathways that regulate swimming patterns of mammalian sperm as they prepare to fertilize. First, I present data on the signaling events that underlie mouse sperm activation and that occur within the first seconds after ejaculation when relatively immotile sperm initiate rapid swimming. I also present data on HCO_3^- induced hyperactivation, another change in motility that occurs late in capacitation.

3.2 Activation

3.2.1 HCO_3^- rapidly increases flagellar beat frequency.

Previous research using subjective estimates of sperm motility implicated HCO_3^- as the physiological signal that awakens sperm from quiescence at mating, a step called activation (Hamner and Williams 1965; Okamura et al. 1985). I now have a quantitative method that documents and quantifies this action of HCO_3^-, and allows study of its kinetics (Fig. 2-1). In the absence of HCO_3^-, basal flagellar beat frequency of sperm obtained from mouse epididymides and vasa deferentia in medium Na7.4 is approximately 3 Hz. Acutely treating single sperm with medium Na7.4 supplemented with 15 mM $NaHCO_3$ activates sperm and results in large elevations of beat frequency (Fig. 3-1*A*). Fig. 3-1*A* shows that HCO_3^- action is rapid (a change in beat is observed 7 s after HCO_3^- exposure) and robust (the beat frequency is increased by nearly 3-fold).

Additional experiments used bath exposure of populations of sperm to Na7.4 with 0 or 15 mM $NaHCO_3$ for 5-20 min. Without HCO_3^- the beat frequencies ranged from 1.9 to 3.9 Hz with a mean of 2.7 Hz. With HCO_3^- the range was 3.5 to 10.4 Hz

with a mean of 7.6 Hz (Fig. 3-1*B*). HCO_3^- activated nearly all examined sperm. I also found that the activating action of HCO_3^- is sustained for at least 30 min.

3.2.2 HCO_3^- action is independent of osmolarity change.

There is a ~10% difference in osmolarity between medium Na7.4 alone or supplemented with 15 mM $NaHCO_3$. Therefore, I explored the possibility that HCO_3^- accelerates beat simply by increasing external osmolarity. Specifically, I monitored sperm beat frequencies in medium Na7.4 alone, or supplemented with an additional 15 mM NaCl or 15 mM $NaHCO_3$. On average, sperm bathed in Na7.4 supplemented with 0 or 15 mM additional NaCl had indistinguishable beats (3.1 ± 0.2 vs 3.0 ± 0.3 Hz) (Fig. 3-2), despite ~10% difference in osmolarity. By contrast, sperm treated with Na7.4 and 15 mM $NaHCO_3$ had a beat of 9.0 ± 0.3 Hz. Hence, ~10% increases in extracellular osmolarity did not increase beat frequency.

3.2.3 HCO_3^- treatment does not alter intracellular pH.

In somatic cells, HCO_3^- often decreases intracellular pH. I considered the possibility that HCO_3^- evokes activation by changing intracellular pH, and asked whether HCO_3^- acidifies sperm cytoplasm by monitoring intracellular pH with the ratiometric fluorescent indicator BCECF. Sperm were loaded with BCECF-AM and the intracellular pH of single sperm cells was determined before and during exposure to 15 mM $NaHCO_3$. Sperm in Na7.4 had an average intracellular pH of 6.80 ± 0.03, and 30 s after exposure to 15 mM $NaHCO_3$, sperm had an average pH of 6.82 ± 0.04 (Fig. 3-3*B*). I did not find a HCO_3^- evoked pH change (Fig. 3-3*A&B*). To verify that this method can report alterations of sperm intracellular pH, I monitored the pH of sperm before and during local exposure to Na7.4 with 15 mM sodium propionate, which on average decreased pH by 0.1 pH units, or to 15 mM ammonium chloride, which increased pH by 0.1 pH units (Figs. 3-3*C&D*). These results support the interpretation that HCO_3^- actions on sperm activation are pH-independent and the HCO_3^- can cross the membrane without acidifying the cytoplasm.

3.2.4 PKA is required for HCO_3^- action.

Discovery of the HCO_3^- -sensitive cyclase sAC and demonstration that sAC is prominent in sperm suggested that HCO_3^- may employ a cAMP-signaling path to speed flagellar beat and activate motility (Chen et al. 2000). The cAMP produced by HCO_3^- could accelerate flagellar beat by several possible mechanisms. For many years after the discovery of cAMP, it was thought that PKA was its only target. Later, evidence of ion channels gated directly by cyclic nucleotides (CNG channels) was found, and most recently, cAMP was found to control a new family of guanine nucleotide exchange factors (EPACs) (Branham et al. 2006; Kinukawa et al. 2006). To dissect the HCO_3^- signaling path of sperm activation, I took a bottom-up approach. I began by asking whether PKA is required for HCO_3^- action on sperm. Specifically, I treated sperm with 30 μM of the PKA inhibitor H89 alone or with 15 mM $NaHCO_3$ (Fig. 3-4*A*). When H89 was present, HCO_3^- did not increase the beat frequency (7.6 ± 0.4 Hz without H89 vs 2.0 ± 0.1 Hz with H89). These data provide pharmacological evidence that HCO_3^- acts through a PKA pathway. Further I found that H89 treatment alone did not affect the beat frequency (2.6 ± 0.1 vs 2.4 ± 0.1 Hz in 0 or 30 μM H89 respectively), indicating that PKA is not required for the 'resting' beat present in the absence of HCO_3^-.

3.2.5 HCO_3^- acceleration of beat frequency is cAMP specific.

Although I now had evidence that PKA was *required* for the HCO_3^- activation of sperm, it remained possible that HCO_3^- had some additional action on sperm that also is required for the elevation of beat. To verify that an increased cAMP content is *sufficient* to activate mouse sperm, I asked whether cell-permeant cAMP analogs could mimic HCO_3^- actions on beat. I monitored the beat frequency of sperm with the following treatments: Na7.4 alone, or with 60 μM cAMP-AM, 50 μM cBiMPS, or 15 mM $NaHCO_3$. The charged phosphate of cAMP is masked by the AM-ester, which makes it cell permeant (Schultz et al. 1994; Schultz et al. 1993). Once inside sperm, esterases

cleave the AM group to free the charged and impermeant parent molecule. I chose to use cBiMPS in addition to cAMP-AM because it is relatively cell-permeant, highly resistant to hydrolysis by phosphodiesterases, and has a much higher affinity for PKA than for protein kinase G (Holt and Harrison 2002; Sandberg et al. 1991).

Sperm in medium Na7.4 had a beat frequency of 3.1 ± 0.2 Hz that increased to 9.0 ± 0.3 Hz when in Na7.4 with 15 mM $NaHCO_3$ (Fig. 3-4*B*). Similar to HCO_3^- treatment, loading sperm with cAMP-AM or cBiMPS dramatically increased the flagellar beat frequency (6.4 ± 0.4 and 7.7 ± 0.7 Hz respectively) (Fig. 3-4). I also included the control treatment of cGMP-AM. The cyclic nucleotide cGMP does not activate PKA, however, it should bind to and open the CNG channels reported in mammalian sperm (Weyand et al. 1994). The flagellar beat frequency of sperm loaded with cGMP-AM, was not significantly different from untreated controls (3.1 ± 0.2 Hz). This indicates that cGMP does not signal activation. These results indicate that cAMP specifically increases the beat of sperm, and provide additional support for the sole involvement of cAMP in the HCO_3^- signaled activation pathway.

3.2.6 HCO_3^- action requires sAC.

I now had pharmacological evidence that HCO_3^- induced activation requires PKA and that increased cAMP replicates the accelerating action of HCO_3^- on beat frequency. The HCO_3^- -responsive adenylyl cyclase of sperm, sAC, seemed a good candidate for linking the HCO_3^- to cAMP/PKA pathway. To test whether the HCO_3^- evoked beat acceleration requires sAC, I used both genetic and pharmacologic approaches. For a genetic approach, I asked whether HCO_3^- accelerates the beat frequency of sAC-null sperm (Esposito et al. 2004). With the help of Sonya M. Schuh, I compared sperm from sAC-null and wild-type mice. In medium Na7.4, the beat frequencies were similar (2.6 ± 0.1 for null vs 3.1 ± 0.2 for wild-type) (Fig. 3-5*A*), but HCO_3^- failed to elevate the beat frequency of sAC null sperm as it did for wild-type sperm (2.8 ± 0.2 vs 9.0 ± 0.3 Hz). As a control, I asked whether cAMP could increase beat rate by loading wild-type and sAC null sperm with cAMP-AM. The ester-generated cAMP indeed increased the beat of both wild-type (8.5 ± 0.3 Hz) and sAC-

null (6.3 ± 0.4 Hz) sperm, indicating that the pathway downstream of cAMP is intact in sAC-null sperm.

As an additional test of the involvement of sAC in the HCO_3^- signaling pathway, I used 2' hydroxyestradiol (2'OH-E), an inhibitor of sAC. In the early 1990's, Theodor Braun documented that 100 µM 2'OH-E almost completely inhibits the Mn^{2+}-sensitive cyclase of sperm (Braun 1990). We now know that the Mn^{2+}-sensitive adenylyl cyclase of sperm is sAC. I asked whether 2'OH-E blocked the HCO_3^- -evoked elevation of flagellar beat. Treatment with 2'OH-E attenuated HCO_3^- actions on sperm in a concentration-dependent manner (Fig. 3-5*B*). Sperm treated with 3 mM $NaHCO_3$ alone had a beat of 8.2 ± 0.5 Hz, whereas after a 30 min pre-incubation with 100 µM 2'OH-E, sperm treated with 3 mM $NaHCO_3$ together with 100 µM 2'OH-E had a beat of only 3.7 ± 0.3 Hz. In conclusion, pharmacologic and genetic experiments indicate that HCO_3^- -evoked acceleration of beat frequency requires sAC.

3.2.7 HCO_3^- enters sperm by CO_2 diffusion.

If HCO_3^- activates sAC, then it must enter the sperm. There are two possible mechanisms for HCO_3^- entry: the anion can be transported across the cell membrane via a carrier or it can diffuse through the membrane as CO_2. The CO_2 dissolved in a solution is in equilibrium with HCO_3^-. Whereas gases freely permeate cellular membranes and CO_2 and H_2O in solution are in slow equilibrium, CO_2 that is created by the dehydration of HCO_3^- outside the cell enters sperm freely. The CO_2 that enters the cell will rehydrate to increase HCO_3^- content.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \qquad \text{[Equation 1]}$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \qquad \text{[Equation 2]}$$

The first reaction (equation 1) is facilitated by the enzyme carbonic anhydrase, and the second reaction occurs spontaneously and instantaneously. The enzyme carbonic anhydrase has an exceptionally high turnover rate, and is primarily limited by the diffusion rate of its substrates. I used a pharmacologic approach to discriminate between HCO_3^- entry by diffusion of CO_2 or by transport of the HCO_3^- anion.

Specifically, I asked whether I could slow the rate of HCO_3^- action by inhibiting anion exchangers in sperm. To do so, I treated sperm with the broad-spectrum inhibitor of anion exchangers, the stilbene derivative 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) (Fig. 3-6). A 60 s pretreatment with 1 mM SITS did not alter the rate of 3 mM $NaHCO_3$ action and sperm under both conditions reached near maximal beat frequency by 35 s (Fig. 3-6).

I also tested the alternative hypothesis that CO_2 enters by diffusion and is re-hydrated there to give HCO_3^- responsible for sperm activation. Specifically, I asked whether inhibiting carbonic anhydrase altered the rate of HCO_3^- actions on beat frequency. Similar to the experiments with the anion exchanger inhibitors, I monitored the time course of the HCO_3^- elevation of the beat frequency for sperm treated with the carbonic anhydrase inhibitor acetazolamide (ACZ). The rate of HCO_3^- action on beat was dramatically slower in sperm treated with 1 mM ACZ compared to untreated cells (Fig. 3-7). Whereas untreated sperm reach maximal beat frequency by ~35 s, sperm treated with ACZ required ~60 s to reach maximal beat rate (Fig. 3-7). At pH 7, acetazolamide has a pKi for carbonic anhydrase of 6.8 (Lindskog and Thorslund 1968), meaning that 158 nM ($10^{-6.8}$ M) should inhibit half of the enzyme. Therefore, most carbonic anhydrase enzymes should be inhibited when sperm were treated with 1 mM, ACZ even if it is not fully membrane-permeant. The ability of HCO_3^- to elevate the beat frequency in the absence of carbonic anhydrase activity is facilitated by the spontaneous hydration of CO_2. At 25°C, this reaction has a rate constant of 0.037 s^{-1} (Roughton 1941), meaning that it should take 27 s to achieve 1/e of the maximal response. Indeed I find that 27 s after the maximal response seen when treating with HCO_3^- alone, the beat frequency of sperm in 1 mM ACZ and HCO_3^- is approximately 6 Hz.

3.2.8 HCO_3^- action requires extracellular Ca^{2+}.

Early work found that HCO_3^- and extracellular Ca^{2+} are required together for large elevations in the cAMP content of guinea pig sperm (Garbers et al. 1982). My own data indicated that cAMP mediates activation of sperm motility by HCO_3^-.

Therefore I hypothesized that this action of HCO_3^- on mouse sperm also requires extracellular Ca^{2+}. To test this hypothesis, I treated sperm with media differing only in their HCO_3^- and Ca^{2+} content and monitored the flagellar beat rate (Fig. 3-8*A*). I found that sperm treated with HCO_3^- and Ca^{2+} together beat approximately three-times faster than sperm treated with Ca^{2+} alone (7.6 ± 0.3 vs 2.7 ± 0.1 Hz). Treatment with medium lacking both HCO_3^- and Ca^{2+} produced a basal beat frequency of 2.7 ± 0.1 Hz. However, the beat frequency of sperm treated with HCO_3^- alone (3.4 ± 0.3 Hz) (Fig. 3-8*A*) was not dramatically different than the resting rate of sperm in medium Na7.4. This indicates that Ca^{2+} is required to signal in the HCO_3^- path, and that the inability of HCO_3^- to elevate beat frequency is not due to a general suppressive effect of Ca^{2+} deprivation.

The next series of experiments determined the concentration-dependence for the extracellular Ca^{2+} requirement in HCO_3^- action on sperm. Sperm were bathed in media with 15 mM $NaHCO_3$ and various extracellular Ca^{2+} concentrations. Fitting the data with the Hill equation, I found that HCO_3^- required ~0.5 mM Ca^{2+} to produce a half-maximal increase in beat frequency (Fig. 3-8*B*).

Additional experiments used local perfusion to change the bathing media composition abruptly. Fig. 3-9 shows that perfused HCO_3^- could increase beat frequency only when supplied together with Ca^{2+}. When sperm were exposed to a 0 mM Ca^{2+} solution with 15 mM HCO_3^-, the beat frequency remained around 2.5 Hz (Fig. 3-9*A*). When I immediately changed to a solution containing 2 mM Ca^{2+} but no HCO_3^-, no elevation of beat occurred. Together these results indicate that HCO_3^- does not initiate a pathway that is temporarily arrested in the absence of external Ca^{2+}. Rather the Ca^{2+} and HCO_3^- are required at the same time to elevate beat frequency. I also found that extracellular Ca^{2+} was required to maintain an elevated beat evoked by simultaneous application of both Ca^{2+} and HCO_3^-. When extracellular Ca^{2+} was removed, the beat elevated by Ca^{2+} and HCO_3^- together slowly returns to that of sperm bathed in Na7.4 (Fig. 3-9*B*). These perfusion experiments reinforce the requirement of extracellular Ca^{2+} in HCO_3^- sperm activation. These results indicate that external Ca^{2+} is a cofactor in the HCO_3^- signaling path.

3.2.9 Ca^{2+} is required for cAMP production, not for cAMP action.

I hypothesized that the Ca^{2+} requirement for HCO_3^- to activate motility of mouse sperm results from a requirement of Ca^{2+} for HCO_3^- to elevate cAMP content. Therefore I monitored the cAMP content of sperm extracts prepared 30 s after treatment with media differing only in Ca^{2+} and HCO_3^- content. In Na7.4 with 2 mM Ca^{2+}, the cAMP content of sperm was 1.6 ± 0.2 pmol/10^7 cells. If 15 mM $NaHCO_3$ was present, the content increased > 2-fold to 3.4 ± 0.7 pmol/10^7 cells. In the absence of external Ca^{2+}, HCO_3^- did not elevate cAMP, 0.9 ± 0.1 and 0.9 ± 0.1 pmol/10^7 cells with 0 and 15 mM $NaHCO_3$ respectively (Fig. 3-10). These results indicate that indeed activation of sAC requires both external HCO_3^- and Ca^{2+}.

To exclude the possibility that extracellular Ca^{2+} is required for action downstream of cAMP production, I examined the Ca^{2+} requirements for some additional activation of sperm by cAMP-AM. Sperm with cAMP-AM in medium lacking Ca^{2+} were compared to sperm treated with cAMP-AM in 2 mM Ca^{2+}. The cAMP-AM elevated the flagellar beat both in the presence (6.8 ± 1.3 Hz) and absence (6.6 ± 1.3 Hz) of extracellular Ca^{2+} (Fig. 3-11). Lack of a difference between these two treatments indicates that extracellular Ca^{2+} is not needed downstream of cAMP production. Together these results indicate that Ca^{2+} is required for cAMP production but not for its subsequent action, and suggest that Ca^{2+} is required for stimulation of sAC by HCO_3^- or for some hypothetical required upstream event.

3.2.10 HCO_3^- does not evoke Ca^{2+} entry.

I reasoned that measuring the Ca^{2+} concentration of sperm during treatments with media of varying Ca^{2+} and HCO_3^- content should allow me to sort through these possibilities. For instance, if external Ca^{2+} is required for HCO_3^- to activate sAC and this occurs without a detectable increase in intracellular Ca^{2+}, then extracellular Ca^{2+} likely acts from outside of the cell. Conversely, if HCO_3^- actions on sAC are accompanied by an increase in intracellular Ca^{2+}, then I would need to document that intracellular Ca^{2+} increases and establish its linkage to cAMP. Alternatively, HCO_3^-

treatment may evoke no intracellular Ca^{2+} increase, but removal of extracellular Ca^{2+} might decrease intracellular Ca^{2+}. This finding would indicate that extracellular Ca^{2+} is required to maintain intracellular Ca^{2+} stores. This seems unlikely because HCO_3^- acts in seconds while depletion of internal Ca^{2+} stores in somatic cells occurs over minutes. A final possibility is that a similar increase in intracellular Ca^{2+} might occur when HCO_3^- is applied with 0 or 2 mM Ca^{2+} media. This would suggest that HCO_3^- signaling involves the release of Ca^{2+} from intracellular stores.

To explore these possibilities, I used photometry with ratiometric fluorescent indicators to monitor the intracellular Ca^{2+} concentration of small groups of sperm, before and during local exposure to Na7.4 with 0 or 15 mM $NaHCO_3$. The fura-2 indicator did not report a detectable HCO_3^- -evoked elevation of Ca^{2+} (Fig. 3-12*A*). However, sperm have little cytoplasm and it is possible that sequestration of fura-2 into intracellular organelles might interfere with my ability to detect a small change in cytoplasmic Ca^{2+} concentrations. Thus in a follow-up experiment, I looked for changes in Ca^{2+} concentration near the plasma membrane using the near-membrane Ca^{2+} indicator FFP18 (Fig. 3-12*B*). This Ca^{2+} indicator is derived from fura-2 and has a large hydrophobic group composed of an eighteen-carbon chain, which acts to anchor the molecule near the plasma membrane (Etter et al. 1996; Vorndran et al. 1995). As for the fura-2 experiments, the FFP18 reports no HCO_3^- -evoked elevation of intracellular Ca^{2+} near the membrane (Fig. 3-12*B*).

As an alternative test of the possibility that an elevation of intracellular Ca^{2+} is required for HCO_3^- to accelerate beat frequency, I asked whether HCO_3^- in 0 mM Ca^{2+} could increase the beat when I used UV photolysis to increase Ca^{2+}. Sperm were loaded with the AM ester of o-nitrophenyl EGTA (NP-EGTA-AM). Once inside sperm, NP-EGTA binds cytosolic Ca^{2+} and external Ca^{2+} will leak into sperm to replenish intracellular free Ca^{2+}. Upon photolysis with UV light, NP-EGTA's affinity for Ca^{2+} decreases greatly, resulting in the rapid release of bound Ca^{2+} (Ellis-Davies and Kaplan 1994). The amount of intracellular Ca^{2+} released by UV photolysis depends on the amount of Ca^{2+} bound to NP-EGTA prior to photolysis and on the rate of clearance of cytoplasmic Ca^{2+} by the pumps and exchangers. Therefore I monitored the Ca^{2+} release after uncaging using fura-2 photometry in sperm loaded with fura-2 and NP-EGTA. I

find that sperm loaded with NP-EGTA and fura-2, increase their intracellular Ca^{2+}, but not their beat frequency, in response to UV photolysis (Fig. 3-13).

3.2.11 Intracellular Ca^{2+} slowly declines with removal of external Ca^{2+}.

To explore the possibility that extracellular Ca^{2+} is required simply to maintain intracellular Ca^{2+} content, I monitored internal calcium concentrations during exposure to a 0 mM Ca^{2+} version of medium Na7.4. Upon removal of external Ca^{2+} the intracellular Ca^{2+} content slowly declined (Fig. 3-14). This decline occurs over tens of seconds, whereas HCO_3^- actions on beat frequency are evident within 7 s of exposure (Fig. 3-1). My findings indicate that although Ca^{2+} concentration does decline with removal of external Ca^{2+}, the rate is too slow to account for the near instantaneous blockage of response to activation by HCO_3^- (Figs. 3-9*A*&*B*).

