THE GANGLIOSIDE GM1 IN MAMMALIAN SPERM FUNCTION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2010

THE GANGLIOSIDE GM1 IN MAMMALIAN SPERM FUNCTION

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Cornell University 2010

G_{M1} is known to influence several signaling pathways in somatic cells that are also important in sperm functional maturation, such as "capacitation" and fertilization. These pathways include tyrosine phosphorylation and exocytosis of the acrosomal vesicle (AE). I report that the ganglioside G_{M1} is enriched in a stable, micron-scale sub-domain in the plasma membrane overlying the acrosome of the sperm head in several species. This membrane sub-domain is conserved across several mammalian species. The domain appears to be specifically masked by seminal vesicle fluid secretions during the process of insemination. I also report that the acrosomal vesicle membrane is enriched in G_{M1}. This enrichment may act to increase plasma membrane G_{M1} during point-fusion events between the acrosomal and plasma membranes. I investigated the impact of modulating the G_{M1} concentrations in sperm membranes. I found that exogenous G_{M1} stimulated an increase in intracellular calcium and accelerated tyrosine phosphorylation cascades in sperm through activation of the TrkA kinase pathway, by-passing the need for sterol efflux in this pathway. Additionally, I found that changing G_{M1} focal enrichments in the plasma membrane, either through the addition of exogenous G_{M1} or using the pentameric B subunit of cholera toxin to cross-link endogenous G_{M1}, induced AE. The effect of G_{M1} clustering appeared to be mediated by the sialic acid residue of G_{M1} because ceramide and asialo-G_{M1} were unable to induce such effects. G_{M1} influences many calcium dependent processes in other cell types, and I hypothesized that a similar calcium-dependent process was

active in sperm. AE was inhibited in a concentration-dependent manner by inhibiting calcium channel activity either with Ni^{2+} or store-operated calcium channels and $Ca_V2.3$ calcium channel inhibitors. Sperm lacking the $Ca_V2.3$ channel displayed impaired fertility and were unable to undergo AE in response to progesterone, zona pellucida, or G_{M1}, despite apparently normal capacitation and membrane fusion. Together, these data suggest a model in which an increase in plasma membrane fluidity during sterol efflux, together with point fusions between the plasma membrane and acrosomal membrane, allow the clustering of endogenous G_{M1}. This clustering then stimulates calcium influx through the $Ca_V2.3$ channel and signaling pathways necessary for mammalian fertilization.

BIOGRAPHICAL SKETCH

Danielle Elise Buttke was born in Milbank, South Dakota on May 7, 1982. She grew up on a dairy farm where her constant contact with animals and nature quickly grew into a deep love and curiosity for the natural environment and veterinary world. Danielle's first purchase from her savings as a child was a plastic microscope, and science has been a part of her life since. Danielle graduated valedictorian from high school with a love for science and went on to the University of South Dakota as a Mickelson-Daschle scholar. While there, she pursued concurrent majors in biology and political science with a minor in chemistry. As an undergraduate, Danielle led a 4-year research project on tallgrass prairie restoration that earned two NSF-REU research grants and developed into the Cotton Park prairie project, an active community park that has served as a pilot for additional prairie restoration projects. Danielle was an adamant environmental activist, and became a two-time recipient of the prestigious national US Department of State-sponsored Udall scholarship for juniors and seniors dedicated to the environment. These and a U.S. State Department Gilman scholarship allowed Danielle to spend a semester in Kenya where she studied the human-wildlife conflict and rangeland degradation on Maasailand. These experiences cemented her love for science and dedication to environmental stewardship, ultimately leading her to pursue a combined DVM/PhD at Cornell University. During the DVM coursework, Danielle was selected to participate in competitive externship opportunities at the United Nations Food and Agriculture Organization and the United States Department of Agriculture Center for Epidemiology and Animal Health. These and other experiences at Cornell through the International Agriculture and Rural Department and public health groups prompted Danielle to pursue a concurrent Masters in Public Health through Johns Hopkins

University in her final year of PhD studies at Cornell. Danielle has accepted a fellowship at the Centers for Disease Control and Prevention (CDC) through the Epidemiology Intelligence Service where she will study global and environmental health following the completion of the PhD requirements.

For my parents

ACKNOWLEDGMENTS

The past six years at Cornell University have been such a varied and occasionally long journey that I have many, many people and experiences to thank for helping through this educational endeavor. I would not have been able to get through this without the love and support of my wonderful family, no matter where we are geographically located at any point in time. The diversity of interests of their three daughters is a testament to what incredibly skilled and wonderful parents I have, who although they may not have had many opportunities growing up, ensured that I was fearless in formulating and reaching goals. I am truly blessed to have the family I was given.

The roommates and classmates that have come and gone while at Cornell have been very enriching. Amanda Wilding has been the best support, roommate, friend, and colleague anyone could ask for and I owe much of my mental health to her. I could not have asked for a better group of labmates to work with, and have to thank Jacque Nelson, Yoni Kim, Lauren Wu, Atsushi Asano, and those that have come before for all of the memories and hard work. Lori McPartlin has become a lifelong colleague and friend for whom I have to thank for introducing me to the most important person in my life, my incredible husband, Patrick, who deserves a great deal of credit for supporting me and putting up with me through all the ups and downs of graduate life. He makes it all worthwhile.

Most important to this thesis, I have to thank those scientists that have made me the scientist I am today. A love of science must begin at a young age, and I have had many memorable high school science teachers that deserve a great deal of credit for their often-unrecognized talent.

Dr. Karen Olmstead gave me the confidence and skills to put my curiosity to use, and I have no doubt I would not be living life to the fullest if not for her influence. Passion is a difficult thing to teach, but if it can be done, Dr. Olmstead is the most successful educator I have met. Thank you to Drs. Doug McGregor, Ynte Schukken, and Ton Schat for the encouragement, open mentorship, and conversation that ultimately put my on my current path. I have to thank my incredibly thoughtful, understanding, and personable committee members Drs. Michael Kotlikoff, Susan Suarez, and Mariana Wolfner, who have always encouraged and guided me down the best possible path given my varied and sometimes mercurial interests. Working with Dr. Alex Travis is a gift. I am an immeasurably better person and scientist for simply knowing him; every interaction with him is a teaching moment, whether it works in your favor or not. Working with him as a mentor is one of the greatest gifts of my career and intellectual life, and he will be sorely missed as a scientific advisor.

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CHAPTER 1

INTRODUCTION

Membrane organization

Lipid rafts (Fig. 1) are small, heterogeneous, highly dynamic and physiologically active microdomains assembled within the plasma membrane bilayer of mammalian cells. These membrane domains are rich in cholesterol and sphingolipids (see Simons & Vaz, 2004, for review). The shape and rigidity of cholesterol preferentially selects for the inclusion of saturated hydrocarbon chains of lipids in the surrounding membrane architecture, conferring a lipid-ordered property on the membrane that can restrict the lateral diffusibility of membrane components within a nanometer-scale raft (Fan, Sammalkorpi, & Haataja, 2009). A specific cohort of sphingolipids, cholesterol, and proteins are targeted to these membrane rafts. The targeted molecules include gangliosides, or glycosylated sphingolipids, GPI-linked proteins, calcium regulatory molecules, signaling receptors, and structural proteins.

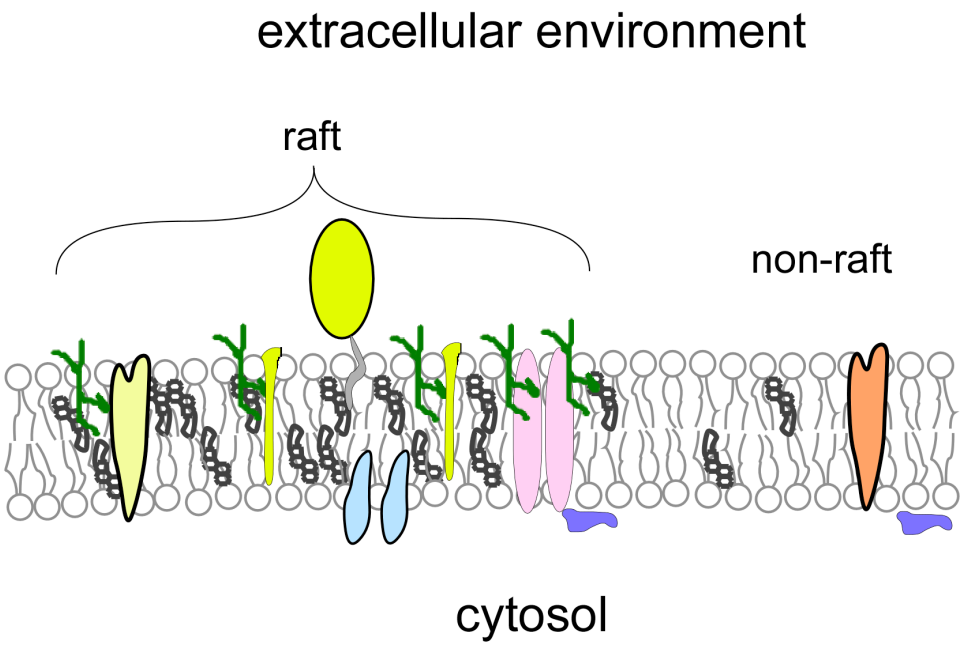
The lipid composition of rafts confers on them the biophysical property of a light buoyant density (Rajendran & Simons, 2005) and resistance to solubilization with detergents at low temperature. These properties allow the isolation of lipid rafts from biologic membranes. However, detergent-based methods can also induce non-physiological, artifactual coalescence of smaller rafts and "patching" artifacts induced by cross-linking reagents such as fixatives (Munro, 2003; Shogomori & Brown, 2003). This has provoked disagreement and controversy among lipid biologists and necessitated greater care in interpreting localization studies. Detergent resistant membranes, or DRM's, have little physiologic significance because their composition is disrupted due to reorganization artifacts induced during the processing. Therefore, DRM's must be interpreted together with alternate methods of disruption and characterization (Pike, 2006). However, density sedimentation properties of in vivo

membrane rafts have allowed a non-disruptive protocol for characterization of physiologic raft properties (Asano et al., 2009; Travis et al., 2001). Together, these studies have generated important information on the composition of membrane rafts and on their formation and maintenance (Lagerholm, Weinreb, Jacobson, & Thompson, 2005). While the inherent tendencies of some lipids to self-aggregate might energetically drive for raft organization, similar to the phenomenon seen in model membranes, protein-lipid and protein-protein interactions likely play a significant role in the formation of heterogeneous membrane sub-domains seen in vivo (see Chichili & Rodgers, 2009, for review). Cells utilize transmembrane proteins, lipid binding proteins, cytoskeletal elements and active vesicular trafficking mechanisms to maintain the lateral heterogeneity of membrane rafts. Together, these interactions contribute to the selective localization of cohorts of molecules influenced by the membrane microenvironment in which they reside.

Within a cell, rafts are usually extremely dynamic and variable in terms of composition, lifespan, and cellular function. A variety of cellular functions and signaling pathways are initiated and regulated by lipid membrane domain formation and function (see Lingwood et al., 2010, for review). These functions include viral entry, cell signaling for migration and differentiation, and more recently, fertilization (Bou Khalil et al., 2006; Nixon & Aitken, 2009; Selvaraj et al., 2006; van Gestel et al., 2005).

One way in which the lipid microenvironment can influence biological activity is through the modulation of ion channels. Membrane sterol and sphingomyelin contents have been found to influence the activity of a variety of ion channels, including

Figure 1. Schematic drawing of a membrane raft and the cohort of molecules typically found in a membrane raft (not to scale). Membrane rafts are cholesterol- and sphingolipid-rich membrane domains that are also enriched in GPI-linked proteins, transmembrane proteins, and other signaling components such as non-receptor tyrosine kinases. Many calcium transporters are also segregated to membrane rafts. Rafts can also function to separate certain signaling components or auxiliary proteins until an initiating membrane-reorganization event occurs such as sterol efflux or receptor cross-linking. The signaling complex can then be assembled outside or inside of a raft, depending on the nature of the complex and cell membrane components. Auxiliary subunits of calcium channels or synaptic fusion proteins are hypothesized to function in this manner, with raft association increasing calcium current or membrane fusion complex formation. The glycosphingolipid G_{M1} is segregated to membrane rafts and often used as a marker for localization of membrane rafts in cellular membranes. G_{M1} also contains extracellular sugar residues that can act to modulate signaling pathways, similar to glycosylated transmembrane proteins of membrane rafts.


extracellular environment
raft
non-raft
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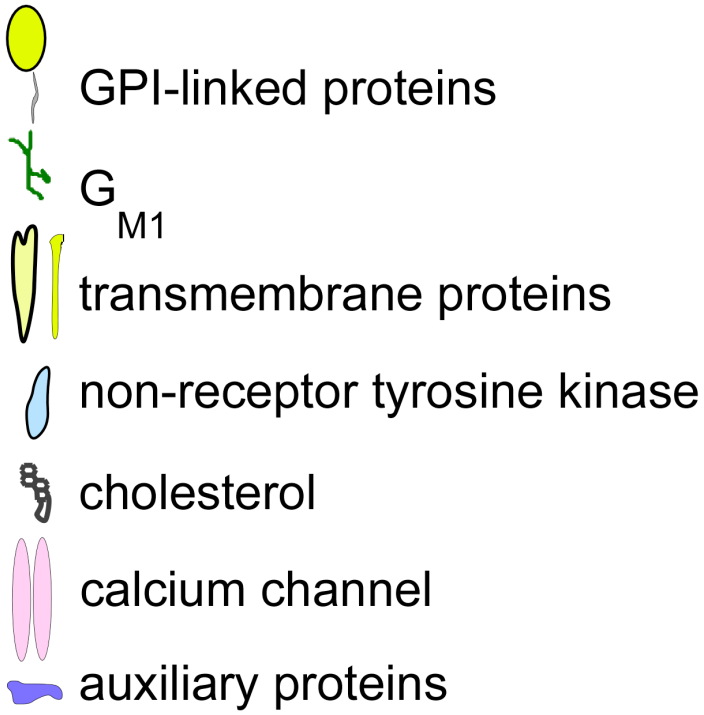
GPI-linked proteins
G
M1
transmembrane proteins
non-receptor tyrosine kinase
cholesterol
calcium channel
auxiliary proteins

nucleotide-gated channels (Brady et al., 2004), voltage-operated calcium channels (Taverna et al., 2004), and epithelial sodium channels (Shlyonsky, Mies, & Sariban-Sohraby, 2003) in a reversible manner. Certain ion channels, including inward rectifying potassium channels (Romanenko et al., 2004; Romanenko, Rothblat, & Levitan, 2004) and transient receptor potential canonical (TRPC) calcium channels (Brazer, Singh, Liu, Swaim, & Ambudkar, 2003; Torihashi, Fujimoto, Trost, & Nakayama, 2002), are assembled in membrane raft signaling complexes where their activity is influenced by the sterol composition of the raft environment.

Sphingomyelin-enriched membrane domains have been reported to determine the efficacy of calcium-triggered membrane fusion (Rogasevskaia & Coorssen, 2006), and many synaptic receptors are targeted to rafts by palmitoylation (Golub, Wacha, & Caroni, 2004; Kanaani, Diacovo, El-Husseini Ael, Bredt, & Baekkeskov, 2004), where the receptor can modulate calcium influx.

Membrane organization and functional maturation in sperm

Gametes, particularly the highly polarized mammalian spermatozoon, provide especially pronounced examples of membrane compartmentation that is important to their function. A spermatozoon released into the female reproductive tract is not able to fertilize an egg (Austin, 1951; Chang, 1951). It must instead reside within the reproductive tract of the female for a species-specific amount of time to undergo the process of capacitation (Austin, 1952; Kopf, Visconti, & Galantino-Homer, 1999; Visconti et al., 2002). Capacitation involves changes in membrane composition in which sterols, phospholipids, and inhibitory seminal plasma components are removed from the plasma membrane. These same stimuli translate to different responses

depending on the region of a spermatozoon despite the fact that a sperm is transcriptionally and translationally quiescent (see Dadoune, Siffroi, & Alfonsi, 2004, for review), leaving the sperm to rely heavily on compartmentation to streamline and regulate its signaling pathways.

Although the events of capacitation and fertilization are highly predictable and reproducible in vitro under defined conditions (Pike 2006), the molecular underpinnings of these signaling pathways are not yet fully elucidated. It is thought that membrane organization and the compartmentalization of signaling pathways to different plasma membrane regions equip a spermatozoon to respond to the same extracellular stimuli in different ways (Bou Khalil et al., 2006; Selvaraj et al., 2006; van Gestel et al., 2005). Membrane rafts may help to accomplish this exquisite regulation by scaffolding or sequestering pre-assembled signaling components in these domains. Indeed, an increase in oocyte-binding ability has been reported for isolated, detergent-resistant membranes from capacitated sperm (Bou Khalil et al., 2006). Additionally, membrane-association of machinery needed for fertilization, such as secretion molecule Rab3A and putative egg-interaction molecules, increases with cholesterol depletion during sterol efflux from sperm (Belmonte et al., 2005; Bou Khalil et al., 2006; Rogasevskaia & Coorssen, 2006). This can allow rapid, precise responses by sperm to stimuli in a localized manner.

Alternatively, the segregation of signaling molecules by membrane components can play an important role in the regulation of cellular responsiveness. Separating molecules of multi-component signaling pathways can prevent premature responses, such as inappropriate vesicular release, which would be devastating to these cells, which has only a single secretory vesicle to release. Changes in membrane

architecture, such as sterol efflux, may then allow association of components in these signaling complexes, such as those for membrane fusion or for calcium signaling, only when the sperm are in close proximity with the egg (van Gestel et al., 2005). In this way, membrane rafts can play a crucial role in both sperm quiescence as well as activity.