3.3 Hyperactivation

3.3.1 Hyperactivation requires PKA.

It is well established that mouse sperm display the hyperactivated waveform following 90-min incubation in a capacitation medium containing HCO_3^-, Ca^{2+}, and BSA at 37°C. My recent work shows that HCO_3^- -evoked activation, an early event in capacitation, requires PKA. Therefore I asked whether PKA is also required for hyperactivation, a late event of capacitation. I used a pharmacological approach; I treated mouse sperm with capacitating incubations known to produce the hyperactivated waveform alone or with 30 μM of the PKA inhibitor H89. I used flagellar beat symmetry to quantify hyperactivation (Fig. 3-15). Before capacitating incubations, sperm have a relatively symmetric beat (absolute asymmetry <0.5 radians at 40 μm along the flagellum) and after capacitating incubations, asymmetry is much greater (absolute asymmetry >1.0 radians at 40 μm along the flagellum) (Fig. 3-16*A*). Sperm incubated with H89 retained an absolute asymmetry of <0.5 radians at 40 μm along the flagellum (Fig. 3-16*B*). These results provide pharmacologic evidence that

PKA activity is required to develop or sustain the large asymmetry associated with hyperactivation.

3.3.2 The sAC null sperm do not hyperactivate.

Establishing the PKA requirement for the asymmetry associated with the hyperactivated waveform implicates a cAMP signaling pathway. I tested the hypothesis that the cAMP is the product of sAC activity. Thus I treated sperm from sAC null males with capacitating incubations known to produce hyperactivation, and compared their beat symmetry to that of wild-type sperm treated identically. Before capacitating incubations, wild-type sperm display a relatively symmetric beat and have an absolute asymmetry of <0.3 radians at 40 μm along the flagellum. Absolute asymmetry increased to >1.2 radians following capacitating incubations (Fig. 3-17*A*). In contrast, sAC null sperm display a relatively symmetric beat both before and after capacitating incubations (absolute asymmetry <0.25 at 40 μm along the flagellum for both) (Fig. 3-17*B*). These results indicate that sAC is required for the asymmetry associated with hyperactivation, presumably because it is the source of the cAMP-messenger that activates PKA.

3.3.3 The HCO_3^- requirement for hyperactivation is partially fulfilled by cBiMPS.

I now had evidence that both sAC and PKA are required for production of the asymmetric beat characteristic of hyperactivation, presumably via the HCO_3^- signaling pathway. It remained possible that HCO_3^- had some additional action on sperm that was required for the display of hyperactivation. To investigate whether an increased cAMP content is sufficient to replace HCO_3^-, I asked whether the cell-permeant cAMP analog cBiMPS could mimic HCO_3^- actions on beat asymmetry. Wild-type mouse sperm were treated with either capacitating incubations (consisting of Na7.4 with 15 mM $NaHCO_3$ and BSA) or with Na7.4 with 50 μM cBiMPS and BSA (Fig. 3-18). I find that cBiMPS only partially replaces HCO_3^- in the production of the asymmetry. This finding leaves open the possibility that HCO_3^- may have additional action in signaling

beat asymmetry. However, it is also possible that a particular extent or duration of elevated cAMP is required to produce the hyperactivated waveform.

3.4 Discussion

I have found that HCO_3^- signals two events of capacitation, the early activation and the later hyperactivation of motility. The HCO_3^- signaling pathway for both flagellar waveforms employs sAC, cAMP, and PKA.

3.4.1 HCO_3^- activates sperm.

I have found that HCO_3^- provided by local perfusion or by bath exposure activates mouse sperm by accelerating their flagellar beat frequency. This HCO_3^- activation of sperm is rapid, near-maximal beat frequencies are produced within 20 s with exposure to 15 mM $NaHCO_3$. Importantly, HCO_3^- is a physiologically-relevant stimulus for activation. The luminal fluid of the epididymis and vas deferens have a low HCO_3^- concentration, which is thought to help maintain sperm in a quiescent state during their maturation and storage (Jones and Murdoch 1996). Consistent with this proposed role, active HCO_3^- reabsorption occurs in the caput epididymis (Breton 2001). At mating, sperm move from a low HCO_3^- environment and encounter high HCO_3^- in the male and female reproductive fluids (Boatman and Robbins 1991; David et al. 1969; David et al. 1973; Okamura et al. 1985). The importance of HCO_3^- was recently reinforced by the demonstration that endometrial epithelial cells express the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel (Wang et al. 2003b). It was further demonstrated that co-culture of sperm with endometrial cells expressing this CFTR increased their capacitation status and their ability to fertilize (Wang et al. 2003b). These experiments were interpreted to indicate HCO_3^- transported via CFTR plays an important role in the *in vivo* capacitation of mammalian sperm, and that low uterine HCO_3^- content may explain the female infertility in cystic fibrosis patients (Sutton et al. 2003; Wang et al. 2003b). Thus, several

lines of evidence indicate that HCO_3^- may be the principle physiologic signal that activates sperm at mating.

3.4.2 HCO_3^--evoked activation is independent of pH and osmolarity.

I explored alternative explanations of HCO_3^- action. Specifically, I asked if HCO_3^- elicits mouse sperm activation by changing extracellular osmolarity or intracellular pH. I found that that increasing the osmolarity of the extracellular medium by supplementing with 15 mM NaCl does not increase the flagellar beat frequency whereas medium supplemented with 15 mM $NaHCO_3$ does. I also found that a 30-s treatment with HCO_3^- does not alter intracellular pH, indicating that HCO_3^- action on sperm activation is pH independent. However, increased intracellular pH has been associated with mammalian sperm capacitation (Cross and Razy-Faulkner 1997; Zeng et al. 1996). In fact, sperm fail to capacitate when internal alkalinization is prevented (Parrish et al. 1989). These reported pH changes are associated with long term (~90 min) capacitating incubations at 37°C, whereas I was looking at HCO_3^- actions only within the first 30 s of exposure at room temperature. It is probable that I would have observed increased intracellular pH following a longer capacitating incubation with HCO_3^--containing medium. Whereas an increased intracellular pH may not signal activation, it might be required for signaling the hyperactivated waveform.

3.4.3 HCO_3^--evoked activation employs sAC/cAMP/PKA pathway.

Numerous past reports proposed roles for cAMP as a regulatory messenger in sperm (Garbers et al. 1983). The recent identification of the sperm adenylyl cyclase sAC and demonstration of its stimulation by HCO_3^- provided an explanation for how HCO_3^- treatment could increase internal cAMP content. However, the details of the HCO_3^- signaling path were not completely understood.

Here I have used pharmacological evidence to show a requirement of PKA activity for the HCO_3^- signaled activation. This interpretation includes the observation

that cAMP still stimulates the motility of demembranated sperm after their reactivation with Mg^{2+}-ATP (Lindemann et al. 1987). This observation would not be possible if CNG channels were required for HCO_3^- signaling. Additional direct evidence of the importance of PKA comes from a knockout mouse produced in Stan McKnight's lab, here at the University of Washington. Targeted mutagenesis was used to specifically disrupt the gene for the sperm-specific catalytic subunit of PKA, Cα2. $PKA_{C\alpha 2}$ null males are infertile and their sperm do not respond to HCO_3^- (Nolan et al. 2004).

PKA presumably phosphorylates motility-related proteins to increase flagellar beat frequency. One such PKA-phosphorylated protein has been identified as the 36 kDa, pyruvate dehydrogenase E1 component β subunit of the mitochondrial sheath of the middle piece (Fujinoki et al. 2003). It is also known that PKA phosphorylates itself, specifically the regulatory RII subunit (Paupard et al. 1988), originally described as axokinin (Tash et al. 1984). However, it is likely that PKA phosphorylates axonemal motor proteins that more directly regulate flagellar motility.

I found that incubation with the cell permeant cAMP-AM or cBiMPS strongly increases flagellar beat frequency of wild-type sperm, presumably by effectively elevating cAMP without involvement of adenylyl cyclase. The ability of cAMP to speed the beat in the absence of HCO_3^- suggests that HCO_3^- actions in sperm activation are strictly the result of elevated cAMP. My results indicate that cAMP likely signals sperm activation via PKA activation. The CNGA3 cyclic nucleotide-gated channel reported as a component of bovine sperm (Weyand et al. 1994) hass not yet been documented in mouse sperm. For these channels, cGMP is the preferred agonist, they bind cGMP with higher affinity than they do cAMP. The presence of the CNG3 channel in sperm suggests it may have a functional role and therefore that it may be involved in the cyclic nucleotide-signaled sperm activation. A few groups have reported a cGMP-evoked Ca^{2+} entry in mammalian sperm, and that cGMP analogs evoke entry more effectively than cAMP analogs, e.g. (Kaupp et al. 1998; Kobori et al. 2000). However, I found that cGMP-AM does not increase the beat frequency of mouse sperm. Further, reduced fertility has not been reported for male CNG3 null mice (Biel et al. 1999), indicating that this channel has no required role in sperm.

Here I report two pieces of evidence that the atypical adenylyl cyclase of sperm is required for HCO_3^- activation. Most directly, I find that HCO_3^- does not increase the beat of sAC null sperm. Importantly, the sAC null sperm is accelerated by cAMP-AM. This rescue of rapid beat shows that the inability of HCO_3^- to act is not due to a defect in the flagellar motor, rather it is in the mechanisms that control it. Rescue of sAC null sperm motility by cAMP analogs has also been observed but not quantified by other groups (Esposito et al. 2004; Hess et al. 2005). Although sAC null sperm increase their beat rate in response to cAMP-AM, they do not respond as well as sperm from wild-type mice. It is possible that sAC is required during spermatogenesis and that the sAC-null sperm have a development defect. Therefore I followed up these experiments using the sAC inhibitor 2'OH-E.

In vitro experiments using recombinant rat sAC documented that 2'OH-E effectively inhibits sAC with an EC_{50} of 10 μM (Pastor-Soler et al. 2003). Braun similarly reported that the soluble cyclase from rat germ cells was inhibited by several catechol estrogens. The EC_{50} for 2'OH-E was 2-6 μM, with almost complete inhibition at 30-100 μM (Braun 1990). Consistent with the hypothesis that HCO_3^- requires sAC to increase beat frequency, I found that sAC inhibitor 2'OH-E attenuates HCO_3^- action on sperm. A slightly more potent sAC inhibitor, KH7, has been recently been reported (Hess et al. 2005). Similar to 2'OH-E, KH7 inhibits sAC purified from mouse testis with an EC_{50} of 3-10 μM, and near complete inhibition at 50 μM (Hess et al. 2005). My own experiments should be followed up with experiments examining the inhibition of HCO_3^- sperm activation by KH7 and of other more potent inhibitors.

It has been hypothesized that some ACIII variants may be activated by HCO_3^- (Cann et al. 2003) suggesting that any ACIII present in mature sperm might mediate the HCO_3^- -evoked elevation of cAMP. The mRNA and protein of the type III adenylyl cyclase (ACIII) are found in rat round spermatids (Gautier-Courteille et al. 1998). Western blot analysis and immunocytochemistry suggested that epididymal sperm may retain ACIII (Baxendale and Fraser 2003; Wade et al. 2003). The possible role of ACIII in sperm motility, however, is not clear. Sperm from the ACIII null males have reduced sperm motility and fertility (Livera et al. 2005), but these mutant mice also have poor general health and reduced survival (Wong et al. 2000), which may itself

affect spermiogenesis. Therefore, poor sperm motility may not be the result of a defect with the signaling pathway that is associated with capacitation. My own findings that sAC null sperm do not increase their beat frequency in response to HCO_3^-, and that sAC inhibitor 2'OH-E attenuates the HCO_3^- response indicate that even if ACIII is present in mature sperm, it is not the source of cAMP elevated by HCO_3^- in activation.

Together these data indicate HCO_3^- produces sperm activation by a signaling pathway that uses sAC, cAMP, and PKA (Fig. 3-19).

3.4.4 HCO_3^- enters sperm via diffusion of CO_2.

My data suggest that the predominant route of HCO_3^- entry into sperm is via diffusion of CO_2 followed by its intracellular re-hydration facilitated by carbonic anhydrase. High concentrations of the stilbene inhibitors of anion exchangers do not alter the onset rate of HCO_3^- action, suggesting that anion exchangers are not involved in HCO_3^- entry for sperm activation.

The role of anion exchangers in sperm capacitation is not clear. Several groups have reported that SITS treatment can block the actions of HCO_3^- on sperm capacitation (Visconti and Kopf 1998), including inhibition of the enhanced cAMP accumulation promoted by phorbol esters (Visconti et al. 1990), the acrosome reaction, and even HCO_3^- transport (Okamura et al. 1988). In contrast, others report that treatment of porcine sperm with DIDS *enhanced* porcine sperm motility and respiration (Tajima and Okamura 1990). This enhancement of motility by DIDS was interpreted as inhibition of anion exchanges that allow efflux of endogenous HCO_3^- derived from metabolic CO_2. The same group further showed an enhanced elevation of cAMP in sperm treated with another stilbene derivative 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 10 mM $NaHCO_3$ together compared to $NaHCO_3$ alone (Tajima and Okamura 1990). In this context, it is not surprising that I did not see a slowing of HCO_3^- action by SITS; rather I would expect to see this drug speed HCO_3^- action on beat frequency. I considered the possibility that these anion exchangers which allow for the enhancement of HCO_3^- action on cAMP production are localized at the midpiece of mature sperm near the mitochondria rather than along the flagellum.

Other groups have also linked carbonic anhydrase activity to the HCO_3^- regulation of sperm motility (Brook et al. 1996; Tajima et al. 1987), and carbonic anhydrase type II is abundant in mammalian sperm (Mezquita et al. 1999; Parkkila et al. 1991). In spite of carbonic anhydrase inhibition with ACZ, HCO_3^- was still able to increase beat frequency presumably because the CO_2 that had entered the sperm by diffusion can spontaneously re-hydrate to increase HCO_3^- content albeit at a slower rate than if carbonic anhydrase had been present.

My work indicates that the HCO_3^- which activates motility enters sperm via diffusion of CO_2. In theory, this route of entry should be accompanied by a decrease of intracellular pH as the result of the increase of protons released from the net reaction:

$$CO_2 + H_2O \rightarrow HCO_3^- + H^+$$

The lack of an observable pH change with HCO_3^- treatment suggests that internal pH of sperm may be buffered strongly enough to preclude such a HCO_3^- associated decrease in intracellular pH. Indeed sperm are thought to have a strong pH buffering capacity (Babcock 1983). One sperm-specific protein that may play a role in the maintaining a stable internal pH is the putative Na^+/H^+ exchanger (sNHE) (Wang et al. 2003a). Sperm lacking sNHE have reduced motility and are not responsive to HCO_3^-, yet they increase their beat upon loading with cAMP-AM (Wang et al. 2003a). Perhaps mis-regulation of pH in the sNHE null sperm precludes HCO_3^- from activating the sAC, yet the signaling pathway downstream of sAC remains intact. Typically NHE's are inactive at resting and alkaline pH's, and they activate at acidic pHs and extrude protons to restore resting pH. The hypothesized involvement of NHE in HCO_3^- activation is additionally attractive because the NHE sequence includes a cyclic-nucleotide-binding consensus sequence at the carboxy terminus (Wang et al. 2003a). Perhaps the NHE activity is enhanced following the HCO_3^- evoked elevation of cAMP, increasing its rate of proton extrusion.

3.4.5 Ca^{2+} is a cofactor in the HCO_3^- signaling pathway.

Early documentation that HCO_3^- and Ca^{2+} are required together for large elevations of cAMP in guinea pig sperm (Garbers et al. 1982; Hyne and Garbers 1979)

suggested that Ca^{2+} may be a cofactor in the HCO_3^- signaling path. I indeed found that HCO_3^- requires Ca^{2+} to elevate the beat frequency of mouse sperm. Where is Ca^{2+} acting in the HCO_3^- signaling path?

Results from other groups suggested to me that Ca^{2+} is required for enter sperm along with HCO_3^- to stimulate the sAC. In particular it was found that recombinant human and rat sAC is Ca^{2+} -sensitive, and further that the stimulatory actions of HCO_3^- and Ca^{2+} on sAC are synergistic (Jaiswal and Conti 2003). Other groups have also reported a HCO_3^- evoked Ca^{2+} entry, for instance, in mouse sperm using confocal imaging with the single wavelength indicator fluo-4 (Ren et al. 2001). However, in our laboratory, Ca^{2+} photometry with the ratiometric indicators fura-2, FFP18, and indo-1 and also with flou-4 (data not shown) in more than one hundred total trials, we have not detected a HCO_3^- -evoked Ca^{2+} rise (Wennemuth et al. 2003b; Wennemuth et al. 2003d). It is possible that an important intracellular Ca^{2+} event is specifically localized within the sperm in an area unavailable to our indicators. Alternatively, it is possible that we are missing some Ca^{2+} event with photometry that might be documented with imaging. The main advantage of imaging over photometry is increased spatial resolution, the result of simultaneous monitoring of many regions. The main disadvantage of imaging over photometry is reduced sensitivity. We used photometry because of its high sensitivity allows increased temporal resolution. As we do not affix our sperm to the coverslip for examination, parts of the flagellum are constantly moving in and out of the view finder. The increased sensitivity with photometry results in efficient detection of photons even in this challenging experimental design.

My own data suggest that Ca^{2+} may be acting on the extracellular surface of the sperm. It is possible that Ca^{2+} is required to bind to and activate an extracellular Ca^{2+} -sensing receptor. Such Ca^{2+} -sensing receptors are expressed in many tissues (Chattopadhyay and Brown 2000), and regulate diverse functions such as parathyroid hormone secretion by the parathyroid gland, calcitonin secretion by C-cells in the thyroid, calcium and H_2O transport in the mammary gland, and fluid fluxes in the gastrointestinal tract ((Pi et al. 2005) and refs. within). Although the Ca^{2+} sensing receptor of the parathyroid was first cloned and characterized more than ten years ago (Brown et al. 1993), we still have much to learn about their diversity, distribution, and

roles. When a transgenic mouse was engineered such that the Ca^{2+} sensing receptor of the parathyroid was knocked out, no male fertility deficits were reported (Kos et al. 2003). However, the Ca^{2+}-sensing receptor of the parathyroid is not the only Ca^{2+} sensing receptor. A novel Ca^{2+}-sensing receptor was recently reported, and its expression was documented in mouse epididymis (Pi et al. 2005). It is possible that this or another unidentified Ca^{2+} receptor is expressed in sperm and is required for the HCO_3^- signaled activation.

3.4.6 Hyperactivation requires PKA/cAMP/sAC.

Here I summarize the data indicating that several components of the signaling pathway that signals activation are also required for the hyperactivated waveform, as assessed by flagellar beat asymmetry. My results are in agreement with other recent reports that H89 prevents the hyperactivated motilty. Computer assisted semen analysis (CASA) showed that H89 treatment prevented hyperactivation of hamster sperm, assessed with straight-line and curvilinear velocity, parameters (Si and Okuno 1999) or helical and circular motility patterns (Jha and Shivaji 2002). Activation is an early event of capacitation whereas hyperactivation is a late event. Activation might need to precede hyperactivation. Thus, preventing activation by inhibiting PKA with H89, or using sperm from the sAC null males would preclude hyperactivation.

I have also found that cBiMPS is not sufficient to replace HCO_3^- in the capacitating incubations. This finding is in agreement with Cooper (1984) who documented, using a stroboscopic technique to follow changes in mouse sperm movements, that permeant cAMP analogs and PDE inhibitors alone were not sufficient to promote the hyperactivated waveform (Cooper 1984). We know now that the cAMP content of sperm cycles through at least two transient elevations during capacitating incubations (Carlson et al. 2005). Perhaps this oscillatory signaling pattern is important for generation of the hyperactivated waveform.

3.4.7 Conclusions.

In conclusion, I have found that HCO_3^- evokes sperm activation via a pathway that involves CO_2 diffusion through the sperm membrane, carbonic anhydrase stimulated rehydration of CO_2 into HCO_3^-, activation of sAC and subsequent elevation of cAMP and activation of PKA. This signaling pathway requires extracellular Ca^{2+}, but this Ca^{2+} is not required downstream of the elevated cAMP. I have also found that this HCO_3^- pathway is required for the flagellar beat asymmetry associated with hyperactivation.

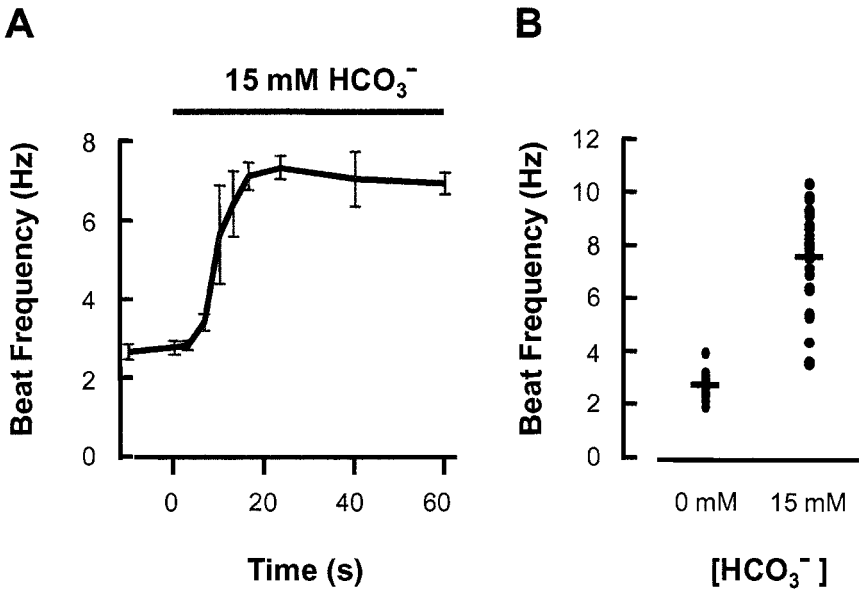

Figure 3-1: **HCO_3^- increases flagellar beat frequency**. **A**: Single sperm (mean ± sem, N=4) locally perfused with Na7.4 supplemented with 0 or 15 mM $NaHCO_3$. Action of HCO_3^- is detectable within 7 s of application. **B**: Data from cells randomly selected while bathed in media with 0 or 15 mM $NaHCO_3$ (N=33-37). Bars mark the mean. Cells are exposed to HCO_3^- 5-20 min before and during examination, indicating that the stimulatory action of HCO_3^- is sustainable.