In support of this hypothesis, distinct sub-domains within the sperm have been described both in fixed cells (Friend & Fawcett, 1974; Friend, 1982) and in live, motile sperm (Selvaraj et al., 2006). For example, the plasma membrane overlying the acrosome is enriched in sterols, G_{M1}, and the protein caveolin-1 (Selvaraj et al., 2006; Travis et al., 2001). When sperm are released from the epididymis, the plasma membrane overlying their acrosome is rich in cholesterol and desmosterol (Bleau & VandenHeuvel, 1974; Elias, Goerke, Friend, & Brown, 1978; Legault, Bouthillier, Bleau, Chapdelaine, & Roberts, 1979). This enrichment promotes the efflux of sterols from the sperm plasma membrane during sperm transit through the reproductive tract. This can dramatically change the fluidity properties of the plasma membrane domain overlying the acrosome (Friend, 1989; van Gestel et al., 2005). Changes in lipid diffusibility within this sub-domain suggest that sterol efflux results in an increase in membrane fluidity, allowing the formation and/or diffusion of smaller raft units within the larger sub-domain (Lin & Kan, 1996; Smith, McKinnon-Thompson, & Wolf, 1998; van Gestel et al., 2005; Wolf, 1995; Wolfe, James, Mackie, Ladha, & Jones, 1998) or potentially disassembling membrane signaling complexes (Bou Khalil et al., 2006; Cross, 2004; Shadan, James, Howes, & Jones, 2004; see Tanphaichitr et al., 2007, for review). Sperm that fail to shed some of this cholesterol and increase the membrane fluidity are unable to fertilize an egg (Buffone et al., 2006; Buffone, Verstraeten, Calamera, & Doncel, 2009).

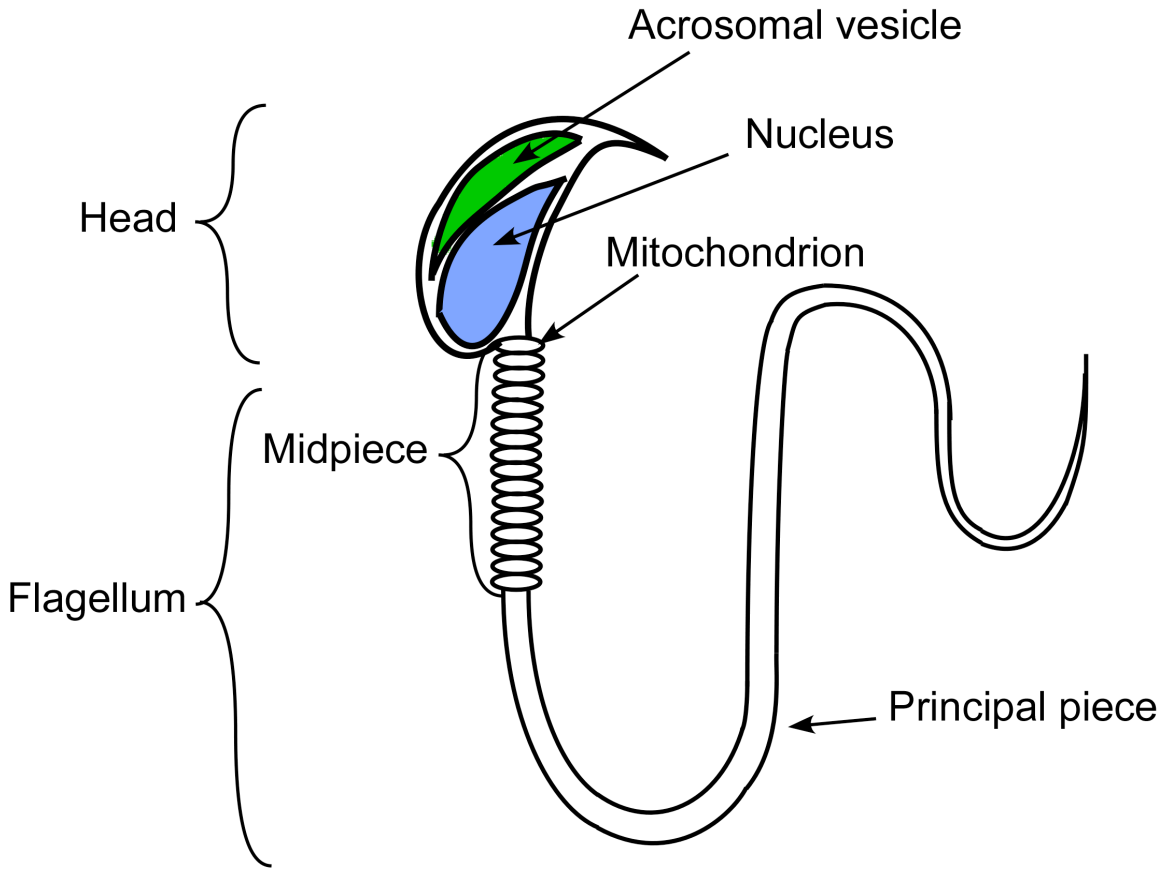

Figure 2. A schematic of a murine sperm depicting the regions and major organelles.

While the events that transpire during this transit and maturation of sperm are well documented (see Visconti et al., 2002, for review), they are less well understood. These events, mediated by separate pathways, are: the acquisition of an asymmetric flagellar beat pattern ("hyperactivation"), and the phosphorylation of a subset of tyrosine residues that change the functional responsiveness of the sperm. The signaling pathway leading to protein tyrosine phosphorylation is not yet fully defined, but involves activation of a soluble adenylate cylase by calcium and bicarbonate resulting in cyclic AMP production and activation of the serine/threonine kinase PKA (see Visconti et al., 2002, for review). Other kinase activity is necessary, as shown by the ability of tyrosine kinase inhibitors to prevent capacitation and acrosomal exocytosis, including protein kinase C (PKC) (Jaiswal, Cohen-Dayag, Tur-Kaspa, & Eisenbach, 1998; Foresta, Rossato, & DI virgilio, 1995; Spungin & Breitbart, 1996).

Concomitant with the changes in kinase activity during capacitation are changes in ion transport in sperm. Upon the efflux of sterols, bicarbonate and calcium enter the sperm (see (Abou-haila & Tulsiani, 2009), for review). In concert with these changes, hyperpolarization of the membrane occurs during capacitation, from -35 to -45 mV in a non-capacitated state to -80 mV (Arnoult et al., 1999; Munoz-Garay et al., 2001). This hyperpolarization occurs in part to activity of the sperm-specific Slo3 potassium channel, although a Na^+/HCO_3^- cotransporter has been localized in sperm and may also be involved, accounting for the change in intracellular bicarbonate levels (Demarco et al., 2003; Santi et al., 2010). It is likely that sterol efflux-mediated Slo3 activity then facilitates activation of the depolarization-sensitive proton pump Hv1, which alkalinizes the sperm, a requirement for successful response to the oocyte (Lishko, Botchkina, Fedorenko, & Kirichok, 2010).

The ultimate consequence of these changes is the functional responsiveness of the sperm to the oocyte. This responsiveness allows the regulated exocytosis of the sperm's single secretory vesicle (the acrosome), in response to contact with the cumulus-oocyte complex and associated glycoproteins, steroid hormones, and structures of the egg (see Bhandari, Bansal, Talwar, & Gupta, 2010, for review). Although a specific, single, and essential sperm receptor for the zona pellucida (ZP) has not yet been described in mammals, both progesterone and ZP glycoproteins are known to induce release of the acrosomal contents (Yanagimachi, 1988; Sutton et al., 2005). The effects of these agonists differ in the initial rise in calcium, which is a single, transient spike for ZP and potentially oscillatory waves for progesterone (Bailey & Storey, 1994; Florman, 1994; O'Toole, Roldan, & Fraser, 1996b). After this initial, voltage-operated calcium entry, phospholipase C (PLC) is activated in both pathways to produce inositol triphosphate (IP_3) and diacylgylerol (DAG) (Fukami et al., 2001; Rice, Parrington, Jones, & Swann, 2000). Intracellular IP_3 then activates IP_3R receptors on the acrosomal membrane to release calcium stores from the acrosome (Herrick et al., 2005; Stamboulian et al., 2005; Sutton et al., 2004). This increase in intracellular calcium mediates calcium influx from a store-operated plasma membrane calcium channel, likely a TRPC channel, although species differences in channel sub-types exist (Jungnickel, Marrero, Birnbaumer, Lemos, & Florman, 2001). This sustained influx of calcium results in SNARE mediated fusion of the plasma membrane and outer acrosomal membrane (De Blas, Roggero, Tomes, & Mayorga, 2005).

Despite the downstream similarities of progesterone and ZP-induced acrosomal excoytosis (AE), progesterone and ZP stimulation of sperm differ in several ways.

Progesterone appears to initiate signaling in part through $GABA_A$ receptors on the sperm (Bhandari et al., 2010; Murase & Roldan, 1996). This differs from the $GABA_A$ insensitive but G-protein dependent ZP-induced membrane fusion and exocytosis (Tesarik, Carreras, & Mendoza, 1993). These differential mechanisms have been suggested to work synergistically, with the lower levels of progesterone found within the oviduct acting to 'prime' the sperm in the event progesterone levels are not sufficient to induce exocytosis on its own. This initial priming with pico- to micromolar concentrations of progesterone may transiently depolarize and further hyperpolarize the sperm membrane, allowing full activation of voltage-operated calcium channels that open upon G-protein coupled responses of the sperm to contact with the ZP (Patrat, Serres, & Jouannet, 2002). Both agonists appear to induce fusion initially via transient, punctate fusion events followed by full-scale exocytosis for release of the enzymes within the acrosome that may aid in penetration of the cumulus matrix and zona pellucida (Kim & Gerton, 2003).

Raft-associated G_{M1} in biologic membranes

Not surprisingly, lipid order alone cannot account for the diverse activities ascribed to membrane rafts and changes in ion fluxes. Cells assemble raft complexes with a variety of structurally distinct and biologically active molecules, including gangliosides. Of these gylcosphingolipids, the most work has been done on the ganglioside G_{M1}. This is largely due to the fact that the B subunit of cholera toxin (CTB) binds to G_{M1} with a remarkable specificity and sensitivity (Cuatrecasas, 1973; Fishman, Pacuszka, & Orlandi, 1993; Lauer, Goldstein, Nolan, & Nolan, 2002),

making it useful for a variety of experimental techniques. Additionally, glycosphingolipids such as G_{M1} can have dramatic influence on the architecture and functionality of biologic membranes (see Westerlund & Slotte, 2009, for review).

G_{M1} is synthesized in the Golgi apparatus and subsequently transferred to the outer leaflet of the plasma membrane (Zeller & Marchase, 1992). The sialic acid residue of G_{M1} projects to the extracellular environment, where it can interact with exogenous molecules, while its hydrophobic ceramide tail remains intercalated into the membrane, where it can interact with intra-membrane and intracellular constituents (Fig. 2) (Svennerholm, 1994; Wiegandt, 1995). These chemical properties make for a very dynamic molecule in terms of the broad array of potential membrane interactions witnessed for G_{M1}. G_{M1} has been shown to segregate preferentially to distinct membrane sub-domains where even a single G_{M1} molecule can exert local effects on the membrane (Goins, Masserini, Barisas, & Freire, 1986; Roy & Mukhopadhyay, 2002). G_{M1} is highly enriched in membrane rafts of neuronal tissues, where it plays a central role in neuronal plasticity, synaptogenesis, response to ischemic and excitatory injury, as well as ionic conductance (Fadda, Negro, Facci, & Skaper, 1993; Ledeen & Wu, 2002; Pedata, Giovannelli, & Pepeu, 1984; Toffano et al., 1983; Wu, Lu, & Ledeen, 1995; Wu et al., 1998).

Studies in somatic cells have shown G_{M1} to be a biologically active molecule, important in transducing extracellular stimuli into intracellular signals (Hadjiconstantinou & Neff, 1998). At the cell surface, G_{M1}'s extracellular sugars can act as receptors for cholera toxin and polyoma and SV40 viruses (Tsai et al., 2003). It can act as a co-receptor for FGF2 (Rusnati et al., 2002), initiating fusion and migration (Chen et al., 2003), and a form of G_{M3} can mediate sperm-egg adhesion in trout

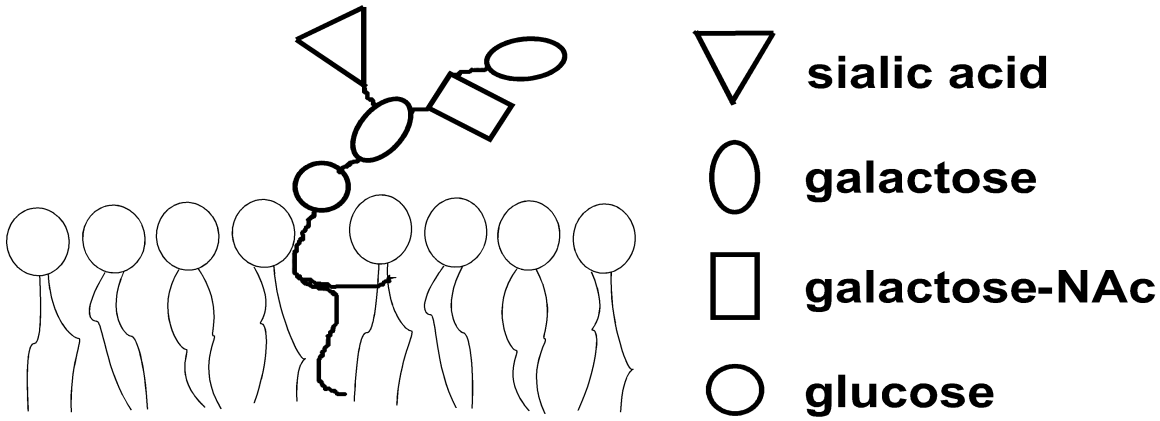

Figure 3. Schematic drawing of the ganglioside G_{M1} (bold face) in the outer leaflet of a phopholipid bilayer. The sugar residues are hydrophilic and exposed to the extracellular environment while the ceramide tail of G_{M1} is hydrophobic and intercalates into the phospholipid and cholesterol-enriched face of a membrane bilayer.

(Yu et al., 2002). The overall amphipathic nature can modulate the open probability of ion channels or alter plasma membrane calcium ATPase (PMCA) (Zhang, Zhao, Duan, Yang, & Zhang, 2005; Zhao, Fan, Yang, & Zhang, 2004), sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) (Xie, Wu, Lu, Rohowsky-Kochan, & Ledeen, 2004), or sodium/calcium exchanger conformation (Xie, Wu, Lu, & Ledeen, 2002; Zhao et al., 2004). The hydrophobic ceramide tail of G_{M1} can serve as a potent intracellular signaling molecule either alone or in concert with sialyl binding partners, such as calmodulin (CaM), and calcium/calmodulin-dependent protein kinase II (CaMKII) to activate tyrosine phosphorylation cascades (Chan, 1989; Duchemin, Neff, & Hadjiconstantinou, 1998; Fukunaga, Miyamoto, & Soderling, 1990; Higashi, Omori, & Yamagata, 1992; Hilbush & Levine, 1991). Sometimes, these properties can work synergistically. G_{M1} clustering through its extracellular sugars can alter the conformation and activity of the palmitoylated, raft-targeted plasma-membrane calcium ATPase (PMCA) (either alone or in a CaM dependent manner), which is essential for calcium homeostasis in most cell types, including sperm (Kanaani et al., 2004; Pang, Zhu, Wu, & Chen, 2005; Wennemuth, Babcock, & Hille, 2003). The effect of G_{M1} on calcium channels and calcium influx has been shown to lead to a variety of functional responses, including cell-cell adhesion, neuronal synapse formation (Silva, Felicio, Nasello, Vital, & Frussa-Filho, 1996), osteoclast differentiation (Fukumoto et al., 2006), and hepatocyte synchronization (Brodsky et al., 2003; see Ledeen & Wu, 2002, for review).

Though the biological effects of G_{M1} vary depending upon cell type, many of its ascribed functions involve calcium as an integral part of signal transduction. The exact molecular underpinnings of this relationship are not fully understood. Crosslinking of G_{M1} molecules by either the pentameric B subunit of cholera toxin

(Fang, Xie, Ledeen, & Wu, 2002) or the pentameric anti-G_{M1} IgM antibody (Quattrini et al., 2001) can stimulate calcium influx through a voltage operated calcium channel. Fang et al. characterized this apparent voltage-operated calcium channel to be gated by the ganglioside G_{M1} and permeable to calcium and barium but not manganese (2002). This current is sensitive to blockade by nickel, cadmium, and lanthanum, qualities shared by T-type, or Ca_V3, and R-type, or $Ca_V2.3$, calcium channels (Ertel, 2004; Fang et al., 2007). The activity of this channel upon cholera toxin B (CTB) binding was responsible for calcium influx and neuritogenesis observed with gangliosides in neurons. Other groups have identified an apparent L-type calcium current that is stimulated by gangliosides (Carlson, Masco, Brooker, & Spiegel, 1994) and inhibited by serum from patients with anti-ganglioside antibiodies (Buchwald et al., 2007; Nakatani et al., 2009), but these studies relied upon calcium channel inhibitors that can non-specifically affect other alpha subunits such as Ca_V 2.3 (Ertel, 2004). Similarly, G_{M1}-induced currents in Neuro2A cells were identified as T-type currents because of their sensitivity to amiloride, which was used at concentrations that will also inhibit the Ca_V 2.3 channel (Ertel, 2004).

G_{M1} in sperm

In sperm, G_{M1} is highly enriched in and segregated to the plasma membrane overlying the acrosome as well as the outer acrosomal membrane in sperm of mouse, bull, horse, and human (Buttke, Nelson, Schlegel, Hunnicutt, & Travis, 2006; Selvaraj et al., 2006). This is in stark contrast to somatic cell membrane organization, where very little G_{M1} is found in vesicular membranes (Zeller & Marchase, 1992). G_{M1} of the sperm plasma membrane is bound specifically by the seminal plasma component SVS2 upon ejaculation when sperm mix with seminal plasma (Kawano, Yoshida, Iwamoto, & Yoshida, 2008). The binding of SVS2 has a de-capacitation effect on

sperm, preventing tyrosine phosphorylation cascades, acrosomal exocytosis, and the fertilization of oocytes in vitro (Kawano & Yoshida, 2007; Kawano et al., 2008). However, as sperm transit through the female reproductive tract, SVS2 is progressively lost from the sperm plasma membrane. At the same time, cholesterol efflux results in an increase in membrane fluidity, and G_{M1} is found to diffuse more freely within the plasma membrane overlying the acrosome (Cross, 2004; Jones et al., 2010; Selvaraj et al., 2007; Shadan, James, Howes, & Jones, 2004). It is plausible then that SVS2 may exert its decapacitation effects by sequestering G_{M1} and preventing its movement around or interaction with target signaling molecules important for sperm responsiveness, such as a calcium channel, similar to the mechanisms seen in somatic cells and described above.