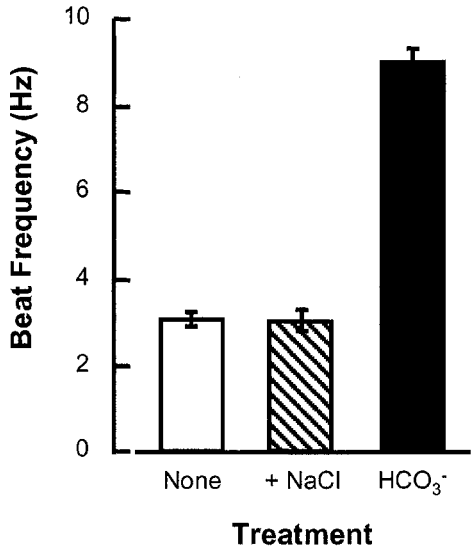

Figure 3-2: **Increasing extracellular osmolarity does not increase beat frequency.** Beat frequency of sperm bathed in Na7.4 with the following modifications: none, supplemented with an additional 15 mM NaCl, or with 15 mM $NaHCO_3$ (mean ± sem, N=8-13, 3 independent experiments).

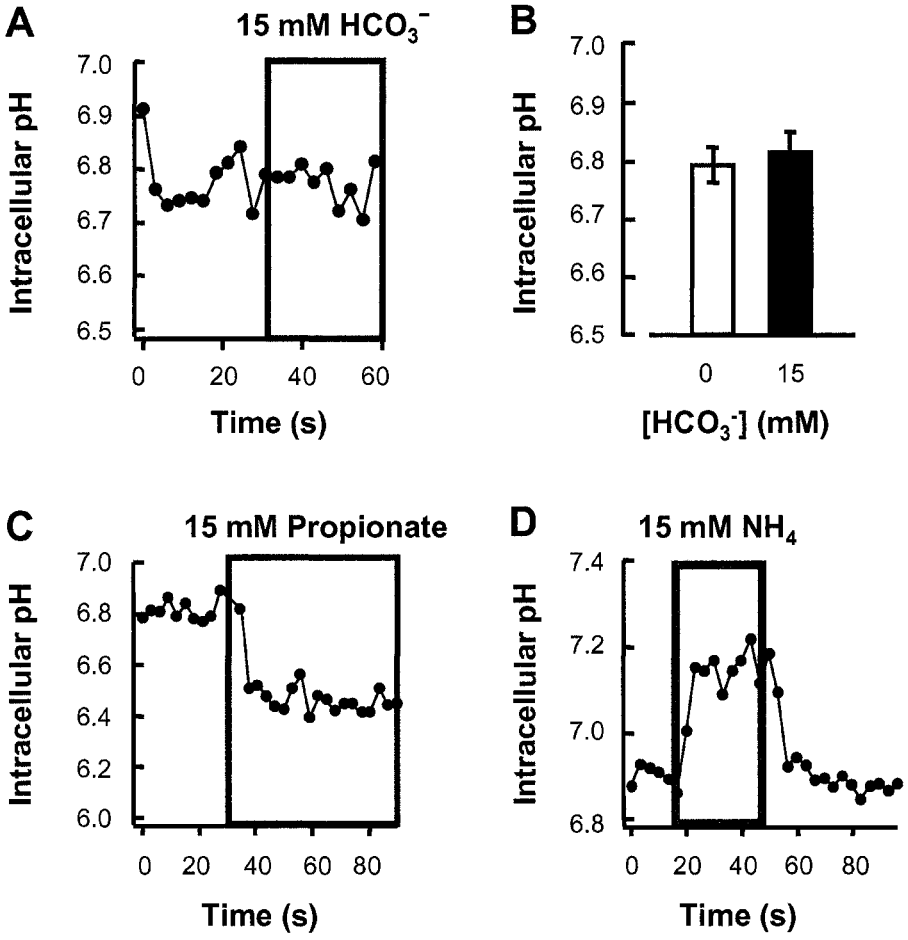

Figure 3-3: **HCO_3^- does not alter intracellular pH**. Intracellular pH of single sperm indicated by BCECF. **A**: Intracellular pH of an example sperm before and during local perfusion with 15 mM $NaHCO_3$. **B**: Average intracellular pH of single sperm before and during exposure to 15 mM $NaHCO_3$, as in **A** (mean ± sem, N=24, 3 independent experiments). Example traces from control experiments validating probe responses were validated by local treatment with either 15 mM sodium propionate (**C**) or 15 mM ammonium chloride (**D**).

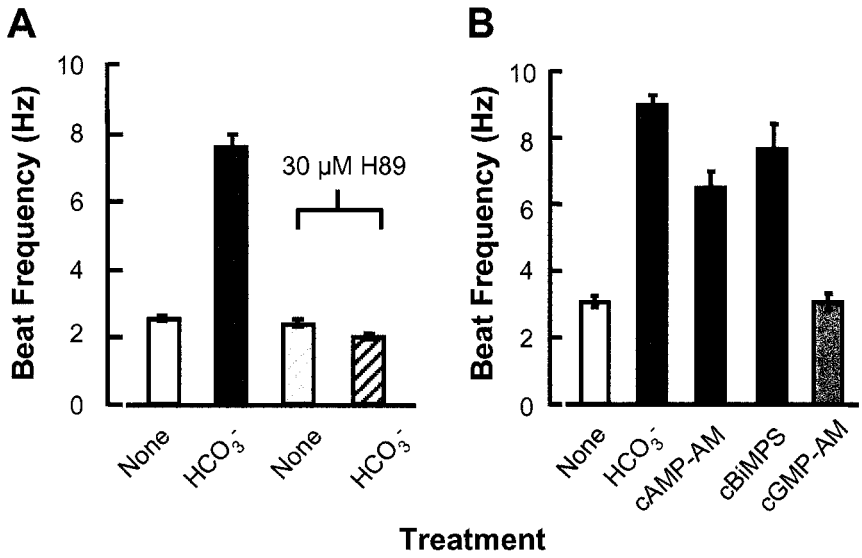

Figure 3-4: **HCO_3^- evoked beat acceleration is mediated by PKA and cAMP**. Flagellar beat frequency (mean ± sem, N=6-19) of sperm bathed in Na7.4 with the following modifications: **A:** 0 or 15 mM $NaHCO_3$ applied after 5-10 min incubation with 0 or 30 µM H89. **B**: none, 15 mM $NaHCO_3$, or pre-loaded (30 min) with 60 µM cAMP-AM or cGMP-AM, or (5 min) 50 µM cBiMPS.

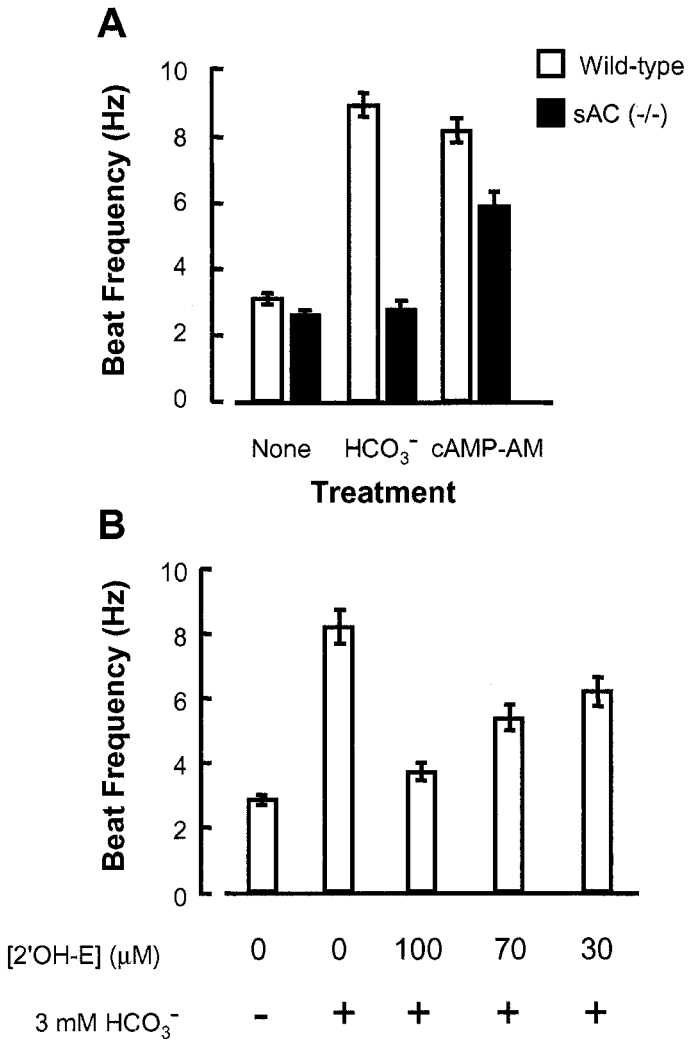

Figure 3-5. **HCO_3^- requires sAC for acceleration of beat. A:** Flagellar beat frequencies of wild-type and sAC null mouse sperm bathed in Na7.4 alone or 15 mM HCO_3^-, or pre-loaded (30 min) with 60 μM cAMP-AM (mean ± sem, n=20-45 cells, 2 independent experiments). **B:** Putative sAC-inhibitor 2'hydroxyestradiol (2'OH-E) attenuates the HCO_3^- elevation of flagellar beat. Sperm are incubated with indicated concentrations of inhibitor for 20 min before exposure to 3 mM HCO_3^- (mean ± sem, N=5-16 cells, 2-3 independent experiments).

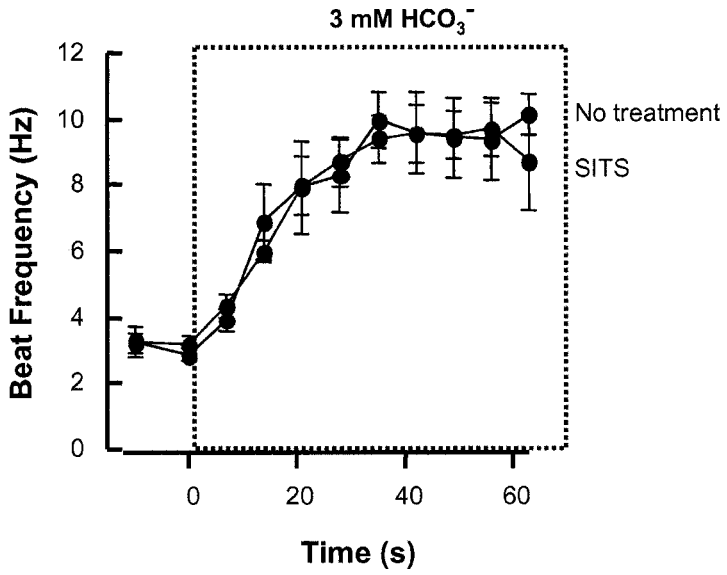

Figure 3-6. **Anion exchange inhibitor does not alter rate of HCO_3^- action.** Sperm incubated with 0 (black trace) or 1 mM SITS (red trace) 60 s before and during application of 3 mM $NaHCO_3$ provided by local perfusion (mean ± sem, N=6-7, 3 independent experiments).

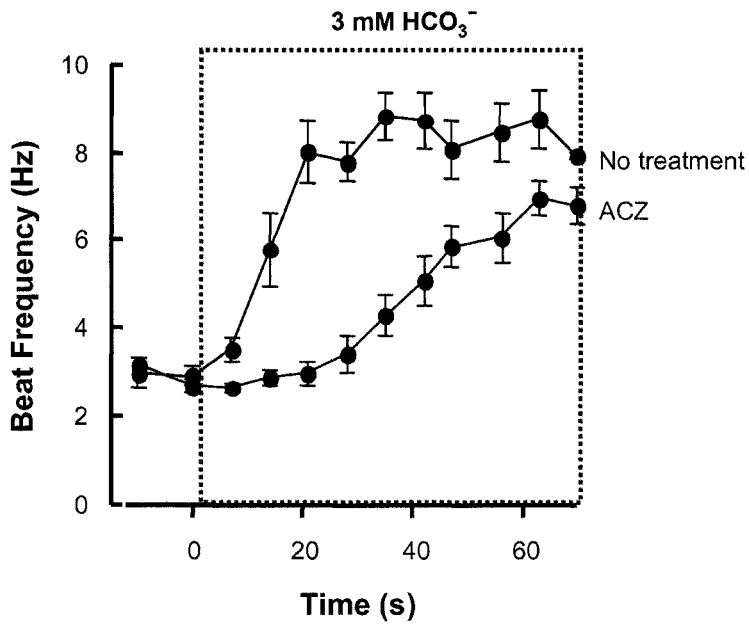

Figure 3-7. **Carbonic anhydrase inhibitor slows HCO_3^- action.** Sperm incubated with 0 (black trace) or 1 mM of the carbonic anhydrase inhibitor acetazolamide (ACZ) (red trace) applied for 30 s before and during exposure to 3 mM $NaHCO_3$ slows provided by local perfusion. The HCO_3^- -action on flagellar beat frequency is compared to no treatment (black trace) (mean ± sem, N=6-11, 3 independent experiments).

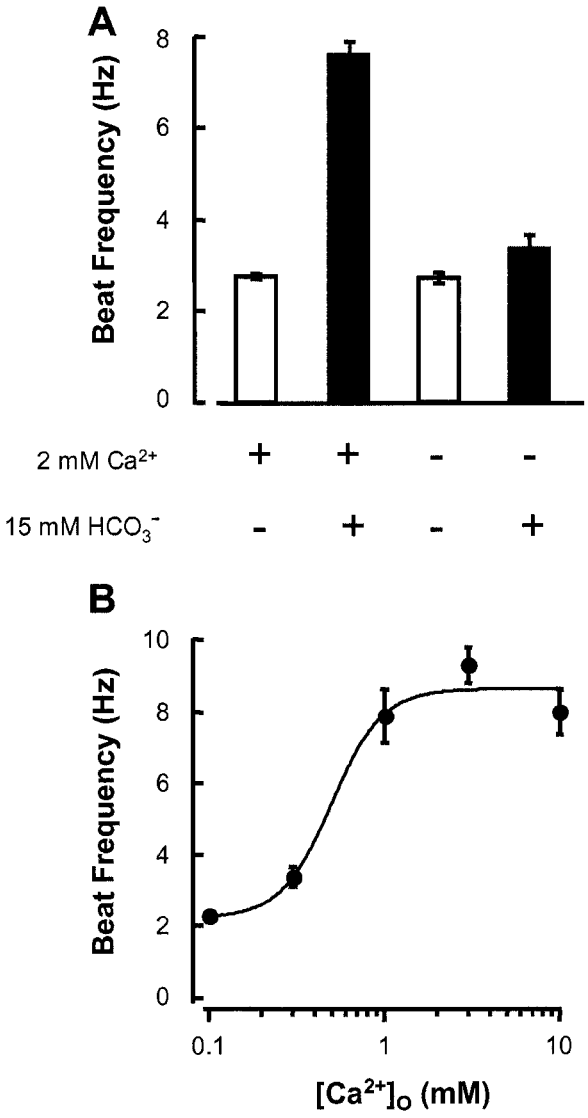

Figure 3-8: **HCO_3^- action requires external Ca^{2+}.** **A**: Flagellar beat frequency of sperm bathed in medium Na7.4 with 0 or 2 mM Ca^{2+} and 0 or 15 mM $NaHCO_3$ as indicated (mean ± sem, N=26-37, 3 independent experiments). **B**: The concentration-response relation for the extracellular Ca^{2+} requirement in HCO_3^- action on sperm. Sperm bathed in media with 15 mM $NaHCO_3$ and various Ca^{2+} concentrations as indicated, and motility assessed at 1-10 min (mean ± sem, N=12-14, 3 independent experiments). Data fitted with a Hill curve with the following parameters: base = 2.2, maximum = 8.6, n= 3.03, and EC_{50} = 499 μM.

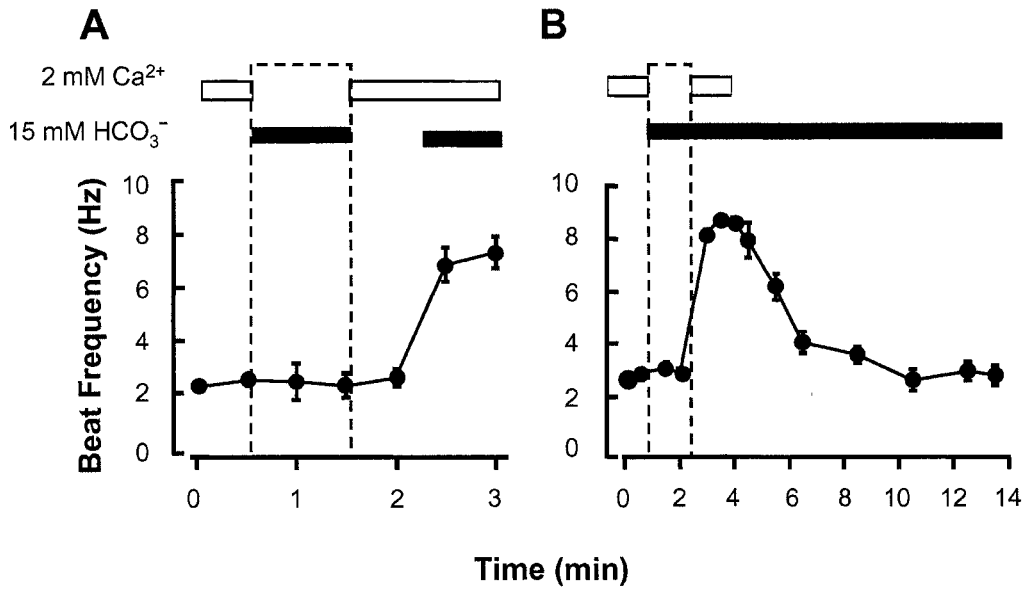

Figure 3-9: **HCO_3^- and Ca^{2+} are required concurrently to increase and maintain increased beat. A&B**: Beat frequencies (mean ± sem, N=3-4) of sperm locally perfused with Na7.4 containing 0 or 2 mM Ca^{2+} and 0 or 15 mM $NaHCO_3$ as indicated.

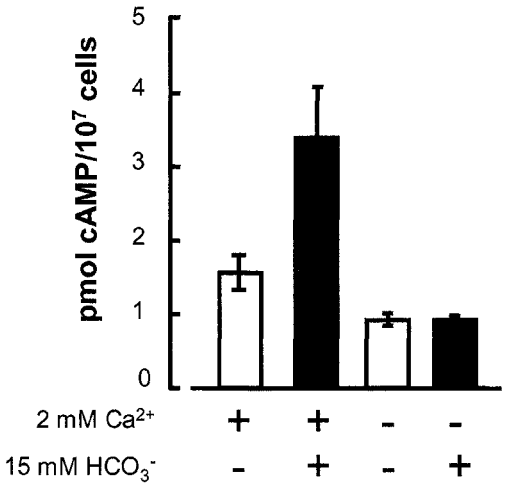

Figure 3-10: **Ca^{2+} is required for cAMP production**. The cAMP content of sperm (mean ± sem, N=11-15 samples, 3 repetitions) extracted after a 30 s treatment with 0 or 2 mM Ca^{2+} and 0 or 15 mM $NaHCO_3$ as indicated. The cAMP concentrations were determined by an enzyme immunoassay kit (Assay Designs Inc.).

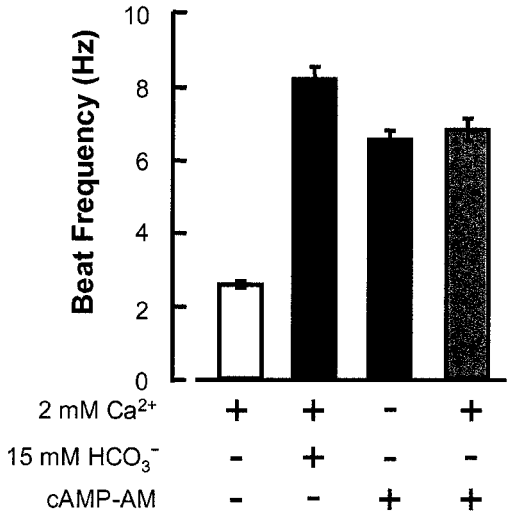

Figure 3-11: **Ca^{2+} is not required for cAMP action on beat frequency**. Beat frequency of sperm (mean ± sem, N=17-28 cells, 3 animals) after 30 min pre-incubation with 60 µM cAMP-AM in medium with 0 or 2 mM Ca^{2+}. Cell exposed to medium Na7.4 containing either 0 or 15 mM $NaHCO_3$ were used for comparisons. Data from cAMP-AM loaded cells were collected within 3 min after transfer to the experimental chamber to preclude loss of response due to dilution external cAMP-AM and metabolism of intracellular cAMP.