Calcium flux in sperm

Calcium homeostasis and flux in sperm is a dynamic, complex, and highly regulated messaging system that is poorly understood. In the flagellum, increased calcium is important to sperm motility, specifically evident in homozygous null mouse models lacking the calcium channel CatSper or calcium transporter PMCA4, which display aberrant motility (Carlson et al., 2003; Quill et al., 2003; Schuh et al., 2004). Downstream, calcium/Calmodulin (CaM) specifically stimulates hyperactivation through CaMKII (Ignotz & Suarez, 2005; see Suarez & Ho, 2003, for review). In the sperm head, calcium influx leading to AE in response to solubilized ZP glycoproteins or progesterone is likely more complex (Abou-haila & Tulsiani, 2009; H. M. Florman, Lemos, Arnoult, Kazam, & O'Toole, 1998; O'Toole, Arnoult, Darszon, Steinhardt, & Florman, 2000). The increase in intracellular calcium in the sperm head occurs in discrete steps, including an elevation in resting calcium levels during capacitation

(Florman, 1994) that is dependent upon the pH-dependent CatSper channel (Olson, Suarez, & Fauci, 2010; Xia & Ren, 2009), and a second, unidentified but likely voltage-operated calcium channel allowing transient calcium elevation upon ZP3 binding that coincides with activation of PLCδ4 and production of IP_3 for the third and store-operated Ca^{2+} entry (Arnoult, Zeng, & Florman, 1996).

The transient rise in calcium that initiates AE has been hypothesized to be facilitated by a voltage dependent calcium channel or voltage operated calcium channel (VOCC) that is activated by membrane depolarization and appears to be regulated by phosphorylation with PKC (Breitbart et al., 1997; Spungin & Breitbart, 1996). Low levels of progesterone can transiently depolarize the sperm plasma membrane, but this is followed quickly by an additional hyperpolarization (Patrat et al., 2002). Mechanisms for this non-genomic progesterone effect are unknown. Mice lacking the Slo3 potassium channel are infertile due to their inability to undergo membrane hyperpolarization during capacitation (Santi et al., 2010). This hyperpolarization appears necessary to transition voltage-operated calcium channels from an inactive state to a closed but voltage-operated state (Rossato, Di Virgilio, Rizzuto, Galeazzi, & Foresta, 2001). Inhibition of the sperm sodium-potassium ATPase using pharmacological inhibitors can invoke membrane depolarization, but a physiological mechanism for its inhibition leading to acrosomal exocytosis is not known (Thundathil, Anzar, & Buhr, 2006).

Several candidates for the ZP-induced membrane depolarization exist. Efflux of Cl^- stimulated by $GABA_A$ receptors or Glycine receptors could be responsible for the ZP induced membrane depolarization (Garcia & Meizel, 1999; Melendrez & Meizel, 1995). Alternatively, G-protein dependent muscarinic receptors have been

hypothesized to be essential for sperm penetration of the zona (Florman & Storey, 1981) as well as zona-induced acrosomal exocytosis (Brandelli, Miranda, & Tezon, 1996; Bray, Son, Kumar, Harris, & Meizel, 2002; Ward, Storey, & Kopf, 1994) because inhibitors of muscarinic receptors can inhibit zona penetration and acrosomal exocytosis. It has been hypothesized that G_i-proteins must be released from muscarinic receptors, allowing their activation and a spike in intracellular calcium (Bray et al., 2002). The spike in calcium produced by muscarinic receptor activity could contribute to the membrane depolarization required for VOCC activation.

The need for G-protein activity in acrosomal exocytosis can be overcome by artificially raising the intracellular pH of sperm (Florman, Tombes, First, & Babcock, 1989; Florman, Corron, Kim, & Babcock, 1992). Physiologically, the increase in intracellular pH appears dependent upon the activity of the proton pump Hv1, which is activated by membrane depolarization likely induced by Slo3 activity (Lishko et al., 2010). Although G-protein interactions with Hv1 have not been described, G-protein-Hv1 interaction would provide an attractive mechanism and straightforward explanation for G-protein dependence of alkalinization and capacitation regulation. The increase in sperm pH is thought to be necessary for the membrane depolarization and activation of VOCC (Fraire-Zamora & Gonzalez-Martinez, 2004). In agreement with this hypothesis, the pH sensitive and ZP-responsive CatSper channel could allow propagation of calcium influx into the head from its activity in the flagellum, allowing the depolarization required for VOCC activation (Olson, Suarez, & Fauci, 2010). This VOCC activity results in a transient influx of calcium that ceases within 50 milliseconds of opening (Jungnickel et al., 2001). In summary, this model links initial sterol-efflux activation of Slo3 to activation of Hv1, which allows CatSper activity and responsiveness for the VOCC calcium entry and AE.

As mentioned above, the transient, VOCC-mediated rise in calcium during initial contact with the zona pellucida is followed closely by a final sustained elevation in intracellular calcium (Arnoult et al., 1999). This sustained influx has been characterized and includes 1) activation of the acrosomal IP_3R by phospholipase C δ4 (PLCδ4) generated IP_3, which depletes the acrosomal calcium store and 2) a plasma membrane store operated TRPC channel, definitively TRPC2 in the mouse, that interacts with a CaM-binding protein to open in response to acrosomal calcium depletion ((Jungnickel et al., 2001; Rice et al., 2000; see Darszon et al., 2005, for review). These changes in intracellular calcium then allow SNARE-mediated fusion of the plasma membrane with the acrosomal membrane in a Rab3A dependent manner (Michaut, Tomes, De Blas, Yunes, & Mayorga, 2000; Michaut et al., 2001; Yunes et al., 2002).

Despite recognition of the chronology and pattern of calcium influx in the sperm head, the nature and regulation of the channels involved in AE remain unclear, specifically the channel responsible for the initial rise in calcium during acrosomal exocytosis. This is due to many factors, such as the high redundancy of pathways in sperm, which converge downstream to elicit similar functions. Pharmacologic inhibitors of calcium channel targets are notoriously non-specific and vary in their effectiveness from one cell-type to the next (Ertel, 2004). Additionally, ablation of certain calcium channel genes can alter expression of other calcium-associated genes in a compensatory manner (Carlson et al., 2005). Further confounding analysis, many types of channels with unknown function have been identified in sperm (see Darszon et al., 2005, for review).

Although several subtypes of voltage-operated calcium channels have been described in sperm, their in vivo activity is unknown. Initial studies on AE suggested the involvement of an L-type channel based on sensitivity to dihydropyridines (DHP) (Florman, 1994), but the concentration and interaction of DHP was later found to affect other channels. Patch-clamp recordings from developing male germ cells detected low voltage activated (LVA), T-type currents, which was in agreement with pharmacological characterization (Arnoult, Villaz, & Florman, 1998). However, mice lacking key T-type calcium channels Cav 3.1 and 3.2 are also fertile, and the current remaining in these sperm differs from somatic cell T-type currents (Stamboulian, De Waard, Villaz, & Arnoult, 2002). Pharmacological studies of T-type channels in mature sperm often require non-specific concentrations to elicit effects, affecting sodium and non-target calcium channels and leading some to the conclusion that T-type channels are not involved in acrosomal exocytosis (Bonaccorsi, Forti, & Baldi, 2001). Additionally, work characterizing depolarization-induced calcium influx to simulate ZP-induced calcium rise in mature mouse sperm proved to be insensitive to blockers of L-, P/Q-, and T-type channels (Wennemuth, Westenbroek, Xu, Hille, & Babcock, 2000). One common thread throughout reported studies on mature sperm is the inhibition of AE by nickel and cadmium ions and the intermediate membrane potentials at which voltage operation occurs, which together support the involvement of a high voltage activated (HVA), R-type current (Westenbroek & Babcock, 1999).

The voltage-operated $Ca_V2.3$ channel

The $Ca_V2.3$ alpha subunit of voltage gated calcium channels is thought to be responsible for the residual, or R-type current, so named for its resistance to traditional inhibitors of L-, N- and P/Q-type calcium channel inhibitors (Fang et al., 2007). This subunit is expressed in neurons, heart tissue, testis and pituitary, with physiologic

roles elucidated in neurotransmitter exocytosis and repetitive firing. Mice lacking the $Ca_V2.3$ gene retain a portion of the R-type current, suggesting that the R-type current actually reflects a heterogenous mixture of channel subunits (Wilson et al., 2000). These mutant mice survive to adulthood and are fertile; however, they exhibit altered pain responses, aberrant eleptogenesis, and sperm abnormaltities including abnormal flagellar waveform and decreased responsiveness to mannosylated-BSA, an agent that induces calcium influx into sperm of some species (Matsuda, Saegusa, Zong, Noda, & Tanabe, 2001; Sakata, Saegusa, Zong, Osanai, Murakoshi, Shimizu, Noda, Aso, & Tanabe, 2001; Sakata et al., 2002; Wilson et al., 2000).

The $Ca_V2.3$ subunit shares pharmacologic and gating properties with T-type calcium channels, making it difficult to differentiate between the two (Bourinet et al., 1996). Like T-type channels, the $Ca_V2.3$ channel containing the α1E subunit is activated and inactivated at negative membrane potentials, sensitive to Ni^{2+}, and permeable to Ba^{2+} and Ca^{2+} (see (Dolphin, 2006), for review; (Bourinet et al., 1996). The $Ca_V2.3$ channel differs from the T-type channels in its higher sensitivity to Cd^{2+}, larger conductance, slower inactivation, and lower sensitivity to amiloride (Ertel, 2004). Like L-type channels, the $Ca_V2.3$ channel can be transiently activated at low to moderate voltages, but is inactivated faster than L- or N-type channels (Miljanich & Ramachandran, 1995).