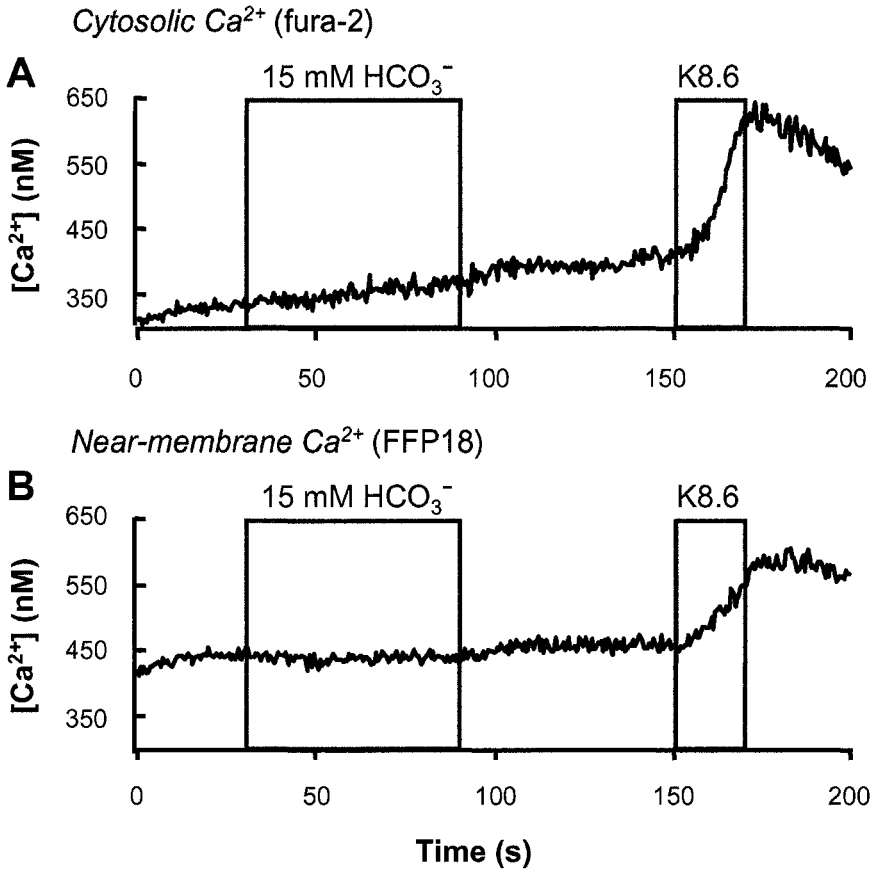

Figure 3-12: **HCO_3^- does not evoke Ca^{2+} entry**. Mean intracellular Ca^{2+} concentration of small groups of 3-5 sperm loaded with the AM ester form of ratiometric indicators **A**: fura-2 (N=24, 2 independent experiments) and **B**: near-membrane indicator FFP18 (N=44, 3 independent experiments). Sperm are locally perfused with a 2 mM Ca^{2+} Na7.4 containing 0 or 15 mM HCO_3^- as indicated. The high potassium, high pH solution K8.6 depolarizes sperm to open voltage-gated calcium channels, and was used as a positive control.

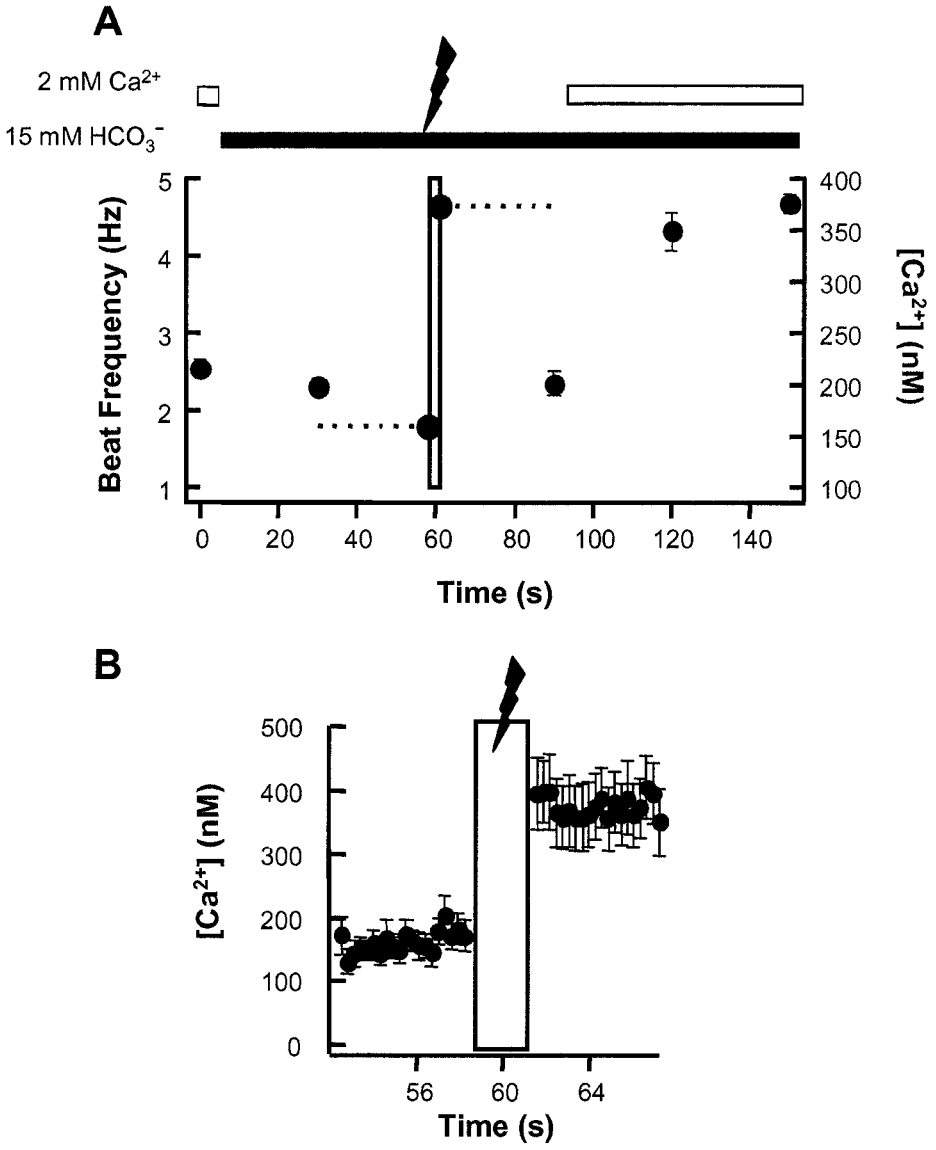

Figure 3-13: **Increased intracellular Ca^{2+} does not activate sperm.** **A:** Beat frequency (black markers and left axis) and intracellular Ca^{2+} concentration (red markers and right axis) of single sperm before and after uncaging Ca^{2+}. Red markers indicate averaged Ca^{2+} concentrations before and after uncaging, and dashed lines indicated predicted Ca^{2+} concentrations during beat frequency measurements. Sperm loaded with the AM ester forms of fura-2 and NP-EGTA in the presence of 2 mM Ca^{2+} (N=9, 2 independent experiments). Exposure to 3 s of 365 nm light increases free Ca^{2+}. **B:** Expanded view of Ca^{2+} concentrations before and after uncaging.

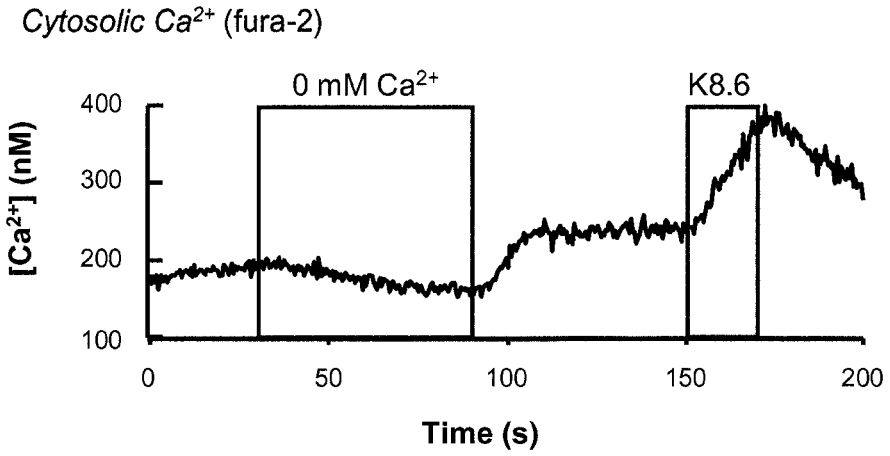

Figure 3-14: **Removal of external Ca^{2+} leads to a slow decrease of intracellular Ca^{2+}.** Sperm are locally perfused with medium Na7.4 with 0 or 2 mM $CaCl_2$ or with K8.6 and intracellular Ca^{2+} concentration assed with fura-2 (N=16, 3 independent experiments).

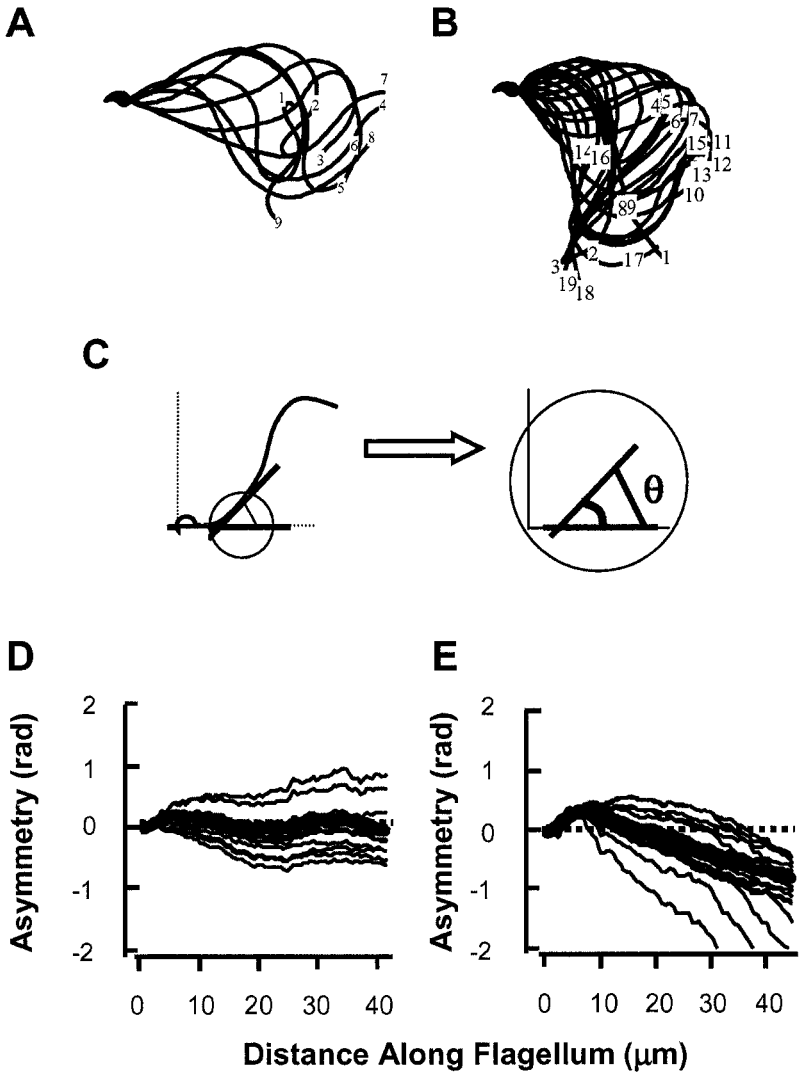

Figure 3-15: **Flagellar beat asymmetry reports hyperactivation.** **A&B**: Aligned flagellar waveform traces for sperm before (**A**) and after (**B**) capacitating incubations known to produce hyperactivation. **C**: For each trace, the tangent angle is determined at 0.5 μm intervals along the flagellum. **D & E**: Asymmetry is the time-averaged angular deciation of the flagellar beat. Its running average converges on a single value over several beat cycles (black). In medium 7.4, sperm have a symmetrical flagellar beat (**D**), and this beat become asymmetrical following capacitating incubations (**E**).

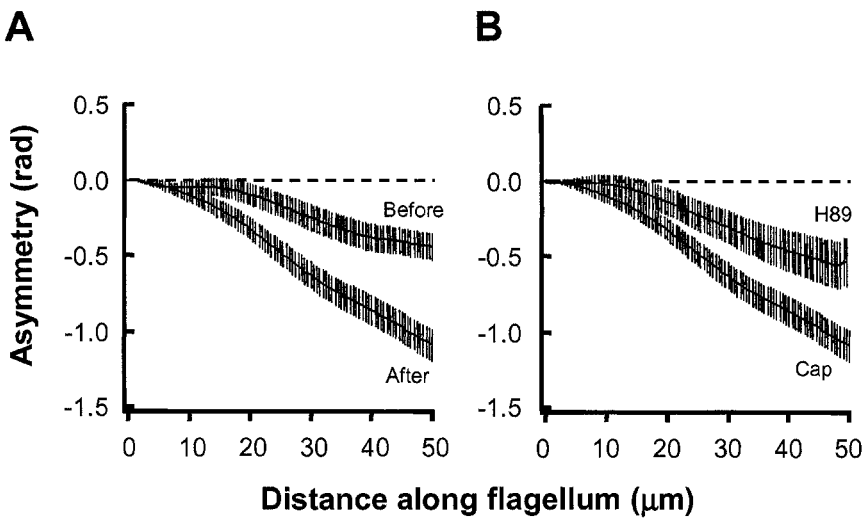

Figure 3-16: **PKA activity is required for hyperactivation-associated beat asymmetry.** **A**: Flagellar beat asymmetry before and after exposure to capacitating incubation. **B**: Beat asymmetry of sperm with and without exposure to 30 μM H89 during capacitating incubations (mean ± sem, N=11-12, 2-5 animals).

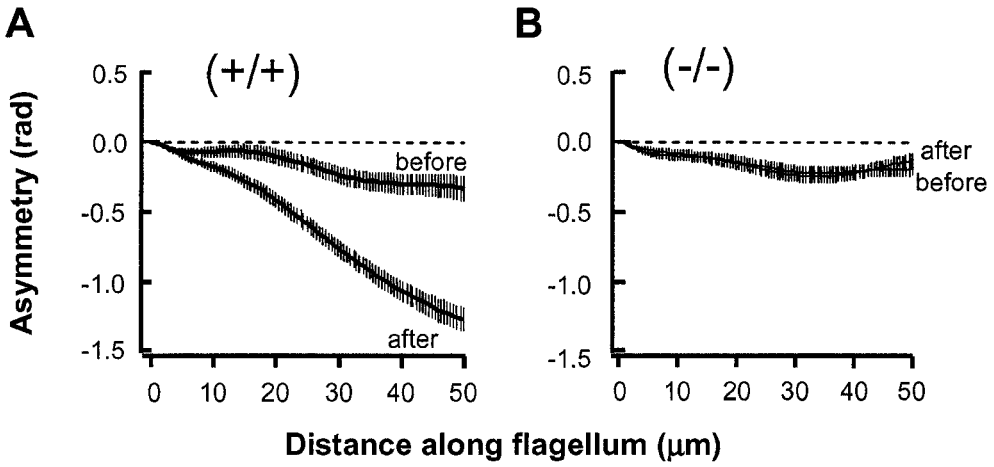

Figure 3-17. **sAC null sperm do not develop the beat asymmetry of hyperactivation.** Flagellar beat asymmetry before (blue) and after (black) exposure to capacitating incubations of wild-type (+/+) (**A**) and sAC null (-/-) (**B**) sperm.

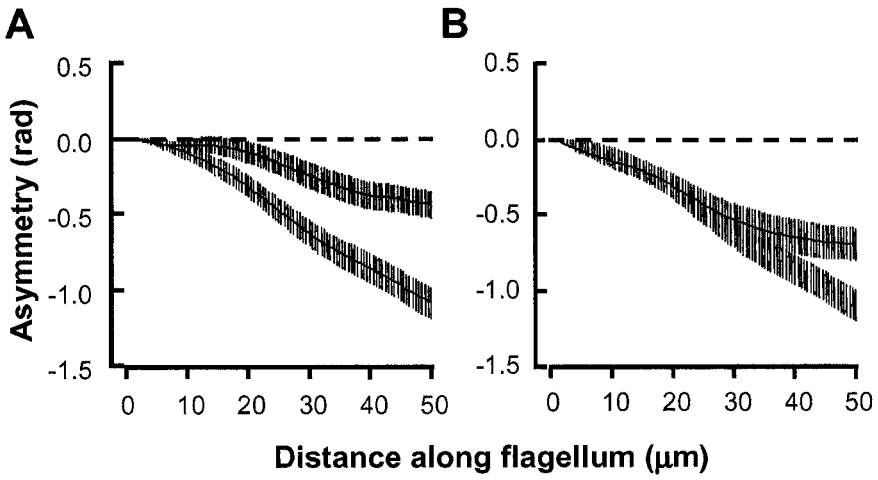

Figure 3-18: **The cAMP-analog cBiMPS partially replaces HCO_3^- in capacitating incubations**. **A**: Flagellar beat asymmetry before (blue) and after (black) exposure to capacitating incubation (from Fig. 3-16). **B**: Beat asymmetry of sperm exposure to capacitating incubations with 15 mM $NaHCO_3$ (black) or 50 μM cBiMPS (green) (mean ± sem, N=11-12, 2-5 animals).

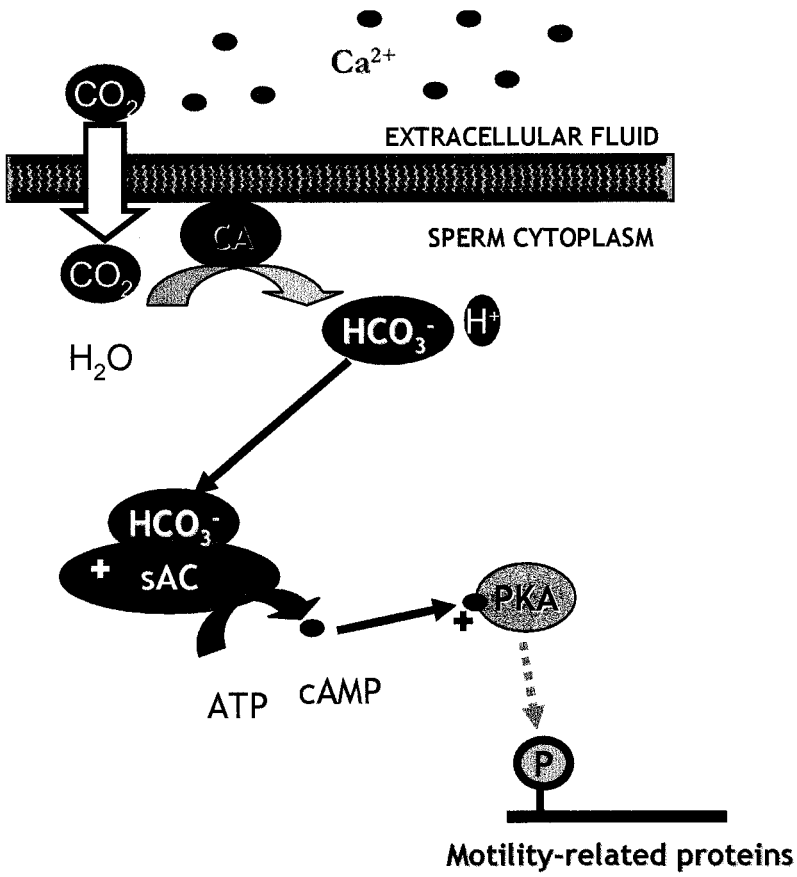

Figure 3-19: **Proposed signaling pathway of HCO_3^- actions on sperm.** Extracellular HCO_3^- and CO_2 should be equilibrium, and CO_2 should readily permeate the sperm membrane. Once inside sperm, carbonic anhydrase (CA) will combine CO_2 and H_2O to increase the intracellular HCO_3^- content. HCO_3^- will then bind to and activate the sperm adenylyl cyclase (sAC) to increase cAMP content, and activity of the cAMP-responsive protein kinase (PKA). PKA presumably then phosphorylates motility-related proteins.

Chapter 4

CATSPER1 AND CATSPER2 FUNCTION AS A CALCIUM CHANNEL TO REGULATE FLAGELLAR WAVEFORM

4.1 Introduction

In 2001, database mining identified two new Ca^{2+} channel-like proteins, CatSper1 and CatSper2 (for Cation channel of Sperm) (Quill et al. 2001; Ren et al. 2001). The CatSper genes are unique as they encode a single, six-transmembrane-spanning repeat (similar to that of voltage-gated K^+ channels), but with a pore region and overall homology closest to a single domain of the larger four-repeat voltage-gated Ca^{2+} channels. The CatSper1 and CatSper2 proteins are exclusively expressed in the testes and are localized on the principal piece of the sperm flagellum. The first report on CatSper1 documented that targeted disruption of CatSper1 results in male sterility in otherwise healthy mice (Ren et al. 2001). Sperm from CatSper1 nulls were initially described as having reduced motility. This motility deficit suggested to me that this putative Ca^{2+} channel may have a required role in the regulation of capacitation. In collaboration with the two laboratories of David Clapham (Children's Hospital, Harvard Medical School), and of David Garbers (University of Texas Southwestern Medical Center) who engineered these transgenic mice, I performed experiments focusing on characterizing the phenotypes of CatSper1- and CatSper2-null sperm. Some of the experiments with the CatSper2 null sperm were done with the help of Sonya M. Schuh. This chapter concerns the function of a complex of CatSper1 and CatSper2 as Ca^{2+} channels that have specific required role in mouse sperm capacitation.