These properties, however, differ significantly between cell type examined, and are highly influenced by secondary binding and signaling molecules that vary between cell types and among cell-signaling stages. Activation of current by exogenously expressed $Ca_V2.3$ subunits has been reported to vary from -30 to -10 mV using rat subunits in *Xenopus* oocytes to 0 to +5 mV using the human α1E subunit expressed in

HEK 293 cells (Ertel, 2004). These differences may be accounted for by the potential differences in membrane architecture between cells types, as well as differences in protein kinase C (PKC) and G-protein activity (Bannister, Melliti, & Adams, 2004; Klockner et al., 2004; Yassin, Zong, & Tanabe, 1996). Heat shock protein 70 (Hsp70) interacts with the II-III intracellular loop of the α1E subunit to allow binding of PKC and allow subsequent phosphorylation of the channel (Krieger et al., 2006). Phosphorylation of the α1E subunit by PKC and PKA changes its gating properties and can dramatically increase calcium current through this channel (Hell, Yokoyama, Breeze, Chavkin, & Catterall, 1995; Kamatchi et al., 2003). PKC activity is necessary for $Ca_V2.3$ currents in brain cells in vitro and in vivo (Tai, Kuzmiski, & MacVicar, 2006). G-protein coupled muscarinic receptors such as M1, M3, and M5 have been found to interact with the α1E subunit upon activation and stimulate open-gating properties of the channel (Bannister et al., 2004). α1E binding partners have not been studied in sperm.

The α1E ($Ca_V2.3$) subunit is especially interesting for sperm cell biology. Its limited tissue distribution (restricted to brain, heart, and testis), combined with the relative abundance of α1E message in testis, makes the $Ca_V2.3$ an intriguing candidate for depolarization-induced calcium entry (Westenbroek & Babcock, 1999). The mRNA for the α1E subunit is the most abundant of all calcium channel subunit mRNA in developing spermatocytes, suggesting a potential role for this protein in mature sperm. Additionally, two common signaling pathways are necessary for both zona pellucida-induced voltage-dependant channel activation leading to exocytosis, as well as voltage-operation of $Ca_V2.3$ channels. G_i-protein activity is necessary for open-gating of the α1E subunit (Toro-Castillo, Thapliyal, Gonzalez-Ochoa, Adams, & Meza, 2007) and zona-induced calcium entry (Ward, Storey, & Kopf, 1992). Furthermore,

protein-kinase A and C activities are absolute requirements for capacitation and later progesterone and zona-induced acrosomal exocytosis (Brener et al., 2003; Endo et al., 1989; O'Toole, Roldan, & Fraser, 1996a; Tomes, Roggero, De Blas, Saling, & Mayorga, 2004). The requirement of PKC activity in AE has been attributed in part to the PKC phosphorylation-dependence of a membrane voltage-operated calcium channel for calcium influx at the initiation of exocytosis (Breitbart et al., 1997). The $Ca_V2.3$ channel is phosphorylated by PKC, which significantly increases current through this channel upon voltage operation (Kamatchi et al., 2003; Krieger et al., 2006), making it a very attractive candidate for the voltage-operated channel in sperm.

Despite the similarities between the $Ca_V2.3$ channel and the unidentified VOCC required for acrosomal exocytosis in sperm, there has been limited investigation of $Ca_V2.3$ as a candidate for the sperm VOCC. Additionally, the few published reports on $Ca_V2.3$ in sperm are contradictory. No R-type current was detected from developing spermatocytes, leading the authors to conclude that this channel is not active in sperm cells (Arnoult et al., 1998), and spermatocytes from α1E null mice have normal LVA calcium currents (Sakata, Saegusa, Zong, Osanai, Murakoshi, Shimizu, Noda, Aso, & Tanabe, 2001). However, the lack of α1E current in immature sperm cells and morphologically abnormal sperm, such as sperm with cytoplasmic droplets used for patch clamp techniques, does not rule out the potential for activity of this channel at a later stage in morphological or functional

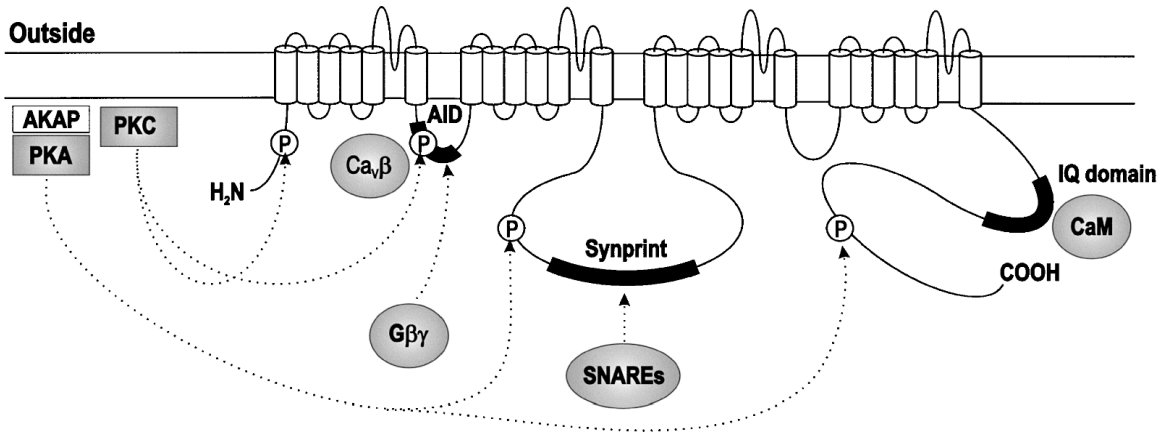

Figure 4. A schematic of the alpha 1 subunit of a voltage-operated calcium channel and the potential intracellular regions of modification and interaction domains and their respective partners. Not all modifications depicted have been confirmed to occur with the α1E subunit of the $Ca_V2.3$ calcium channel, but the intracellular target regions are conserved across all alpha 1 subunits. Reprinted with permission from Felix, 2005.

development. Indeed, the dependence of α1E subunit current on the activation of signaling molecules such as chaperone proteins, PKA, PKC, and G-protein coupled muscarinic receptors, supports the hypothesis that $Ca_V2.3$ currents would not be functional in sperm at least until the onset of capacitation and tyrosine phosphorylation of the channel allows open gating. In agreement with this interpretation, the VOCC in sperm is not functional until the maturational process of capacition. It may then be necessary to evaluate such currents in mature, capacitated sperm to definitively address this question. Patch-clamp techniques are difficult in capacitated sperm (Florman, pers. comm.), making another investigative approach necessary.

The recent generation of mice lacking the α1E pore-forming subunit of the $Ca_V2.3$ calcium channel provides an excellent way to investigate the $Ca_V2.3$ channel in sperm. Functional responsiveness of α1E null sperm has not been fully characterized. However, sperm from these mice were noted to have a more straight flagellar waveform and path velocity in vitro (Sakata et al., 2002). Additionally, these sperm had lower and significantly slower calcium influx in response to mannosylated-BSA, despite normal intracellular resting and capacitated calcium levels. No other agonists for acrosomal exocytosis have been characterized, and a description of acrosomal exocytosis competence of the $Ca_V2.3$ null sperm has not been reported. This is an important next step in addressing the role of the $Ca_V2.3$ channel in sperm function, and the effects of mannosylated-BSA versus progesterone and zona pellucida proteins have not been characterized in murine sperm.

Conclusions and Objectives

The following studies address the question of how form translates into function for a mammalian sperm. The ganglioside G_{M1} has historically been considered a membrane-organizing lipid associated with membrane raft formation, but not necessarily the signaling molecule that many studies are beginning to suggest. Considerable gaps exist in our knowledge of how movements or interactions with G_{M1} are translated into the diverse cellular functions and signals ascribed to this molecule. The studies presented in this thesis are initially descriptive in nature. Chapters two and three describe the organization of mammalian sperm membrane and build a picture of a highly compartmentalized cell with unique membrane organizing principles. It is the peculiar quality, dynamics, and nature of the G_{M1}-enriched membrane domain described in chapters two and three that lays the foundation for the question, '**Why have mammalian sperm evolved such conserved enrichment of G_{M1} in such a highly active and interactive membrane region of the sperm?**' This question is addressed in three parts. In chapter four, I address the issue of membrane dynamics and species diversity of G_{M1} changes as a sperm functionally matures. In conducting these experiments, I discovered the responsiveness of a spermatozoon to changes in focal concentrations of membrane G_{M1}. The work described in chapter five tells of the regulation of calcium channel activity through focal enrichment of G_{M1} in the plasma membrane overlying the acrosome, which mimics sperm responses to agonists of acrosomal exocytosis. This work identifies the voltage-operated calcium channel responsible for the initial depolarization-evoked rise in intracellular calcium caused by zona pellucida as the $Ca_V2.3$ channel formed by the α1E subunit. Chapter six presents a mechanism by which G_{M1} dynamic might regulate signaling processes important to $Ca_V2.3$ and exocytotic function. That chapter also gives clinical

significance to current methods of cryopreservation that may be limiting sperm viability by re-capitulating G_{M1} clustering that is physiologically relevant to sperm-oocyte interaction. The final chapter summarizes the significant findings presented here and suggests future directions for the field of sperm biology and ganglioside function.