4.2 CatSper1 and CatSper2 regulate flagellar waveform

4.2.1 HCO_3^- activates CatSper null sperm.

Activation of motility is one of the earliest events of capacitation, occuring shortly after ejaculation and signaled by the high HCO_3^- concentrations of male and

female reproductive fluids. *In vitro*, the flagellar beat frequency increases several-fold within seconds of exposing sperm to HCO_3^- (Fig. 2 and (Carlson et al. 2003; Wennemuth et al. 2003d)). I have found that this HCO_3^- mediated action is Ca^{2+}-dependent (Carlson et al. 2003) and occurs by a pathway employing sAC followed by cAMP-mediated activation of PKA (Figs. 3-4, 3-5, 3-10). I hypothesized that the Ca^{2+} dependence of HCO_3^- action results from a required entry of Ca^{2+} that occurs through CatSper channels. By comparison of sperm from CatSper1 or CatSper2 null males with sperm from their wild-type littermates, I asked if the HCO_3^- signaling pathway requires CatSper. I found that both CatSper1 and CatSper2 null sperm increased their beat frequency nearly 3-fold upon exposure to 15 mM $NaHCO_3$ (3.6 ± 0.2 to 9.5 ± 0.4 Hz for CatSper1 nulls and 3.8 ± 0.1 to 9.8 ± 0.4 Hz for CatSper2 nulls) or upon incubation with the membrane-permeant cAMP-AM ester (7.3 ± 0.3 Hz for CatSper1 nulls and 7.5 ± 0.3 Hz for CatSper2 nulls), as for sperm from their wild-type littermates (e.g. 2.9 ± 0.1 and 7.6 ± 0.4 Hz for 0 or 15 mM $NaHCO_3$, and 5.3 ± 0.2 Hz for cAMP-AM for wild-type littermate pairs from CatSper2 experiments) (Fig. 4-1). Thus, neither CatSper1 nor CatSper2 is required for signaling events downstream of cAMP in the bicarbonate-evoked acceleration of the flagellar beat frequency.

4.2.2 Increased basal beat frequency of CatSper2 null sperm is due to elevated cAMP.

Fig. 4-1 also shows that CatSper1- and CatSper2-null sperm have an elevated basal (before exposure to HCO_3^-) beat frequency (for instance, 3.8 ± 0.1 for CatSper2 nulls vs. 2.8 ± 0.2 Hz for wild-type sperm). This elevated beat frequency suggested that these CatSper null sperm have elevated cAMP and PKA activity. I used a pharmacological approach to test the hypothesis that increased PKA activity increases the beat frequency of CatSper null sperm. Specifically, I used the PKA inhibitor H89, which blocks the HCO_3^--evoked acceleration of the beat frequency of wild-type sperm (Fig. 3-4*A* and (Wennemuth et al. 2003d)). Fig 4-2*A* compares the beat frequency before and during exposure to 30 μM of the PKA inhibitor H89. The H89 had little or no effect on the 2.5 ± 0.2 Hz basal beat of wild-type sperm, but decreased the beat of

CatSper2 null sperm from 4.1 ± 0.2 to 2.5 ± 0.2 Hz, similar to the basal beat of wild-type sperm (Fig. 4-2*A*).

Our collaborator Timothy Quill (University of Texas Southwestern Medical Center) measured the cAMP content of CatSper2 null and wild-type to ask whether increased cAMP accompanied the accelerated beat. The CatSper2 null sperm indeed had an elevated basal cAMP content (Fig. 4-2*B*) (Carlson et al. 2005). Together these data indicate that as for wild-type sperm, the increased basal cAMP content of CatSper2 null sperm raises the resting beat frequency by PKA-mediated phosphorylation presumably of a flagellar motor protein. These results strongly suggest that CatSper have a previously unknown required role in setting the basal cAMP content.

4.2.3 No hyperactivated motility for CatSper null sperm.

I also asked whether CatSper2 is required for the highly asymmetrical flagellar waveform that is a hallmark of sperm hyperactivation. Specifically, I monitored the waveform asymmetry before and after capacitating incubations known to produce hyperactivation in wild-type sperm. For wild-type sperm bathed in medium Na7.4, asymmetry was low initially, and became greater after incubation under capacitating conditions (Fig. 4-3*A*). The absolute value for asymmetry at 40 μm along the flagellum increased from <0.3 to >1.0 radians. In contrast, the asymmetry of CatSper2-null sperm changed little after capacitating incubations (Fig. 4-3*B*). The mean value for asymmetry at 40 μm along the flagellum remained <0.2 radians. I have similarly documented the absence of hyperactivated motility in the CatSper1 null sperm (Carlson et al. 2003).

The hyperactivated waveform also is characterized by increased beat amplitude, here measured by the maximal excursion from the flagellar beat axis for each point along the flagellum (Figs. 4-3*C*&*D*). The amplitude at 30 μm for wild-type sperm in Na7.4 increased from 28 ± 2 to 35 ± 2 μm after capacitating incubations. The amplitude for CatSper2 null sperm decreased only slightly from 25 ± 1 to 23 ± 2 μm after capacitating incubations.

4.2.4 Procaine partially rescues flagellar beat asymmetry in CatSper2 null sperm.

I asked whether CatSper2 null sperm lack an asymmetrical waveform after capacitating incubations due to a defective flagellar motor or due to defects in the mechanisms that control it. As a test, I applied an alternative, pharmacological method to induce flagellar asymmetry. Past work reports that local anesthetics such as procaine induced hyperactivation in sperm (Singh et al. 1983; Vijayaraghavan et al. 1985; Lindemann et al. 1987). Procaine action requires external Ca^{2+}but reportedly is independent of cAMP and tyrosine phosphorylation (Marquez and Suarez 2004). We find that with 5 min exposure to 10 mM procaine, both wild-type and CatSper2 null sperm display a highly asymmetrical flagellar beat (Fig. 4-4*A*). Thus the inability of CatSper2 null sperm to display the beat asymmetry characteristic of hyperactivation is not due to a defect in the flagellar motor, rather it is in the signaling pathways that control the motor. The demonstration that procaine evokes beat asymmetry in CatSper2 null sperm means that I can document every flagellar waveform characteristic of wild-type sperm in CatSper null sperm. This indicates that the CatSper null sperm do not have developmental deficits that may preclude a flagellar waveform and strongly suggests that the CatSper complex does not have a required role in the normal development of mature sperm.

The waveform of procaine-treated sperm was similar but not identical to that of the hyperactivated waveform of wild-type sperm. Whereas capacitating incubations decreased the beat frequency of wild-type sperm (from 3.1 ± 0.1 before to 2.0 ± 0.1 Hz after capacitating incubations), the beat of procaine-treated sperm remained slightly elevated (4.0 ± 0.4 Hz) (Fig. 4-5*A*). For CatSper2 null sperm, capacitating incubations marginally increased beat frequency (4.1 ± 0.2 vs 4.9 ± 0.3 Hz before and after capacitating incubation) and procaine marginally decreased (3.4 ± 0.2 Hz) beat frequency. In addition, procaine treatment decreased beat amplitude at 30 μm along the flagellum for both wild-type (18 ± 1 μm) and CatSper2 null (19 ± 1 μm) sperm (Fig. 4-4*B*), whereas hyperactivating conditions increased only the beat amplitude of wild-type sperm (Fig. 4-4*B*). I further characterized the procaine-induced waveform by

measuring the maximal curvature of the flagellar midpiece, in both the pro-hook and anti-hook bend directions (i.e. in the same or opposite direction as the hook of the head (Ishijima et al. 2002)). After capacitating incubations, wild-type sperm displayed increased pro-hook curvature (arbitrarily assigned a negative value) of the midpiece (-1.1 ± 0.1 radians before and -1.6 ± 0.1 radians after capacitating incubations). Procaine treatment did not increase pro-hook curvature (-1.2 ± 0.1 radians), but decreased anti-hook curvature (indicated by a positive value) of both wild-type (1.1 ± 0.1 radians before and 0.5 ± 0.1 radians after procaine) and CatSper2 null (1.1 ± 0.1 before and 0.7 ± 0.1 radians after procaine) sperm (Fig. 4-5*B*). Therefore, the similar asymmetry produced by incubation under capacitating conditions or by procaine (cf. Figs. 4-3*A* and 4-4*A*) was produced by different distributions of bending in pro- and anti-hook directions.

4.3 CatSper1 and CatSper2 as a Ca^{2+} channel

4.3.1 CatSper null sperm lack evoked Ca^{2+} entry.

To explore the possible role of CatSper as a functional Ca^{2+} channel, Donner Babcock and I asked whether evoked Ca^{2+} entry is altered in CatSper1 and CatSper2 null sperm. Ca^{2+} entry in sperm was evoked by treatment with the high-potassium, high-pH medium K8.6 (Babcock and Pfeiffer 1987a), and the rate of depolarization-evoked Ca^{2+} entry, reported by Ca^{2+} photometry, indicated the relative number of open, voltage-gated Ca^{2+} channels (Wennemuth et al. 2003d). In Fig. 4-6, fura-2 was used to monitor spatially-averaged Ca^{2+} concentration in small clusters of 3-7 motile sperm loosely adhered to a coverslip. The cells were perfused with medium Na7.4 alone or with 15 mM $NaHCO_3$, except during 30-s depolarizing stimuli with medium K8.6. For wild-type sperm (Fig. 4-6*A*), intracellular Ca^{2+} rose abruptly during each stimulus, then returned slowly towards the initial resting level. As in past work (Carlson et al. 2003; Nolan et al. 2004; Wennemuth et al. 2003d; Wennemuth et al. 2000), channel opening was facilitated by incubation with the bicarbonate anion. The K8.6-evoked rate of rise was 21 ± 3 nM s^{-1} before and 33 ± 6 nM s^{-1} after conditioning with HCO_3^- (Fig 4-6*C*).

For CatSper2 null sperm, depolarization evoked little or no increase in Ca^{2+} before or after conditioning with HCO_3^- (Fig. 4-6*B*). Rates of Ca^{2+} rise were <1 nM s^{-1} under both conditions. We have similarly documented the absence of K8.6-evoked Ca^{2+} entry in CatSper1 null sperm (Carlson et al. 2003). Thus, both CatSper1 and CatSper2 are required for depolarization-evoked Ca^{2+} entry in sperm. These findings support the hypothesis that the CatSper proteins form a functional Ca^{2+} channel complex.

4.3.2 Unaffected regional distributions of Ca_V channels in CatSper1 and CatSper2 null sperm.

A requirement for CatSper1 and CatSper2 on K8.6 evoked Ca^{2+} entry suggests, but does not demonstrate, that the CatSper2 protein functions as a Ca^{2+} entry channel. Therefore, we considered the alternate hypothesis that the CatSper2 protein instead is required for membrane targeting and functional expression of conventional voltage-gated Ca^{2+} channels, which also are candidates for the route of depolarization-evoked Ca^{2+} entry in sperm (Wennemuth et al. 2000; Westenbroek and Babcock 1999). In collaboration with Ruth Westenbroek (University of Washington, Department of Pharmacology), I found indistinguishable regional localizations of $Ca_V1.2$, $Ca_V2.2$, and $Ca_V2.3$ immunoreactivity in wild-type and CatSper2 null sperm (Fig. 4-7). Results with CatSper1 null sperm were essentially the same (Carlson et al. 2003). The loss of Ca^{2+} entry channel function in CatSper1 and CatSper2 null sperm apparently does not result from disrupted regional distributions of voltage-gated Ca^{2+} channel (Ca_V) proteins.

4.3.3 Inhibitor blocks evoked Ca^{2+} entry.

In collaboration with the pharmaceutical company Hydra Biosciences (Cambridge, MA), we received a candidate compound, and which we will call for now 'CatSper Inhibitor'. I asked whether this CatSper Inhibitor could inhibit the K8.6-evoked Ca^{2+} entry. Specifically, I used fura-2 photometry to monitor changes of free Ca^{2+} concentrations of sperm challenged with a paired-pulse protocol. Each cell received an initial ~15 s stimulus with medium K8.6, and after a 2 min recovery, a

second ~15 s stimulus. During recovery, sperm were exposed to medium Na7.4 alone or supplemented with the CatSper Inhibitor. At concentrations of 3 μM, the CatSper Inhibitor dramatically reduced the evoked Ca^{2+} entry, and at 10 μM it nearly abolished the evoked rise (Fig. 4-8). Using a protocol with two exposures to K8.6, I found that K8.6 evoked an average 130 and 90 nM increase of intracellular Ca^{2+} during each exposure when no inhibitor was present, and an average 100 and 40 nM increase before and after exposure to 3 μM CatSper inhibitor (Fig. 4-8*A*). In another series of experiments, I found that each K8.6 exposure evoked a 120 nM elevation of intracellular free Ca^{2+} (Fig. 4-8*B*). K8.6 evoked an average 100 nM increase of intracellular free Ca^{2+} prior to exposure to 10 μM of the CatSper inhibitor, and <10 nM increase after (Fig. 4-8*B*). These results provide further support for the hypothesis that the CatSper protein complex acts as a Ca^{2+} channel that is the route for K8.6-evoked Ca^{2+} entry.

4.3.4 Removal of external Ca^{2+} results in an elevation of internal Na^+.

It is possible that some CatSper channels are open, letting Ca^{2+} enter unstimulated sperm bathed in medium Na7.4. Many Ca^{2+} channels readily pass Na^+ in the absence of their preferred ion Ca^{2+}, therefore I asked whether local exposure to 0 mM Ca^{2+} evokes an intracellular Na^+ rise. Specifically, I used the ratiometric Na^+ indicator SBFI to monitor the intracellular Na^+ concentration of small groups of 5-7 sperm before and during exposure to Na7.4 with 0 or 2 mM Ca^{2+} (Fig. 4-9). I found that upon exposure to 0 mM Ca^{2+}, the intracellular Na^+ concentration increased. Probe responses were verified by treating sperm with 10 μM of the Na^+/H^+ -exchanging ionophore monensin. Monensin treatment with medium Na7.4 (containing 120 mM Na^+) evoked a rise of intracellular Na^+, whereas monensin in NMDG solution (nominally Na^+-free) evoked a decline of intracellular Na^+ (Fig. 4-9*B*&*C*).

4.3.5 K8.6 increases intracellular pH.

Simply depolarizing sperm with a high-potassium medium does not evoke much Ca^{2+} entry. Rather a high-potassium and high-pH solution, such as medium K8.6, is required for to evoke Ca^{2+} entry (Babcock and Pfeiffer 1987b; Florman et al. 1992). I have found that CatSper1 and CatSper2 also are required for this evoked Ca^{2+} entry. One explanation for these findings would be that the putative CatSper channel complex is regulated by intracellular pH. This hypothesis is consistent with the fact that the amino terminus of CatSper1 is histidine-rich (83 histidines in the 446-residue N-terminus (Ren et al. 2001)), which would make it highly susceptible to reversible protonation around physiologic pH. Therefore, I asked whether medium K8.6 indeed alters intracellular pH in 20 s. Using BCECF, I found that small groups of 5-7 sperm increased their pH upon exposure to medium K8.6 (from 7.1 ± 0.01 in Na7.4 to 7.2 ±0.01 pH units in K8.6) (Fig. 4-10). These data, along with the requirement of CatSper for evoked Ca^{2+} entry are consistent with the hypothesis that the CatSper complex Ca^{2+} channel is modulated by pH.

4.4 Co-dependent expression of CatSper1 and CatSper2 proteins

In independent experiments, I found that the CatSper1- and CatSper2- null sperm appear to have identical phenotypes. The simplest interpretation for the apparent identical phenotypes is that expression of CatSper1 and CatSper2 is co-dependent, and that these two proteins together form a functional Ca^{2+} channel in mature sperm. Our collaborator Timothy Quill (University of Texas Southwestern Medical Center) has verified the requirement of CatSper1 and CatSper2 for each others expression. Specifically, he first asked if CatSper2 transcription is required for subsequent production of CatSper1 mRNA and vice versa, by examining adult CatSper1 and -2 null testes by quantitative real-time PCR. Compared to wild-type testes, neither the relative content of CatSper1 mRNA in the CatSper2 null testes (1.6 ± 1.6 -fold; n = 3), nor the CatSper2 mRNA content of CatSper1 null testes (1.0 ± 1.1 -fold; n = 3) was significantly altered. In contrast, immunoblot analysis documents that

CatSper1 and -2 proteins stabilize each other through an interaction at the protein level. Fig. 4-11*A* shows immunoblots of wild-type sperm and CatSper1 and -2 null sperm probed with antibodies directed against the CatSper1 and -2 proteins. The wild-type sperm show prominent immunoreactive ~82 kDa CatSper1 and ~72 kDa CatSper2 bands. Neither of these bands was detected in either the CatSper1 or -2 null sperm examined at similar protein loading as verified by probing with an antibody for α–tubulin (Fig. 4-11*A*). Fig. 4-11*B* shows PCR analysis of genomic DNA from the animals used in the immunoblotting experiments, providing confirmation of the assigned genotype. These data indicate that CatSper1 null sperm lack the CatSper2 protein and that CatSper2 null sperm similarly lack the CatSper1 protein, and suggest that the CatSper proteins form a complex required for proper trafficking or stabilization against degradation.

4.5 Discussion

The putative Ca^{2+} channel subunits CatSper1 and CatSper2 are expressed exclusively in the testes, and localized on the principal piece of sperm. The CatSpers are members of the voltage-gated ion channel superfamily that includes voltage-gated K^+, Na^+, and Ca^{2+} channels, and the CNG and transient receptor potential channels (Quill et al. 2006). Both CatSper1 and CatSper2 null engineered mice have male infertility. These are the only two Ca^{2+} channel proteins thus far known to be absolutely required for male fertility. A spontaneous deletion that affects four genes in humans including a portion of the CatSper2 resulted in male infertility (Avidan et al. 2003) and thus CatSper was suggested to have a possible role in heritable male infertility (Nikpoor et al. 2004). Since the identification of CatSper1 and CatSper2, two additional CatSper genes have been identified in the human and mouse genomes, CatSper3 and CatSper4 (Lobley et al. 2003).

Whereas CatSper1 and CatSper2 null males are infertile, they are otherwise healthy (Quill et al. 2001; Ren et al. 2001). Spermatogenesis is normal in CatSper1 and CatSper2 nulls as judged by testicular histology, sperm counts, and sperm morphology

(Quill et al. 2001; Ren et al. 2001). These qualities attracted me to study the role of putative CatSper Ca^{2+} channel in signaling capacitation.

4.5.1 CatSper is required for K8.6-evoked entry.

Immunological evidence indicates that the pore-forming α subunits of numerous conventional voltage-gated (Ca_V) channels are regionally distributed in distinctive patterns on both the sperm head and flagellum (Serrano et al. 1999; Westenbroek and Babcock 1999; Wennemuth et al. 2000), and that depolarization-evoked entry of Ca^{2+} has a pharmacological sensitivity profile consistent with involvement of $Ca_V2.2$ and $Ca_V2.3$ channels (Wennemuth et al. 2000). Unexpectedly, we have found that both CatSper1 (Carlson et al. 2003) and CatSper2 (Fig. 4-6) null sperm completely lacked the evoked entry of Ca^{2+}, indicating that the CatSer1 and CatSper2 proteins are required directly or indirectly for functional Ca^{2+} entry channels. In both CatSper1 (Carlson et al. 2003) and CatSper2 (Fig. 4-7) null sperm regional distributions of $Ca_V1.2$, $Ca_V2.2$, and $Ca_V2.3$ channel proteins were indistinguishable from those of wild-type sperm. Thus, the absence of CatSper1 or CatSper2 apparently does not effect expression or trafficking of Ca_V channel proteins. In the simplest interpretation, the CatSper1 and -2 proteins present in the membrane of the principal piece (Quill et al. 2001; Ren et al. 2001) form voltage-gated Ca^{2+} channels that are the major route for depolarization-evoked entry of Ca^{2+} into the flagellum. The photometry methods used here and in past studies of evoked entry of Ca^{2+} in wild-type sperm (Carlson et al. 2003; Wennemuth et al. 2000; Wennemuth et al. 2003a; Wennemuth et al. 2003c) report changes in spatially-averaged [Ca^{2+}] from the heads and proximal flagella of several cells. Evoked Ca^{2+} entry also occurs when the detection window is limited to the flagella of clusters of wild-type sperm (data not shown), and when Ca^{2+} is monitored by imaging of individual immobilized sperm (Fukuda et al. 2004). Thus opening of CatSper channels in the flagellum allows an entry of Ca^{2+} that is communicated to the rest of the cell.

The putative CatSper Ca^{2+} channel is likely regulated by intracellular pH. We find that medium K8.6 evokes Ca^{2+} entry into sperm, and here I document that K8.6

treatment is accompanied by intracellular alkaninization (Fig. 4-10, first shown in (Babcock et al. 1983)). Our data are consistent with the recent experiments from David Clapham and co-workers who reported electrical recordings from wild-type sperm vs. CatSper1 null sperm that suggested CatSper is indeed a pH-sensitive Ca^{2+} channel whose open probability increases with increased intracellular pH (Kirichok et al. 2006). The amino terminus of CatSper1 is histidine rich and a probable candidate for the pH-sensing domain.

4.5.2 CatSper1 and CatSper2 regulate flagellar beat asymmetry.

I have found that CatSper1 (Carlson et al. 2003) and CatSper2 (Fig. 4-3) are required for acquisition of the asymmetrical waveform that underlies hyperactivation. Using computer-assisted semen analysis (CASA) with viscous media, Garbers and co-workers confirmed the absence of hyperactivation in CatSper2 null sperm (Quill et al. 2003). Further, a lack of hyperactivation is consistent with the finding that CatSper1 and CatSper2 null sperm fertilize only zona-free oocytes (Quill et al. 2003; Ren et al. 2001). I have documented that correct expression and trafficking of several other proteins do not to require CatSper1 (Carlson et al. 2003), or CatSper2 (Figs. 4-1, 4-4, 4-5 & 4-7). Therefore, all available evidence is consistent with the hypothesis that the hyperactivation deficit is the critical lesion in the infertility of CatSper1 and -2 sperm.

In permeabilized sperm preparations, Ca^{2+} mediates transition from a symmetrical to an asymmetric flagellar waveform (Lindemann et al. 1987; Brokaw 1979) similar to that of intact sperm hyperactivated during Ca^{2+}-dependent capacitation *in vitro*. In the simplest explanation, CatSper1 and -2 together form functional channels that open to allow entry of Ca^{2+} that is required to initiate or sustain hyperactivation. In a more complicated explanation, CatSper1 and -2 might function to maintain or refill a putative internal store proposed by others (Ho and Suarez 2003) to provide a Ca^{2+} signal for hyperactivation. Experiments to distinguish between these hypotheses have not been done.

In yet another possible explanation for the hyperactivation deficit of CatSper1 and -2 null sperm, these proteins could be required during spermiogenesis for

formation of a hyperactivation-competent flagellar axoneme, capable of responding to the Ca^{2+} signal with waveform asymmetry. Past studies report that procaine (Ho and Suarez 2003; Lindemann et al. 1987; Singh et al. 1983) and other membrane active agents (Gu et al. 2004; Lindemann et al. 1987; Singh et al. 1983) produce Ca^{2+}-dependent, hyperactivation-like responses in sperm. I have found that the loss-of-flagellar-asymmetry phenotype of CatSper2 null sperm can be rescued pharmacologically by procaine treatment (Fig. 4-4*A*), indicating that defects in the responses of the flagellum to Ca^{2+} do not explain the hyperactivation deficit of the mutant sperm. However, procaine-treatment and capacitating incubations of wild-type sperm produce flagellar waveforms differ in several ways (Figs. 4-4*B* and 4-5).

Hyperactivation was first documented in the 1970's, and many hypotheses have been proposed regarding its significance for the movement of sperm through the female reproductive tract. I have found that CatSper1 and CatSper2 are required for sperm to display the hyperactive waveform. Apart from the hyperactived waveform, the other cellular hallmarks of capacitation are intact in the CatSper null sperm suggesting that the inability of CatSper null sperm to fertilize is a result of their inability to hyperactivate. My results indicate that the hyperactived waveform is an essential step of capacitation and is required for fertilization *in vivo*.

4.5.3 CatSper1 and CatSper2 are required for setting the basal cAMP content but not for HCO_3^-- evoked activation.

In contrast to the requirement for CatSper1 (Carlson et al. 2003) and -2 (Fig. 4-3) in Ca^{2+}-mediated control of flagellar asymmetry, CatSper1 (Carlson et al. 2003) and -2 (Fig. 4-1) are not required for the cAMP-mediated activation. Despite this clear separation of functional roles, the cAMP and Ca^{2+} signaling systems of sperm seem to be intricately intertwined. For example, experiments from this laboratory document that a PKA-mediated phosphorylation is required to facilitate Ca^{2+} channel activity (Nolan et al. 2004; Wennemuth et al. 2003d), and to limit the early accumulation of cAMP (Nolan et al. 2004). Conversely, external Ca^{2+}, is required for HCO_3^--evoked increases in cAMP content (Carlson et al. 2005; Garbers et al. 1982) and acceleration of

the flagellar beat frequency (Carlson et al. 2003). Now I have found that a presumptive entry of Ca^{2+} through a CatSper-dependent pathway may determine the set point for resting cAMP content (Fig. 4-2*B*).

It is possible that the CatSper null sperm have elevated cAMP content and beat frequency because they lack a phosphodiesterase activity that is present in the unstimulated wild-type sperm. We believe that some CatSper channels are open in the unstimulated sperm, and this may allow for some Ca^{2+} entry. Calmodulin-dependent PDEs are prominent in epididymal sperm, and provide one of the major PDE activities in mature sperm cells (Yan et al. 2001). PDE1A and 1C are two major CaM-dependent PDE proteins that are highly expressed in the developing male germ cell (Yan et al. 2001). Further, a new variant of PDE1A has been identified here at the University of Washington in the Beavo lab, and is expressed through the length of the flagellum (Vasta et al. 2005). Ca^{2+} that enters via the CatSper channel may bind to CaM and allow for subsequent activation of PDE sufficient to depress basal cAMP concentrations. This hypothesis is in agreement with findings from our laboratory that treatment of sperm with the broad-spectrum PDE inhibitor IBMX in medium Na7.4 elevates the flagellar beat frequency, which itself indicates that basal cAMP content of sperm is determined by a balance of basal adenylyl cyclase and phosphodiesterase activities (Wennemuth et al. 2003d). If Ca^{2+} entry via CatSper to increase PDE activity occurs, we would expect accelerated beat frequency of sperm exposed to medium Na7.4 with 0 Ca^{2+}; experimentally we do not find this (Figs. 3-8 & 3-9).

Each of the four known CatSper sequences contain putative protein interaction domains/motifs near their carboxy terminus (Quill et al. 2006). It is possible that the CatSper subunits not only interact with each other, but act as scaffolding proteins to bring together other signaling proteins. Therefore, disruption of the CatSper protein complex may disrupt other protein interactions required for basal cAMP regulation.

4.5.4 Co-dependent expression of CatSper1 and CatSper2 proteins.

Our collaborator Timothy Quill (University of Texas Southwestern Medical Center) has found a reciprocal requirement of CatSper1 and CatSper2 to express both

proteins in cauda epididymal sperm (Carlson et al. 2005). A simple explanation of this codependency of stable expression is that the two proteins are components of a single heteromeric ion channel that regulates sperm hyperactivation. However, functional channels have not yet been produced by co-transfection of the CatSper1 and -2 proteins in somatic cells (Quill et al. 2001). A possible explanation is that yet another component(s) is required for the appropriate folding, trafficking, or assembly of a functional CatSper channel.

Here I have shown data suggesting that CatSper1 and CatSper2 likely form a pH-sensitive Ca^{2+} channel in mature sperm. I also document a requirement for CatSper1 and CatSper2 in capacitation. Specifically, sperm lacking either CatSper1 or CatSper2 fail to display the flagellar beat asymmetry associated with hyperactivation. In contrast, CatSper1 and CatSper2 are not required for the HCO_3^- evoked activation. Yet the elevated cAMP content and flagellar beat frequencies of the CatSper2 null sperm indicate some functional relationship between this Ca^{2+} channel and cAMP regulation in mature sperm. Because the CatSper proteins are exclusively expressed in the testes, and the channels are absolutely required for male fertility, the CatSper channel presents an ideal target for male contraception.

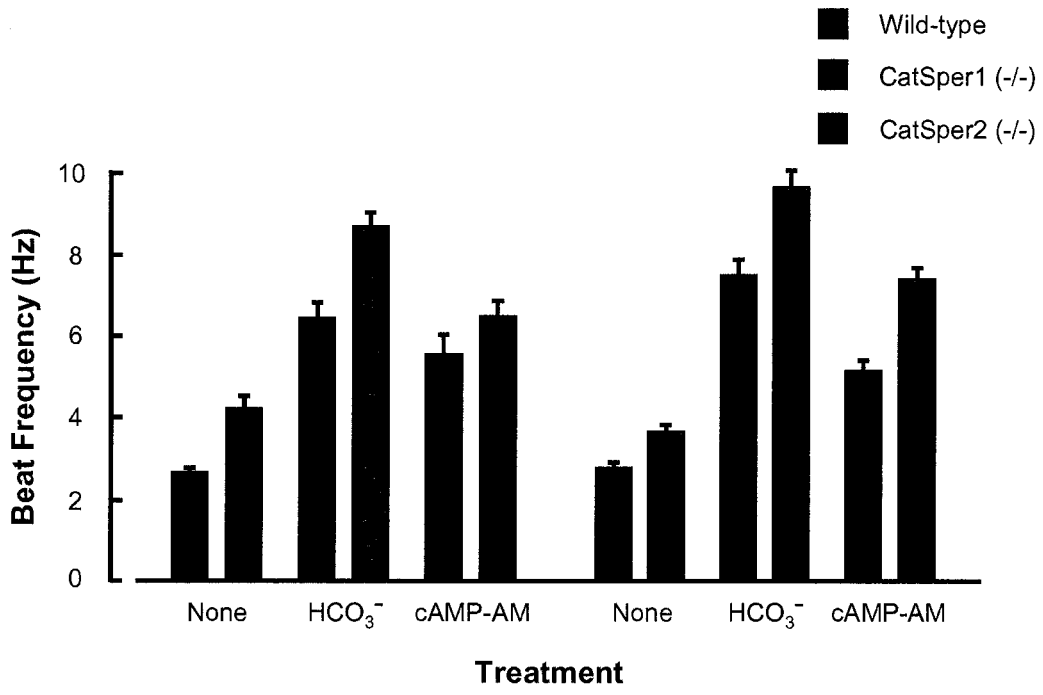

Figure 4-1: **CatSper1 and CatSper2 are not required for HCO_3^- or cAMP-mediated acceleration of beat frequency**. Beat frequency monitored from single sperm cells. Parallel experiments were performed with sperm from CatSper1 (-/-) or CatSper2 (-/-) mice or their wild-type littermates. Sperm were randomly sampled after 1-10 min incubation in Na7.4 lacking of containing 15 mM $NaHCO_3$ to stimulate cAMP production. Ester-generated cAMP was supplied by incubating sperm for 30 min with the membrane-permeant cAMP-AM (60 μM) (mean ± sem, N=16-31 in four independent experiments for each genotype-pair).

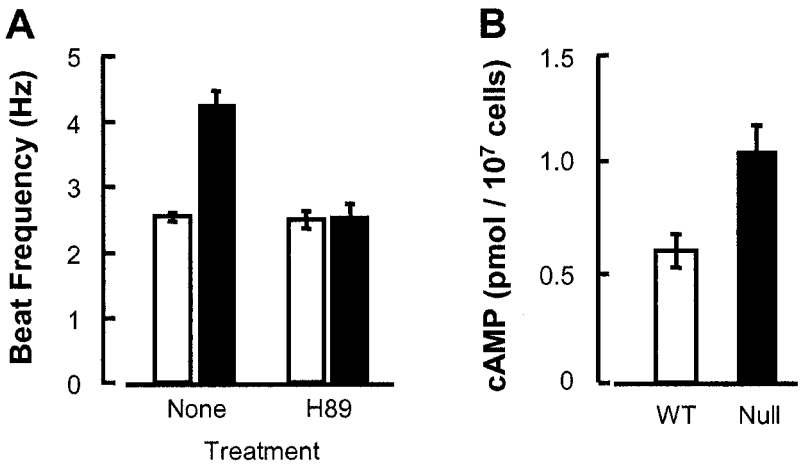

Figure 4-2: **Elevated basal beat of CatSper2 null sperm is due to increased cAMP and PKA-mediated signals**. **A:** The flagellar beat frequency of wild-type (open) or CatSper2 null (closed) sperm randomly sampled after 5 – 10 min incubation in medium Na7.4 lacking or containing 30 μM H89. N=13-14 in two independent experiments. **B**: The cAMP content of wild-type (open) and CatSper2 null (closed) sperm before exposure to 15 mM $NaHCO_3$ (Carlson et al. *JBC* 2005, cAMP assays by Timothy Quill, University of Texas Southwestern Medical Center).

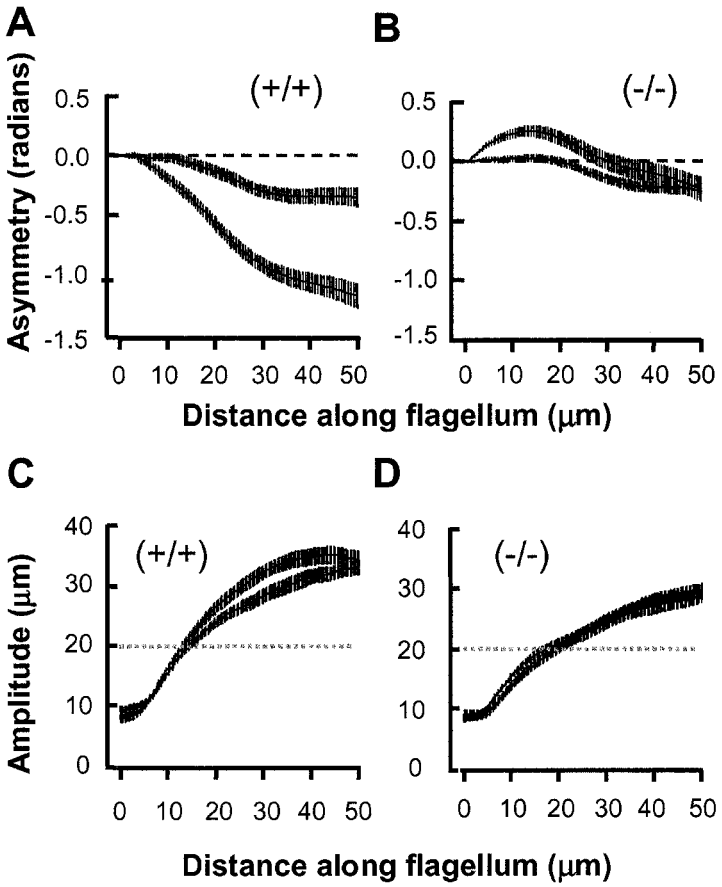

Figure 4-3: **CatSper2 is required for hyperactivated motility.** **A & B:** Flagellar asymmetry reported by time-averaged tangent angles for wild-type (+/+) and null (-/-) sperm, examined before (blue) and after (black) capacitating incubations. **C & D:** The beat amplitude measured by the maximal excursion from the flagellar beat axis traveled by each point along the flagellum during a beat cycle. N=17-23 in four independent experiments.

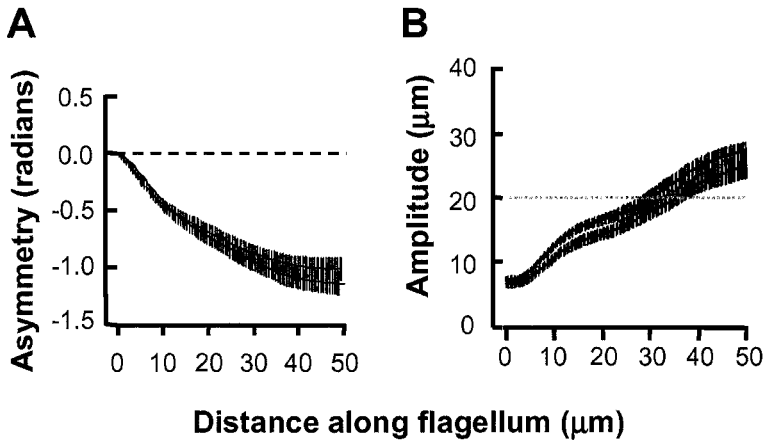

Figure 4-4: **Procaine partially recovers hyperactivation deficit in CatSper2 null sperm**. **A**: Flagellar asymmetry reported by time-averaged tangent angles **B:** and beat amplitude for wild-type (black) and null (red) sperm, examined after 5 min treatment with 10 mM procaine (mean ± sem, N = 12-23 cells in four independent experiments).

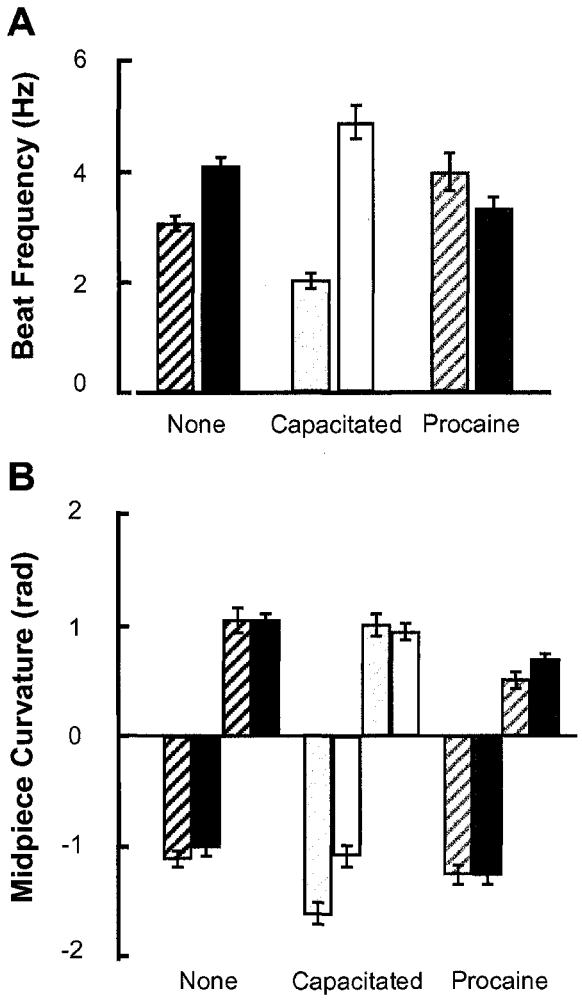

Figure 4-5: **Procaine treatment differs from hyperactivated waveform. A:** Flagellar beat frequency of wild-type (striped) or CatSper2 null (solid) sperm randomly sampled before or after incubation with capacitating conditions, or with 10 mM procaine treatment. **B:** Midpiece curvature of wild-type (striped) and CatSper2 null (solid) sperm before and after treatment with capacitating conditions, or after treatment with 10 mM procaine. Pro-hook curvature indicated by the arbitrary assigned negative value, and anti-hook curvature indicated by a positive value (mean ± sem, N = 12-23 cells in four independent experiments).

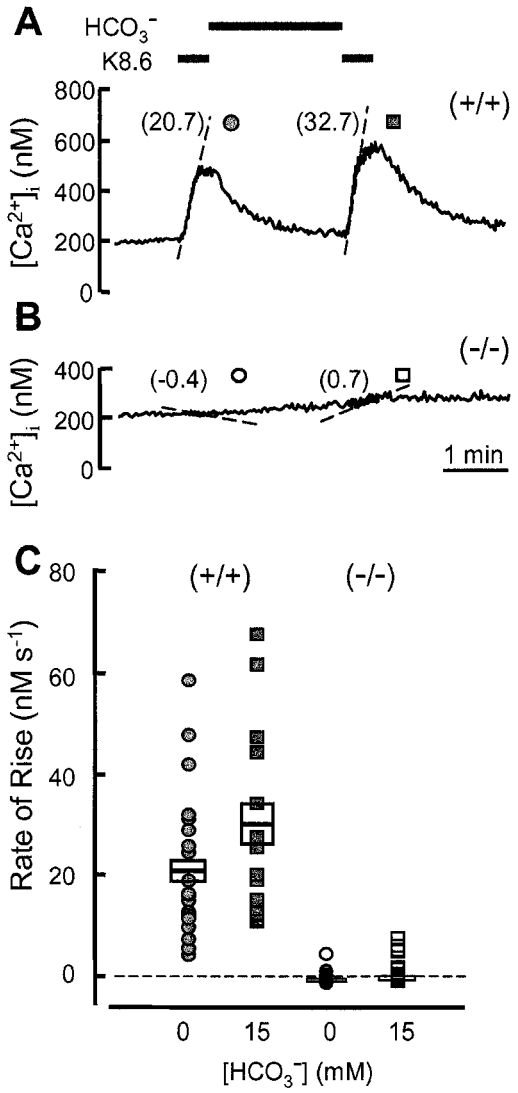

Figure 4-6. **No evoked Ca^{2+} entry in CatSper2 null sperm.** Intracellular free Ca^{2+}-concentration was reported by fura-2 ratiometric photometry. **(A,B)** Averaged responses from 5 small clusters of cells locally perfused with Na7.4, or with Na7.4 supplemented with 15 mM $NaHCO_3$, except during 30 s depolarizing stimuli with medium K8.6, as indicated. **A:** wild-type (+/+) sperm, **B:** CatSper2 null (-/-) sperm. **C:** Rates of rise evoked by depolarization applied before (0 BC) (circles) and after (15 BC) (squares) conditioning with 15 mM $NaHCO_3$. Average and SEM indicated by solid lines. N = 20 clusters of cells, in 4 independent experiments. Boxes indicate the mean and sem.

Figure 4-7: **CatSper2 is not required for regional distribution of Ca_V channel proteins**. Confocal immunofluorescence images shown in reverse contrast for wild-type (+/+) and CatSper2 null (-/-) sperm treated with antibodies directed to the $Ca_V1.2$ (**A** and **B**); $Ca_V2.2$ (**C** and **D**); and $Ca_V2.3$ channel proteins (**E** and **F**). Each panel contains representative images of central optical sections from the head (above) and the proximal flagellum (below) (from Carlson et al. 2005, images collected by Ruth Westernbroek, University of Washington)

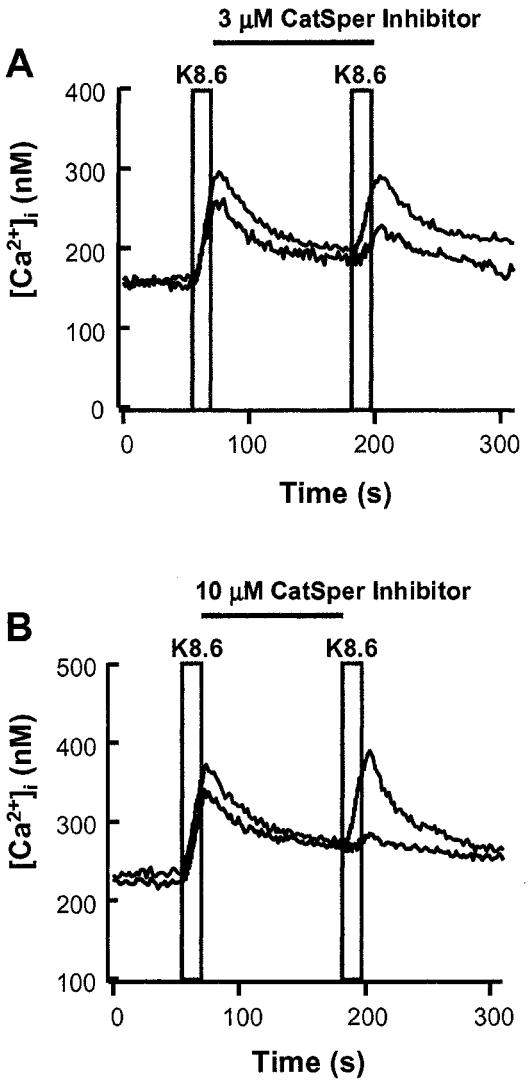

Figure 4-8: **CatSper inhibitor blocks the K8.6-evoked Ca^{2+} entry**. Intracellular Ca^{2+} content of small groups of 3-5 cells in Na7.4 alone (black traces), or supplemented with 3 (**A**) or 10 (**B**) µM of the CatSper inhibitor (blue traces), and treated with the high potassium, high pH solution K8.6 in the presence or absence of the inhibitor, as indicated (N=16-19, 3 independent experiments).