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CHAPTER 2

THE ORGANIZATION AND STABILITY OF G_{M1}-ENRICHED MEMBRANE DOMAINS IN MAMMALIAN SPERM

[1]

[1] This work is the author's contribution to previously published reports "Segregation of micron-scale membrane sub-domains in live murine sperm. Selvaraj V, Asano A, Buttke DE, McElwee JL, Nelson JL, Wolff CA, Merdiushev T, Fornés MW, Cohen AW, Lisanti MP, Rothblat GH, Kopf GS, Travis AJ. (2006) *J Cell Physiol 206(3)*, 636-46." and "Mechanisms underlying the micron-scale segregation of sterols and G_{M1} in live mammalian sperm. Selvaraj V, Asano A, Buttke DE, Sengupta P, Weiss RS, Travis AJ. J Cell Physiol. 2009 Mar;218(3):522-36."

Abstract

The organization of functional signaling pathways within discrete domains of sperm membranes into is an attractive means by which the transcriptionally and translationally quiescent sperm could respond to the same extracellular stimuli in regionally differing ways. Several labs have attempted to visualize membrane heterogeneities through the localization of G_{M1} in fixed sperm membranes with widely varing results. I examined the localization of G_{M1} in live sperm using a fluorophore-conjugated B subunit of cholera toxin (CTB) and witnessed dramatic changes in localization upon the cessation of motility and cell death. Examination of fluorescence intensity suggested that new G_{M1} binding sites were becoming accessible to the extracellular environment upon cell death, and attempts to saturate G_{M1} binding with CTB were only successful when cells were permeabilized, suggesting an intracellular pool of G_{M1} existed. We identified the acrosome of developing male germ cells as being highly enriched in G_{M1} and accounting for the extra source of surface G_{M1} externalized upon cell death. Furthermore, we present evidence that communication occurs between these two membranes during capacitation, as some CTB became internalized and no longer accessible to the extracellular environment during incubation with capacitating stimuli. These findings suggest that dynamic changes in membrane G_{M1} concentrations occur during capacitation and might suggest an important role for this biologically active lipid in sperm function.

Introduction

Mammalian sperm are highly specialized cells, with specific functions and pathways compartmentalized to distinct regions of the spermatozoon (see Travis & Kopf, 2002b, for review). This high degree of specialization is thought to be necessary in order to minimize the size of the cell and maximize mobility and speed, while still maintaining discrete and timely responsiveness to the extracellular environment. The sperm can be divided into two separate macro-regions consisting of the head and flagellum, which can itself be divided into the principal piece and end piece. Aside from the end piece, each is associated with specific signaling and metabolic pathways that perform the distinct functions of each region. The flagellum propels the spermatozoon forward using energy produced by the mitochondria as well as by glyolytic enzymes thethered along the membrane. The midpiece is a rigid region of the spermatozoon containing mitochondria that perform oxidative respiration. The head of the spermatozoon interacts with and penetrates the zona pellucida and then fuses with the oocyte plasma membrane.

Each of these segregated functional pathways in sperm must respond to the same extracellular stimuli as the sperm transits through the female reproductive tract. Penetration of and fusion with the zona pellucida and oocyte is the final step in a spermatozoon's journey. The sperm has evolved numerous mechanisms to ensure the precisely timed success of these events. The use of membrane rafts to organize and scaffold signaling pathways within the membrane is commonly employed in somatic cells (see Helms & Zurzolo, 2004; Rajendran & Simons, 2005; Salaun, James, & Chamberlain, 2004, for review). Evidence of similar membrane organization exists in the sperm (Asano et al., 2009; Travis et al., 2001a; van Gestel et al., 2005). Within the

head, distinct subdomains exist based on differences in lipid/protein composition caused or maintained by diffusion barriers (Asano et al., 2009; Selvaraj et al., 2006; Selvaraj et al., 2009). The post-acrosomal plasma membrane is extremely rigid and sterol-poor (James, Hennessy, Berge, & Jones, 2004). The plasma membrane overlying the acrosome is highly enriched in sterols and sphingolipids, with the subacrosomal ring possessing a high degree of rigidity relative to the apical acrosomal membrane (Friend, 1982; Friend, 1989; Selvaraj et al., 2009). The lipid composition of the plasma membrane overlying the acrosome is further modified by the adsorption of lipids and proteins from seminal plasma during ejaculation (Florman & First, 1988; Metz, Berger, & Clegg, 1990; San Agustin & Lardy, 1990; Tezon, Miller, & Bardin, 1986). These factors include many noted to have a de-capacitating effect on the sperm, maintaining a state of relative quiescence until these factors and sterols are lost or removed from the membranes as the sperm transits the female reproductive tract. Despite a number of elegant studies describing the relative changes in sterol content during the process of capacitation, the precise regulatory and downstream effects of this process remain unknown.

Membrane rafts are small, heterogenous, and highly dynamic regions of the plasma membrane that possess a high molar ratio of saturated sphingolipids and sterols relative to phospholipids, as well as a cohort of proteins that preferentially segregate to these membrane rafts (Pike, 2006). Because of this, proteins, such as caveolin or GPI-anchored proteins, and sphingolipids, such as the ganglioside G_{M1}, can be used as markers to identify membrane rafts. Localization of membrane rafts has increased our knowledge of their prevalence in polarized cells, where rafts increase the responsiveness of the cell for exocytosis, cell migration, differentiation, and

endocytosis and membrane fusion (Asano et al., 2009; Bou Khalil et al., 2006; James et al., 2004; Jones et al., 2010; Tanphaichitr et al., 2007; van Gestel et al., 2005).

Because of this similar membrane composition and functional relevance of membrane rafts to signaling organization, we wanted to investigate whether membrane rafts were present in live spermatozoon in order to address their potential roles in sperm function. Visualization of membrane rafts is difficult due to the small and variable nature of rafts as well as the complications and artifacts that fixatives and aggregating probes can induce in cell membranes (Gaus et al., 2003; Heerklotz, Szadkowska, Anderson, & Seelig, 2003; Lagerholm, Weinreb, Jacobson, & Thompson, 2005). In order to avert some of these potential complications, we utilized the B subunit of cholera toxin, which binds up to five G_{M1} molecules in the plasma membrane with high affinity and specificity.

Materials and Methods

Reagents and animals

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. CTB (Molecular Probes, Eugene, OR) was purchased conjugated with Alexa Fluor 488 or Alexa Fluor 647 as indicated. Male CD-1 mice were from Charles River Laboratories (Kingston, NY).

Preparation of media and incubations of sperm

A modified Whitten's medium (MW; 22 mM HEPES, 1.2 mM MgCl2, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemi-calcium salt, pH 7.35) (Travis et al., 2001) containing 5.5 mM glucose was used for all incubations unless

otherwise indicated. 2-hydroxypropyl--cyclodextrin (2-OHCD; 3 mM) was supplemented as needed. 2-OHCD supports sperm capacitation and in vitro fertilization by functioning as a sterol acceptor, and is preferred over the more potent methyl--cyclodextrin (Visconti et al., 1999). Mature sperm were collected from the cauda epididymides by a swim-out procedure as described previously (Travis et al., 2001). All steps of washing of sperm and all incubations for experiments were performed at 37°C.

Fluorescence localization of lipids

All incubations during localization experiments were carried out under dim lighting at 37°C in a humidity chamber. Sperm (2 × 106) were incubated in 300 µl MW. The localization of G_{M1} was visualized with CTB in live sperm or after fixation under different conditions. In both cases, cells were viewed with a Nikon Eclipse TE 2000-U microscope (Nikon, Melville, NY) equipped with a Photometrics Coolsnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany), and Openlab 3.1 (Improvision, Lexington, MA) automation and imaging software. Assignments of sperm to G_{M1} localization patterns were performed in a blind fashion regarding incubation condition. To compare shifts in population tendencies, the numbers were converted to percentages prior to statistical evaluation. In all cases, 100 cells were counted for each test condition, and every sperm in a given field was counted to avoid potential bias.

For localization in live sperm, a stage-mounted incubation chamber (LiveCell, Neue Product Group, Westminister, MD) was used along with an objective heater (Bioptechs, Butler, PA). Samples were observed using glass bottom culture dishes (MatTek Corporation, Ashland, MA) overlaid with mineral oil, or using small aliquots on slides under coverslips. Samples were incubated for 10 min with CTB (10 µg/ml).

To avoid any membrane damage, some samples were not washed, but viewed with CTB in the final medium as indicated. To study the effect of sterol efflux in live sperm, MW medium supplemented with 3 mM 2-OHCD was used and the sperm were incubated for 45 min before addition of CTB. Images of motile sperm were captured using programmed exposure intervals, and serial images were tethered into QuickTime (Apple Computers, Cupertino, CA) movies.