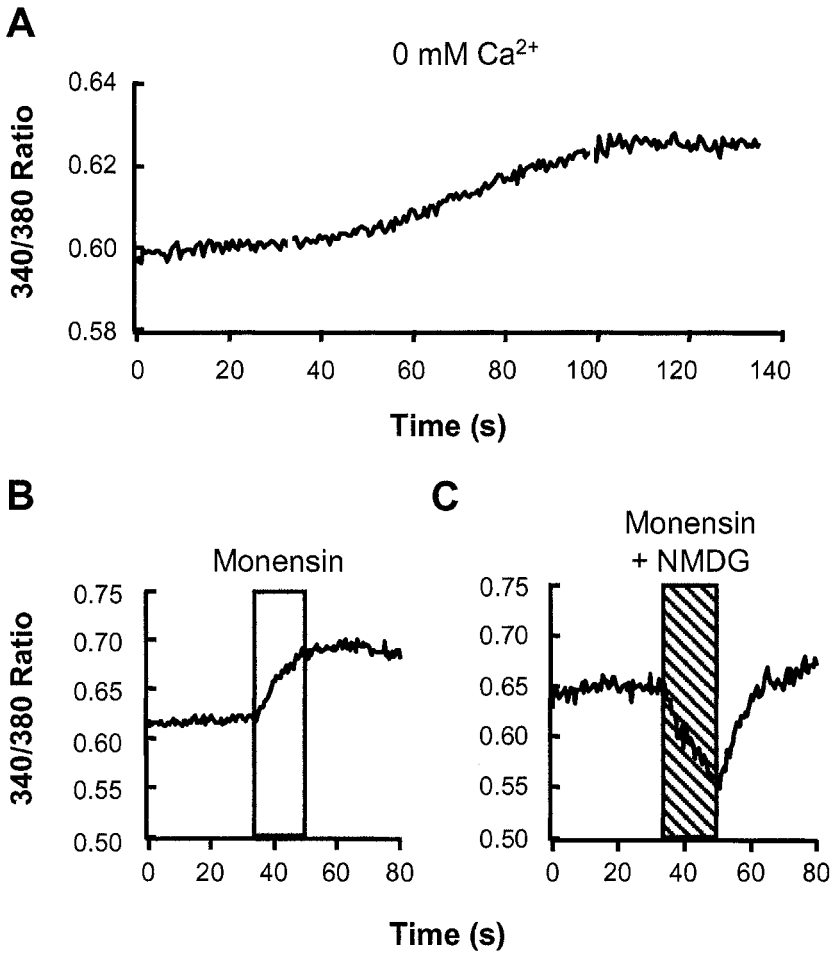

Figure 4-9: **Intracellular Na^+ increases upon removal of external Ca^{2+}.** **A:** Intracellular Na^+, indicated by ratiometric indicator SBFI, of small groups of 3-5 cells. **B** and **C**: Probe responses validated by treatment with the Na^+/H^+ ionophore monensin (10 µM), either in medium Na7.4 or in a Na^+- free/NMDG solution (120 or 0 mM Na^+ respectively).

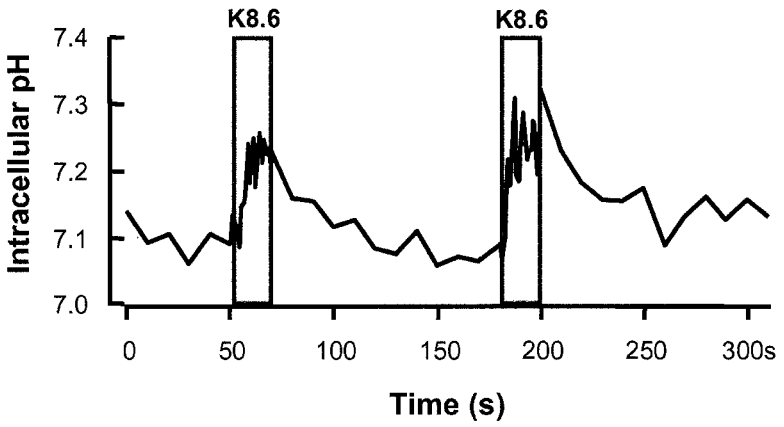

Figure 4-10: **K8.6 treatment increases intracellular pH**. Average intracellular pH, as indicated by BCECF photometry, of small groups of 3-5 cells during exposure to Na7.4 and K8.6, as indicated (N=5, 1 experiment).

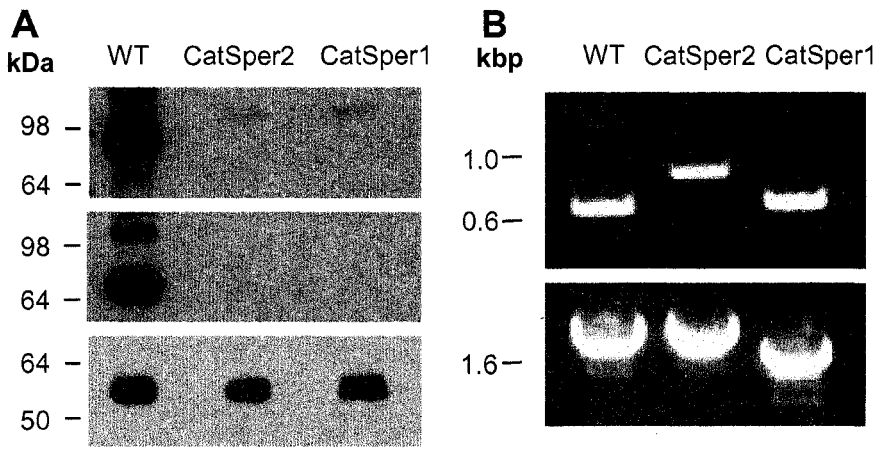

Figure 4-11: **The expression of CatSper1 and CatSper2 in sperm is co-dependent. A:** Duplicate immunoblots were probed with antibodies to CatSper1 (top panel) and CatSper2 (middle panel). Following the removal of the CatSper2 antibody, this blot was re-probed with antibodies to α-tubulin (lower panel). Total cell SDS extract from one million cells was loaded in each lane. **B:** Genotype confirmation of the samples used for immunoblotting. CatSper1 gene PCR analysis (upper panel): wild-type allele – 2100 bp, null allele – 1700 bp; CatSper2 gene PCR analysis (lower panel): wild-type allele – 641 bp, null allele – 831 bp. (from Carlson et al. 2005, gels by Timothy Quill, University of Texas Southwestern Medical Center).

CHAPTER 5

CONCLUSIONS

5.1 Overview

Here I have shown evidence that HCO_3^- activates mouse sperm, the first stage of capacitation, in a signaling pathway that uses carbonic anhydrase, sAC, cAMP, and PKA. I have also found that this same signaling pathway is required for development of the hyperactivated waveform. Further, I have characterized the role of the putative Ca^{2+} channel CatSper in capacitation and provided evidence that CatSper functions as a pH-sensitive Ca^{2+} channel. In this chapter, I will discuss future directions in the study of mouse sperm capacitation and the significance of my findings in the evolution of capacitation, and of possible applications of my findings.

5.2 Future studies

5.2.1 Role of other Ca_V channels in sperm.

CatSpers are the first Ca^{2+} channel proteins proven essential for male fertility; however, sperm possess a variety of other Ca^{2+} channels. Sperm are highly compact and differentiatedcells, and therefore teleologically it is difficult to imagine that they would contain unnecessary proteins. As Ca^{2+} is an important regulator of sperm motility, these channels may be important for sperm regulation and therefore the role of these other Ca^{2+} channels should be further investigated. For example, it would be interesting to examine whether specific inhibition of some of these other channels also precludes development of the hyperactivated waveform

5.2.2 Role of other cellular events of capacitation on flagellar waveforms.

Several cellular changes associated with sperm capacitation have been documented, yet a link between many of these events and the flagellar waveform is not yet known. Both tyrosine phosphorylation (Visconti et al. 1995a) and membrane-

associated changes (Gadella and Van Gestel 2004) occur slowly with capacitating incubations, on a similar time scale as the appearance of the hyperactive waveform. Therefore the possible requirement of either of these two capacitation events for development of the hyperactivated waveform should be investigated. For instance, can we preclude hyperactivation by blocking tyrosine phosphorylation? We know that BSA or another cholesterol-accepting protein is required for membrane-associated events and in our capacitation medium (Gadella and Van Gestel 2004). Therefore to begin investigation of the role of membrane events in the development of the hyperactive waveform, we can experimentally ask whether BSA is required in capacitating medium for development of hyperactivation.

5.2.3 PKA-mediated phosphorylation.

My own findings indicate that PKA is required for both the activated and hyperactivated waveforms. What proteins are phosphorylated by PKA, and which of these phosphorylation events is required for activation and hyperactivation? The application of proteomics is a powerful tool, and in combination with genetic approaches we may be able to determine the proteins phosphorylated by PKA.

5.2.4 Combined use of ionic indicators and waveform analysis.

Here I have established some of signaling pathways underlying flagellar waveforms of mouse sperm capacitation. I have used waveform analysis and photometry independently to understand flagellar movement and ionic dynamics. Combining fluorescent indicators with waveform analysis may allow for detection of important ionic changes that occur in specific subcellular domains. For example, it is possible that intracellular Ca^{2+} concentrations are higher at bending points on the flagellum compared to straighter segments. I cannot document this sort of event with my waveform and photometry setups.

I suggest a few changes that would enable the simultaneous use of waveform analysis and the use of fluorescent indicators. My own photometry experiments were

performed with ratiometric indicators excited by two wavelengths of light. Clear stop-motion images require a brief light-pulse of approximately 1 ms a time duration insufficient for excitation at two wavelengths. Therefore a single excitation and dual emission ratiometric indicator, like indo-1, would be ideal. The T.I.L.L. monochrometer used for my own photometric experiments is not capable of providing excitation stimuli of a sufficiently brief duration. The monochrometer utilizes pulses of electricity to move a galvanometer to select the appropriate wavelength of light, a process accompanied by a 3 ms delay of action. The use of LEDs is preferable for stop-motion images because they allow for very short pulses of light, necessary to acquire stop-motion flagellar images. With the recent creation of LEDs capable of emitting ultraviolet wavelengths of light, it is conceptually possible to perform waveform analysis on sperm loaded with ionic indicators. This experimental setup will allow for dissection of the subcellular ionic events and their study for different flagellar waveforms associated with capacitation.

5.2.5 Relationship between beat frequency and freely swimming sperm.

The waveform analysis system employed by our laboratory provides powerful measurements of flagellar movements. This system exploits the tendency of sperm to adhere to glass spontaneously at the equatorial region of their head. Despite this 'spontaneous-tethering' their flagella still beat. It is difficult to estimate the relationship of how the flagella moves in this confined manner to how the flagellum moves while swimming freely *in vitro*, or while traversing the female reproductive tract. Therefore, the applicability our waveform analysis findings to free swimming sperm should be investigated. For example, it would be informative to monitor free swimming sperm in medium Na7.4 alone or with 15 mM $NaHCO_3$, and track how fast they are swimming. A regression analysis between average track-velocity and flagellar beat frequency may begin to lend insights into how our beat frequency relates to freely swimming sperm.

5.3 Significance of the need for capacitation

Capacitation is not required for several non-mammalian vertebrates, notwithstanding the fact that sperm must traverse the female tract to fertilize internally. The limited comparative evidence seems consistent with the possibility that the need for capacitation has arisen *de novo* in eutherian mammals (Bedford 1983). The driving force for the evolution of capacitation may have been maintenance of precious ATP stores and retention of the capacity to fertilize. Flagellar beating is energetically costly and would be wasteful while mature sperm are stored in the epididymis. Further, sperm are capable of undergoing the acrosome reaction only once and this reaction is required for fertilization, a stable membrane during epididymal maturation and storage should aid in prevention of premature acrosome reaction.

The ubiquitous physiologic presence of HCO_3^- likely facilitated its evolution as the primary physiologic initiator of capacitation. The adult male can actively remove HCO_3^- from the epididymis and vas deferens to decrease the likelihood of inappropriately timed capacitation-associated cellular reactions. High HCO_3^- concentrations will be encountered in both the male and female reproductive fluids as the sperm traverses the female reproductive tract.

5.4 Research applications

Capacitation is a necessary process in the preparation of sperm for fertilization. Further understanding of capacitation may lend to the design of new forms of infertility treatments, or alternatively, contraception.

LIST OF REFERENCES

Austin, C. R. (1951). Observations on the penetration of the sperm into the mammalian egg. *Aust J Sci Res* **4**: 581-596.

Austin, C. R. (1967). Capacitation of spermatozoa. *Int J Fert* **12**: 25-31.

Avidan, N., H. Tamary, O. Dgany, D. Cattan, A. Pariente, M. Thulliez, N. Borot, L. Moati, A. Barthelme, L. Shalmon, T. Krasnov, E. Ben-Asher, T. Olender, M. Khen, I. Yaniv, R. Zaizov, H. Shalev, J. Delaunay, M. Fellous, D. Lancet and J. S. Beckmann (2003). CATSPER2, a human autosomal nonsyndromic male infertility gene. *Eur J Hum Genet* **11**: 497-502.

Babcock, D. F. (1983). Examination of the intracellular ionic environment and of ionophore action by null point measurements employing the fluorescein chromophore. *J Biol Chem* **258**: 6380-9.

Babcock, D. F. and D. R. Pfeiffer (1987a). Independent elevation of cytosolic [Ca2+] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J Biol Chem* **262**: 15041-7.

Babcock, D. F. and D. R. Pfeiffer (1987b). Independent elevation of cytosolic [Ca^{2+}] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J Biol Chem* **262**: 15041-7.

Babcock, D. F., G. A. Rufo, Jr. and H. A. Lardy (1983). Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. *Proc Natl Acad Sci U S A* **80**: 1327-31.

Baxendale, R. W. and L. R. Fraser (2003). Evidence for multiple distinctly localized adenylyl cyclase isoforms in mammalian spermatozoa. *Mol Reprod Dev* **66**: 181-9.

Bedford, J. M. (1983). Significance of the need for sperm capacitation before fertilization in eutherian mammals. *Biol Reprod* **28**: 108-20.

Bedford, J. M. and N. L. Cross (1998). Sperm Capacitation. Encyclopedia of Reproduction. E. Knobil and J. D. Neill. San Diego, Academic Press. **4:** 597-602.

Bedford, J. M. and R. Yanagimachi (1992). Initiation of sperm motility after mating in the rat and hamster. *J Androl* **13**: 444-9.

Biel, M., M. Seeliger, A. Pfeifer, K. Kohler, A. Gerstner, A. Ludwig, G. Jaissle, S. Fauser, E. Zrenner and F. Hofmann (1999). Selective loss of cone function in mice lacking the cyclic nucleotide- gated channel CNG3. *Proc Natl Acad Sci U S A* **96**: 7553-7.

Birkhead, T. R. (2000). Promiscuity: An evolutionary history of sperm competition. Cambridge, Harvard University Press.

Boatman, D. E. and R. S. Robbins (1991). Bicarbonate: Carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol Reprod* **44**: 806-13.

Branham, M. T., L. S. Mayorga and C. N. Tomes (2006). Calcium-induced acrosomal exocytosis requires cAMP acting through a protein kinase A-independent, Epac-mediated pathway. *J Biol Chem* **281**: 8656-66.

Branigan, E. F., M. A. Estes and C. H. Muller (1999). Advanced semen analysis: a simple screening test to predict intrauterine insemination success. *Fertil Steril* **71**: 547-51.

Braun, T. (1990). Inhibition of the soluble form of testis adenylate cyclase by catechol estrogens and other catechols. *Proc Soc Exp Biol Med* **194**: 58-63.

Braun, T., H. Frank, R. Dods and S. Sepsenwol (1977). Mn^{2+}-sensitive, soluble adenylate cyclase in rat testis. Differentiation from other testicular nucleotide cyclases. *Biochim Biophys Acta* **481**: 227-35.

Breton, S. (2001). The cellular physiology of cabonic anhydrases. *JOP* **2**: 159-164.

Brokaw, C. J. (1979). Calcium-induced asymmetrical beating of triton-demembranated sea urchin sperm flagella. *J Cell Biol* **82**: 401-11.

Brokaw, C. J., R. Josslin and L. Bobrow (1974). Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. *Biochem Biophys Res Commun* **58**: 795-800.

Brook, P. F., J. Lawry, I. D. Cooke and C. L. Barratt (1996). Measurement of intracellular pH in human spermatozoa by flow cytometry with the benzo[c]xanthene dye SNAFL-1: a novel, single excitation, dual emission, molecular probe. *Mol Hum Reprod* **2**: 18-25.

Brown, E. M., G. Gamba, D. Riccardi, M. Lombardi, R. Butters, O. Kifor, A. Sun, M. A. Hediger, J. Lytton and S. C. Hebert (1993). Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* **366**: 575-80.

Buck, J., M. L. Sinclair, L. Schapal, M. J. Cann and L. R. Levin (1999). Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc Natl Acad Sci U S A* **96**: 79-84.

Cann, M. J., A. Hammer, J. Zhou and T. Kanacher (2003). A defined subset of adenylyl cyclases is regulated by bicarbonate ion. *J Biol Chem.*

Carlson, A. E., T. A. Quill, R. E. Westenbroek, S. M. Schuh, B. Hille and D. F. Babcock (2005). Identical phenotypes of CatSper1 and CatSper2 null sperm. *J Biol Chem* **280**: 32238-44.

Carlson, A. E., R. E. Westenbroek, T. Quill, D. Ren, D. E. Clapham, B. Hille, D. L. Garbers and D. F. Babcock (2003). CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. *Proc Natl Acad Sci U S A* **100**: 14864-14868.

Carr, D. W., A. Fujita, C. L. Stentz, G. A. Liberty, G. E. Olson and S. Narumiya (2001). Identification of sperm-specific proteins that interact with A-kinase anchoring proteins in a manner similar to the type II regulatory subunit of PKA. *J Biol Chem* **276**: 17332-8.

Carrera, A., J. Moos, X. P. Ning, G. L. Gerton, J. Tesarik, G. S. Kopf and S. B. Moss (1996). Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: identification of A kinase anchor proteins as major substrates for tyrosine phosphorylation. *Dev Biol* **180**: 284-96.

Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into fallopian tubes. *Nature* **168**: 697-698.

Chattopadhyay, N. and E. M. Brown (2000). Cellular "sensing" of extracellular calcium (Ca(2+)(o)): emerging roles in regulating diverse physiological functions. *Cell Signal* **12**: 361-6.

Chen, Y., M. J. Cann, T. N. Litvin, V. Iourgenko, M. L. Sinclair, L. R. Levin and J. Buck (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289**: 625-8.

Cook, S. P., C. J. Brokaw, C. H. Muller and D. F. Babcock (1994). Sperm chemotaxis: egg peptides control cytosolic calcium to regulate flagellar responses. *Dev Biol* **165**: 10-9.

Cooper, T. G. (1984). The onset and maintenance of hyperactivated motility of spermatozoa from the mouse. *Gamete Res* **9**: 55-74.

Cross, N. L. and P. Razy-Faulkner (1997). Control of human sperm intracellular pH by cholesterol and its relationship to the response of the acrosome to progesterone. *Biol Reprod* **56**: 1169-74.

David, A., B. G. Brackett, C. R. Garcia and L. Mastroianni, Jr. (1969). Composition of rabbit oviduct fluid in ligated segments of the Fallopian tube. *J Reprod Fertil* **19**: 285-9.

David, A., D. M. Serr and B. Czernobilsky (1973). Chemical composition of human oviduct fluid. *Fertil Steril* **24**: 435-9.

Demott, R. P. and S. S. Suarez (1992). Hyperactivated sperm progress in the mouse oviduct. *Biol Reprod* **46**: 779-85.

Ellis-Davies, G. and J. Kaplan (1994). Nitrophenyl-EGTA, a photolabile chelator that selectively binds Ca^{2+} with high affinity and releases it rapidly upon photolysis. *Proc Natl Acad Sci U S A* **91**: 187-191.

Esposito, G., B. S. Jaiswal, F. Xie, M. A. Krajnc-Franken, T. J. Robben, A. M. Strik, C. Kuil, R. L. Philipsen, M. van Duin, M. Conti and J. A. Gossen (2004). Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proc Natl Acad Sci U S A* **101**: 2993-8.

Etter, E. F., A. Minta, M. Poenie and F. S. Fay (1996). Near-membrane [Ca^{2+}] transients resolved using the Ca^{2+} indicator FFP18. *Proc Natl Acad Sci U S A* **93**: 5368-5373.

Ficarro, S., O. Chertihin, V. A. Westbrook, F. White, F. Jayes, P. Kalab, J. A. Marto, J. Shabanowitz, J. C. Herr, D. F. Hunt and P. E. Visconti (2003). Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. *J Biol Chem* **278**: 11579-89.

Florman, H. M., M. E. Corron, T. D. Kim and D. F. Babcock (1992). Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Dev Biol* **152**: 304-14.

Fraser, L. R. (1982). Ca^{2+} Is Required for mouse sperm capacitation and fertilization *in vitro*. *J Androl* **3**: 412-419.

Fujinoki, M., T. Kawamura, T. Toda, H. Ohtake, T. Ishimoda-Takagi, N. Shimizu, S. Yamaoka and M. Okuno (2003). Identification of 36-kDa flagellar phosphoproteins associated with hamster sperm motility. *J Biochem (Tokyo)* **133**: 361-9.

Fukuda, N., K. Yomogida, M. Okabe and K. Touhara (2004). Functional characterization of a mouse testicular olfactory receptor and its role in chemosensing and in regulation of sperm motility. *J Cell Sci* **117**: 5835-45.

Gadella, B. M. and R. A. Van Gestel (2004). Bicarbonate and its role in mammalian sperm function. *Anim Reprod Sci* **82-83**: 307-19.

Garbers, D. L. and G. S. Kopf (1980). The regulation of spermatozoa by calcium cyclic nucleotides. *Adv Cyclic Nucleotide Res* **13**: 251-306.

Garbers, D. L., G. S. Kopf, D. J. Tubb and G. Olson (1983). Elevation of sperm adenosine 3':5'-monophosphate concentrations by a fucose-sulfate-rich complex associated with eggs: I. Structural characterization. *Biol Reprod* **29**: 1211-20.

Garbers, D. L., D. J. Tubb and R. V. Hyne (1982). A requirement of bicarbonate for Ca^{2+}-induced elevations of cyclic AMP in guinea pig spermatozoa. *J Biol Chem* **257**: 8980-4.

Gautier-Courteille, C., M. Salanova and M. Conti (1998). The olfactory adenylyl cyclase III is expressed in rat germ cells during spermiogenesis. *Endocrinology* **139**: 2588-2599.

Grynkiewicz, G., M. Poenie and R. Y. Tsien (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440-3450.

Gu, Y., J. C. Kirkman-Brown, Y. Korchev, C. L. Barratt and S. J. Publicover (2004). Multi-state, 4-aminopyridine-sensitive ion channels in human spermatozoa. *Dev Biol* **274**: 308-17.

Hamner, C. E. and W. L. Williams (1965). Composition of Rabbit Oviduct Secretions. *Fertil Steril* **16**: 170-6.

Harrison, R. A., P. J. Ashworth and N. G. Miller (1996). Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol Reprod Dev* **45**: 378-91.

Harrison, R. A. and N. G. Miller (2000). cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Mol Reprod Dev* **55**: 220-8.

Hess, K. C., B. H. Jones, B. Marquez, Y. Chen, T. S. Ord, M. Kamenetsky, C. Miyamoto, J. H. Zippin, G. S. Kopf, S. S. Suarez, L. R. Levin, C. J. Williams, J. Buck and S. B. Moss (2005). The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Dev Cell* **9**: 249-59.

Ho, H. C. and S. S. Suarez (2003). Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biol Reprod* **68**: 1590-6.

Holt, W. V. and R. A. Harrison (2002). Bicarbonate stimulation of boar sperm motility via a protein kinase A-dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. *J Androl* **23**: 557-65.

Hyne, R. V. and D. L. Garbers (1979). Regulation of guinea pig sperm adenylate cyclase by calcium. *Biol Reprod* **21**: 1135-42.

Ishijima, S., S. A. Baba, H. Mohri and S. S. Suarez (2002). Quantitative analysis of flagellar movement in hyperactivated and acrosome-reacted golden hamster spermatozoa. *Mol Reprod Dev* **61**: 376-84.

Jaiswal, B. S. and M. Conti (2001). Identification and functional analysis of splice variants of the germ cell soluble adenylyl cyclase. *J Biol Chem* **276**: 31698-708.

Jaiswal, B. S. and M. Conti (2003). Calcium regulation of the soluble adenylyl cyclase expressed in mammalian spermatozoa. *Proc Natl Acad Sci U S A* **100**: 10676-81.

Jha, K. N. and S. Shivaji (2002). Protein serine and threonine phosphorylation, hyperactivation and acrosome reaction in in vitro capacitated hamster spermatozoa. *Mol Reprod Dev* **63**: 119-30.

Jones, R. C. and R. N. Murdoch (1996). Regulation of the motility and metabolism of spermatozoa for storage in the epididymis of eutherian and marsupial mammals. *Reprod Fertil Dev* **8**: 553-68.

Katz, D. F., E. Z. Drobnis and J. W. Overstreet (1989). Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. *Gamete Res* **22**: 443-69.

Katz, D. F. and R. Yanagimachi (1980). Movement characteristics of hamster spermatozoa within the oviduct. *Biol Reprod* **22**: 759-64.

Kaupp, U. B., C. Dzeja, S. Frings, J. Bendig and V. Hagen (1998). Applications of caged compounds of hydrolysis-resistant analogs of cAMP and cGMP. *Methods Enzymol* **291**: 415-30.

Kaupp, U. B., J. Solzin, E. Hildebrand, J. E. Brown, A. Helbig, V. Hagen, M. Beyermann, F. Pampaloni and I. Weyand (2003). The signal flow and motor response controling chemotaxis of sea urchin sperm. *Nat Cell Biol* **5**: 109-17.

Kinukawa, M., S. Oda, Y. Shirakura, M. Okabe, J. Ohmuro, S. A. Baba, M. Nagata and F. Aoki (2006). Roles of cAMP in regulating microtubule sliding and flagellar bending in demembranated hamster spermatozoa. *FEBS Lett* **580**: 1515-20.

Kirichok, Y., B. Navarro and D. E. Clapham (2006). Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature* **439**: 737-40.

Kirkman-Brown, J. C., K. A. Sutton and H. M. Florman (2003). How to attract a sperm. *Nat Cell Biol* **5**: 93-6.

Kobori, H., S. Miyazaki and Y. Kuwabara (2000). Characterization of intracellular Ca(2+) increase in response to progesterone and cyclic nucleotides in mouse spermatozoa. *Biol Reprod* **63**: 113-20.

Kos, C. H., A. C. Karaplis, J. B. Peng, M. A. Hediger, D. Goltzman, K. S. Mohammad, T. A. Guise and M. R. Pollak (2003). The calcium-sensing receptor is required for normal calcium homeostasis independent of parathyroid hormone. *J Clin Invest* **111**: 1021-8.

Lindemann, C. B., J. S. Goltz and K. S. Kanous (1987). Regulation of activation state and flagellar wave form in epididymal rat sperm: evidence for the involvement of both Ca^{2+} and cAMP. *Cell Motil Cytoskeleton* **8**: 324-32.

Lindskog, S. and A. Thorslund (1968). On the interaction of bovine cobalt carbonic anhydrase with sulfonamides. *Eur J Biochem* **3**: 453-60.

Livera, G., F. Xie, M. A. Garcia, B. Jaiswal, J. Chen, E. Law, D. R. Storm and M. Conti (2005). Inactivation of the mouse adenylyl cyclase 3 gene disrupts male fertility and spermatozoon function. *Mol Endocrinol* **19**: 1277-90.

Lobley, A., V. Pierron, L. Reynolds, L. Allen and D. Michalovich (2003). Identification of human and mouse CatSper3 and CatSper4 genes: Characterisation of a common interaction domain and evidence for expression in testis. *Reprod Biol Endocrinol* **1**: 53.

Marquez, B. and S. S. Suarez (2004). Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod* **70**: 1626-33.

Mezquita, P., C. Mezquita and J. Mezquita (1999). Novel transcripts of carbonic anhydrase II in mouse and human testis. *Mol Hum Reprod* **5**: 199-205.

Moore, H. D. and D. A. Taggart (1995). Sperm pairing in the opossum increases the efficiency of sperm movement in a viscous environment. *Biol Reprod* **52**: 947-53.

Nallella, K. P., R. K. Sharma, N. Aziz and A. Agarwal (2006). Significance of sperm characteristics in the evaluation of male infertility. *Fertil Steril* **85**: 629-34.

Nikpoor, P., S. J. Mowla, M. Movahedin, S. A. Ziaee and T. Tiraihi (2004). CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility. *Hum Reprod* **19**: 124-8.

Nolan, M. A., D. F. Babcock, G. Wennemuth, W. Brown, K. A. Burton and G. S. McKnight (2004). Sperm-specific protein kinase A catalytic subunit Calpha2 orchestrates cAMP signaling for male fertility. *Proc Natl Acad Sci U S A* **101**: 13483-8.

Okamura, N., Y. Tajima, A. Soejima, H. Masuda and Y. Sugita (1985). Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J Biol Chem* **260**: 9699-705.

Okamura, N., Y. Tajima and Y. Sugita (1988). Decrease in bicarbonate transport activities during epididymal maturation of porcine sperm. *Biochem Biophys Res Commun* **157**: 1280-7.

Oko, R. and Y. Clermont (1998). Spermiogenesis. Encyclopedia of Reproduction. E. Knobil and J. D. Neill. San Diego, Academic Press. **4:** 602-609.

Parkkila, S., K. Kaunisto, S. Kellokumpu and H. Rajaniemi (1991). A high activity carbonic anhydrase isoenzyme (CA II) is present in mammalian spermatozoa. *Histochemistry* **95**: 477-82.

Parrish, J. J., J. L. Susko-Parrish and N. L. First (1989). Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. *Biol Reprod* **41**: 683-99.

Pastor-Soler, N., V. Beaulieu, T. N. Litvin, N. Da Silva, Y. Chen, D. Brown, J. Buck, L. R. Levin and S. Breton (2003). Bicarbonate-regulated Adenylyl Cyclase (sAC) Is a Sensor That Regulates pH-dependent V-ATPase Recycling. *J Biol Chem* **278**: 49523-9.

Paupard, M. C., J. MacLeod, W. Wasco and G. A. Orr (1988). Major 56,000-dalton, soluble phosphoprotein present in bovine sperm is the regulatory subunit of a type II cAMP-dependent protein kinase. *J Cell Biochem* **37**: 161-75.

Pi, M., P. Faber, G. Ekema, P. D. Jackson, A. Ting, N. Wang, M. Fontilla-Poole, R. W. Mays, K. R. Brunden, J. J. Harrington and L. D. Quarles (2005). Identification of a novel extracellular cation-sensing G-protein-coupled receptor. *J Biol Chem* **280**: 40201-9.

Pinto-Correia, C. (1997). The ovary of Eve: sperm and egg preformation. Chicago, The University of Chicago Press.

Quill, T. A., D. Ren, D. E. Clapham and D. L. Garbers (2001). A voltage-gated ion channel expressed specifically in spermatozoa. *Proc Natl Acad Sci U S A* **98**: 12527-31.

Quill, T. A., S. A. Sugden, K. L. Rossi, L. K. Doolittle, R. E. Hammer and D. L. Garbers (2003). Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc Natl Acad Sci U S A* **100**: 14869-74.

Quill, T. A., D. Wang and D. L. Garbers (2006). Insights into sperm cell motility signaling through sNHE and the CatSpers. *Mol Cell Endocrinol.*

Ren, D., B. Navarro, G. Perez, A. C. Jackson, S. Hsu, Q. Shi, J. L. Tilly and D. E. Clapham (2001). A sperm ion channel required for sperm motility and male fertility. *Nature* **413**: 603-9.

Roughton, F. J. W. (1941). The kinetics and rapid thermochemistry of carbonic acid. *J. Am. Chem. Soc.* **63**: 2930-4.

Sandberg, M., E. Butt, C. Nolte, L. Fischer, M. Halbrugge, J. Beltman, T. Jahnsen, H. G. Genieser, B. Jastorff and U. Walter (1991). Characterization of Sp-5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole- 3',5'-monophosphorothioate (Sp-5,6-DCl-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem J* **279 (Pt 2)**: 521-7.

Schultz, C., M. Vajanaphanich, H. G. Genieser, B. Jastorff, K. E. Barrett and R. Y. Tsien (1994). Membrane-permeant derivatives of cyclic AMP optimized for high potency, prolonged activity, or rapid reversibility. *Mol Pharmacol* **46**: 702-8.

Schultz, C., M. Vajanaphanich, A. T. Harootunian, P. J. Sammak, K. E. Barrett and R. Y. Tsien (1993). Acetoxymethyl esters of phosphates, enhancement of the permeability and potency of cAMP. *J Biol Chem* **268**: 6316-22.

Serrano, C. J., C. L. Trevino, R. Felix and A. Darszon (1999). Voltage-dependent Ca(2+) channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm. *FEBS Lett* **462**: 171-6.

Shulman, A., R. Hauser, S. Lipitz, Y. Frenkel, J. Dor, D. Bider, S. Mashiach, L. Yogev and H. Yavetz (1998). Sperm motility is a major determinant of pregnancy outcome following intrauterine insemination. *J Assist Reprod Genet* **15**: 381-5.

Si, Y. and M. Okuno (1995). Activation of mammalian sperm motility by regulation of microtubule sliding via cyclic adenosine 5'-monophosphate-dependent phosphorylation. *Biol Reprod* **53**: 1081-7.

Si, Y. and M. Okuno (1999). Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. *Biol Reprod* **61**: 240-6.

Sinclair, M. L., X. Y. Wang, M. Mattia, M. Conti, J. Buck, D. J. Wolgemuth and L. R. Levin (2000). Specific expression of soluble adenylyl cyclase in male germ cells. *Mol Reprod Dev* **56**: 6-11.

Singh, J. P., D. F. Babcock and H. A. Lardy (1983). Motility activation, respiratory stimulation, and alteration of Ca^{2+} transport in bovine sperm treated with amine local anesthetics and calcium transport antagonists. *Arch Biochem Biophys* **221**: 291-303.

Smith, T. T. and R. Yanagimachi (1989). Capacitation status of hamster spermatozoa in the oviduct at various times after mating. *J Reprod Fertil* **86**: 255-61.

Smith, T. T. and R. Yanagimachi (1990). The viability of hamster spermatozoa stored in the isthmus of the oviduct: the importance of sperm-epithelium contact for sperm survival. *Biol Reprod* **42**: 450-7.

Stauss, C. R., T. J. Votta and S. S. Suarez (1995). Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biol Reprod* **53**: 1280-5.

Suarez, S. S. (1996). Hyperactivated motility in sperm. *J Androl* **17**: 331-5.

Suarez, S. S., X. B. Dai, R. P. DeMott, K. Redfern and M. A. Mirando (1992). Movement characteristics of boar sperm obtained from the oviduct or hyperactivated in vitro. *J Androl* **13**: 75-80.

Suarez, S. S., D. F. Katz and S. Meizel (1984). Changes in Motility That Accompany the Acrosome Reaction in Hyperactivated Hamster Spermatozoa. *Gamete Res* **10**: 253-265.

Suarez, S. S., D. F. Katz and J. W. Overstreet (1983). Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. *Biol Reprod* **29**: 1277-87.

Suarez, S. S., D. F. Katz, D. H. Owen, J. B. Andrew and R. L. Powell (1991). Evidence for the function of hyperactivated motility in sperm. *Biol Reprod* **44**: 375-81.

Summers, K. E. and I. R. Gibbons (1971). Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proc Natl Acad Sci U S A* **68**: 3092-6.

Sutton, K. A., M. K. Jungnickel and H. M. Florman (2003). Of fertility, cystic fibrosis and the bicarbonate ion. *Nat Cell Biol* **5**: 857-9.

Suzuki, N. and D. L. Garbers (1984). Stimulation of sperm respiration rates by speract and resact at alkaline extracellular pH. *Biol Reprod* **30**: 1167-74.

Tajima, Y. and N. Okamura (1990). The enhancing effects of anion channel blockers on sperm activation by bicarbonate. *Biochim Biophys Acta* **1034**: 326-32.

Tajima, Y., N. Okamura and Y. Sugita (1987). The activating effects of bicarbonate on sperm motility and respiration at ejaculation. *Biochim Biophys Acta* **924**: 519-29.

Tash, J. S., S. S. Kakar and A. R. Means (1984). Flagellar motility requires the cAMP-dependent phosphorylation of a heat-stable NP-40-soluble 56 kd protein, axokinin. *Cell* **38**: 551-9.

Tash, J. S. and A. R. Means (1983). Cyclic adenosine 3',5' monophosphate, calcium and protein phosphorylation in flagellar motility. *Biol Reprod* **28**: 75-104.

Thomas, J. A., R. N. Buchsbaum, A. Zimniak and E. Racker (1979). Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18**: 2210-8.

Tsien, R. Y. (1981). A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **290**: 527-8.

Vasta, V., W. K. Sonnenburg, C. Yan, S. H. Soderling, M. Shimizu-Albergine and J. A. Beavo (2005). Identification of a new variant of PDE1A calmodulin-stimulated cyclic nucleotide phosphodiesterase expressed in mouse sperm. *Biol Reprod* **73**: 598-609.

Vijayaraghavan, S., L. M. Critchlow and D. D. Hoskins (1985). Evidence for a role for cellular alkalinization in the cyclic adenosine 3',5'-monophosphate-mediated initiation of motility in bovine caput spermatozoa. *Biol Reprod* **32**: 489-500.

Visconti, P., J. Bailey, G. Moore, D. Pan, P. Olds-Clarke and G. Kopf (1995a). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121**: 1129-1137.

Visconti, P., G. Moore, J. Bailey, P. Leclerc, S. Connors, D. Pan, P. Olds-Clarke and G. Kopf (1995b). Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121**: 1139-1150.

Visconti, P. E. and G. S. Kopf (1998). Regulation of Protein Phosphorylation during Sperm Capacitation. *Biol Reprod* **59**: 1-6.

Visconti, P. E., J. P. Muschietti, M. M. Flawia and J. G. Tezon (1990). Bicarbonate dependence of cAMP accumulation induced by phorbol esters in hamster spermatozoa. *Biochim Biophys Acta* **1054**: 231-6.

Vorndran, C., A. Minta and M. Poenie (1995). New fluorescent calcium indicators designed for cytosolic retention or measuring calcium near membranes. *Biophys J* **69**: 2112-24.

Wade, M. A., S. D. Roman, R. C. Jones and R. J. Aitken (2003). Adenylyl cyclase isoforms in rat testis and spermatozoa from the cauda epididymidis. *Cell Tissue Res* **314**: 411-9.

Wang, D., S. M. King, T. A. Quill, L. K. Doolittle and D. L. Garbers (2003a). A new sperm-specific Na(+)/H(+) Exchanger required for sperm motility and fertility. *Nat Cell Biol* **5**: 1117-1122.

Wang, X. F., C. X. Zhou, Q. X. Shi, Y. Y. Yuan, M. K. Yu, L. C. Ajonuma, L. S. Ho, P. S. Lo, L. L. Tsang, Y. Liu, S. Y. Lam, L. N. Chan, W. C. Zhao, Y. W. Chung and H. C. Chan (2003b). Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm. *Nat Cell Biol* **5**: 902-6.

Wennemuth, G., D. F. Babcock and B. Hille (2003a). Calcium clearance mechanisms of mouse sperm. *J Gen Physiol* **122**: 115-128.

Wennemuth, G., D. F. Babcock and B. Hille (2003b). Calcium clearance mechanisms of mouse sperm. *J Gen Physiol* **122**: 115-128.

Wennemuth, G., A. E. Carlson, A. J. Harper and D. F. Babcock (2003c). Bicarbonate actions on flagellar and Ca^{2+} -channel responses: Initial events in sperm activation. *Development* **130**: 1317-26.

Wennemuth, G., A. E. Carlson, A. J. Harper and D. F. Babcock (2003d). Bicarbonate actions on flagellar and Ca^{2+} -channel responses: Initial events in sperm activation. *Development* **130**: 1317-26.

Wennemuth, G., R. E. Westenbroek, T. Xu, B. Hille and D. F. Babcock (2000). $Ca_V2.2$ and $Ca_V2.3$ (N- and R-type) Ca^{2+} channels in depolarization-evoked entry of Ca^{2+} into mouse sperm. *J Biol Chem* **275**: 21210-7.

Westenbroek, R. E. and D. F. Babcock (1999). Discrete regional distributions suggest diverse functional roles of calcium channel alpha1 subunits in sperm. *Dev Biol* **207**: 457-69.

Weyand, I., M. Godde, S. Frings, J. Weiner, F. Muller, W. Altenhofen, H. Hatt and U. B. Kaupp (1994). Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. *Nature* **368**: 859-63.

Wong, S. T., K. Trinh, B. Hacker, G. C. Chan, G. Lowe, A. Gaggar, Z. Xia, G. H. Gold and D. R. Storm (2000). Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. *Neuron* **27**: 487-97.

Yan, C., A. Z. Zhao, W. K. Sonnenburg and J. A. Beavo (2001). Stage and cell-specific expression of calmodulin-dependent phosphodiesterases in mouse testis. *Biol Reprod* **64**: 1746-54.

Yanagimachi, R. (1969). *In vitro* capacitation of hamster spermatozoa by follicular fluid. *J Reprod Fertil* **18**: 275-86.

Yanagimachi, R. (1970). The movement of golden hamster spermatozoa before and after capacitation. *J Reprod Fertil* **23**: 193-6.

Yanagimachi, R. (1994). Mammalian Fertilization. New York, Raven Press.

Zeng, Y., J. A. Oberdorf and H. M. Florman (1996). pH regulation in mouse sperm: Identification of Na^+-, Cl^--, and HCO_3^--dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Dev Biol* **173**: 510-20.

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2. Rosenbaum, T., L.D. Islas, **A.E. Carlson**, and S.E. Gordon. 2003. Dequalinium: A novel, high-affinity blocker of CNGA1 channels. Journal of General Physiology 121: 37-47.

3. Wennemuth, G., **A.E. Carlson**, A.J. Harper, and D.F. Babcock. 2003. Bicarbonate actions on flagellar and Ca^{2+} -channel responses: Initial events in sperm activation. Development 130: 1317-1326.

4. **Carlson, A.E.**, R.E. Westenbroek, T. Quill, D. Ren, D.E. Clapham, B. Hille, D.L. Garbers, and D.F. Babcock. 2003. CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. Proceedings of the National Academy of Science 100: 14864-14868.

5. **Carlson, A.E.**, T. Quill, R.E. Westenbroek, S.M. Schuh, B. Hille, and D.F. Babcock. 2005. Identical phenotypes of CatSper1 and CatSper2 null sperm. Journal of Biological Chemistry 280: 32238-32244.

6. Schuh, S.M., **A.E. Carlson**, G.S. McKnight, M. Conti, B. Hille, D.F. Babcock. 2006. Signaling pathways for modulation of mouse sperm motility by adenosine and catecholamine agonists. Biology of Reproduction 74: 492-500.

7. Xie, F., M.A. Garcia, **A.E. Carlson**, S.M. Schuh, B.S. Jaiswal, M. van Duin, D.F. Babcock, and M. Conti. Soluble adenylyl cyclase is indispensable for sperm function and fertilization. *Submitted to Developmental Biology.*