Alternatively, for experiments designed to quantify the relative fluorescence intensities over the plasma membrane overlying the acrosome (APM) versus the plasma membrane overlying the post-acrosomal area (PAPM), live sperm were allowed to attach to coverslips, incubated in CTB (5 μg/ml) for 10 min, and then washed five times with MW medium. Images of motile sperm were taken before and after changes in pattern of G_{M1} localization. Using image analysis tools in Openlab 3.1, minimum, maximum, mean, and mode fluorescence intensity per pixel (arbitrary units) were recorded for the whole sperm head and for equal-sized circles drawn within the APM and PAPM, before and after change in G_{M1} localization. Background fluorescence intensity was measured in identically-sized circles immediately adjacent to the sperm head, and these values were subtracted from each measurement within the sperm head to adjust for local differences in background and for any signal quenching that might have occurred between images taken of the same cell. Means for the signal intensity over the whole sperm head, the APM, and the PAPM were compared within sperm cells that exhibited a change in localization pattern, using the Wilcoxon-signed rank test for non-parametric data.

Localization of G_{M1} in live cells

For visualizing G_{M1}, cells were incubated with CTB (AlexaFluor 488; 5 μg/ml) for 10 min. For experiments assessing acrosomal status, PNA (5 μg/ml) was used after incubating with CTB as above. For induction of sterol efflux, sperm were incubated in MW medium supplemented with 3 mM 2-hydroxypropyl--cyclodextrin (2-OHCD) for 30 min. For all the above conditions, samples were not washed, but viewed with the respective reagents in the final medium to avoid damage to membranes.

Localization of G_{M1} and sp56 in developing male germ cells

For labeling G_{M1} in developing male germ cells, the cells were spread on coverslips and incubated in KRB in a humidity chamber at 37°C for 10 min to allow attachment. The cells were then fixed using 4% PF for 10 min, permeabilized using 0.1% Triton X-100 for 1 min, washed, and air-dried. They were then rehydrated with PBS and incubated with CTB (5 μg/ml) for 10 min, and washed again using PBS. For dual labeling experiments, these cells were first blocked for 30 min in PBS with 1% bovine serum albumin, and then incubated with anti-sp56 (1:50) for 1 h. The cells were then washed using PBS, incubated with the secondary antibody (1:500) for 30 min, and washed again. In dual labeling experiments, the cells were incubated with CTB (AlexaFluor 488 or 555) as a final step. Coverslips were mounted using a GVA mountant (Invitrogen). A control for non-specific binding of the secondary antibody was performed.

Saturation experiment using labeled CTB

To test whether there was exposure of additional G_{M1} during or after redistribution from the APM to the PAPM, we performed experiments in which G_{M1} was saturated

in murine sperm before and after weak fixation. As a control to demonstrate our ability to saturate all surface-accessible G_{M1}, live sperm were incubated with CTB conjugated with FITC (250 μg/ml) for 1 min, while simultaneously allowing attachment to coverslips. This was followed by fixation using 0.004% PF and then a final addition of AlexaFluor 555-conjugated B subunit of cholera toxin (CTB; 5 μg/ml). If successful, saturation with a 50-fold higher concentration of FITC-conjugated CTB should prevent AlexaFluor 555-conjugated CTB from binding. To investigate if there was exposure of additional G_{M1} upon redistribution, live sperm were incubated with CTB conjugated with FITC (250 μg/ml) for 1 min while simultaneously allowing attachment to coverslips, followed by concurrent addition of AlexaFluor 555-conjugated CTB (5 μg/ml) and 0.004% PF. In this experiment, if new G_{M1} became exposed on the surface, then AlexaFluor 555-conjugated CTB would compete for any new binding sites as they appeared during redistribution. The concentration of FITC-conjugated CTB was selected empirically so that all surface-accessible G_{M1} was saturated in both live and fixed sperm.

Results

Using a fluorescent-probe conjugated B subunit of cholera toxin (CTB), we have found the plasma membrane overlying the acrosome of murine sperm to be highly enriched with the ganglioside G_{M1} in live sperm (Fig. 1A). Importantly, this segregation of G_{M1} to the APM was maintained even after sterol efflux with 2-OHCD (3 mM) for 60 min (Fig. 1B, C), a concentration and duration of incubation sufficient

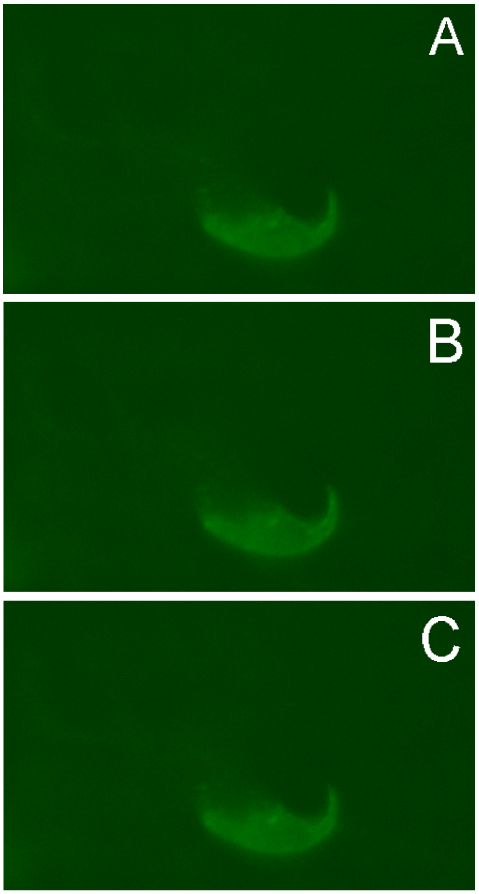


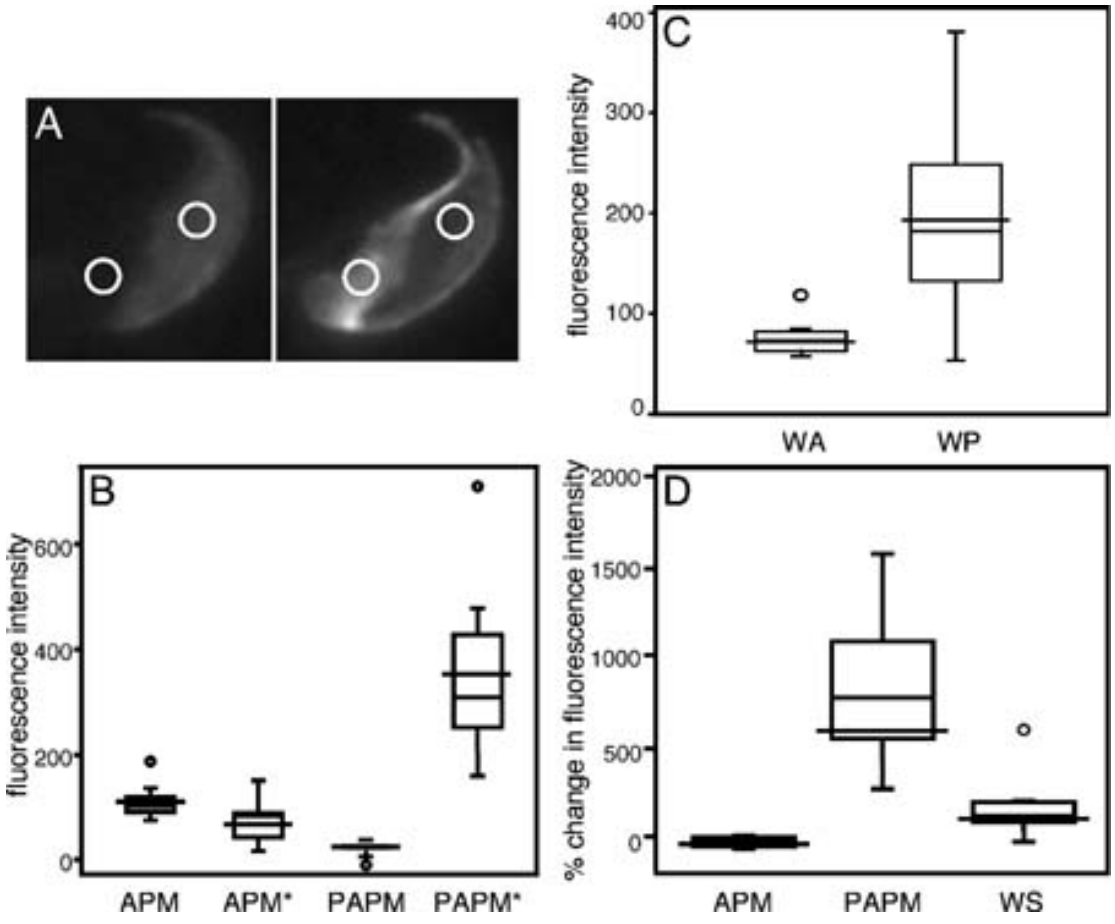
Figure 1. Captured images from a single live sperm showing no change in G_{M1} segregation or appearance with sterol efflux. Sperm were incubated with Alexa 488-CTB and 3 mM 2 OHCD with 10 mM bicarbonate to induce sterol efflux and capacitation. Images were taken at 0 (panel A), 30 (panel B) and 60 (panel C) minutes of incubation.

to induce the level of sterol efflux necessary for sperm capacitation (Visconti et al., 1999).

However, within seconds after the cessation of motility, the pattern of CTB fluorescence changed dramatically, appearing to lessen over the APM and brightening strongly over the PAPM (Fig. 2). First, the signal decreased from within the ES and increased at the borders of the APM (the AA, the SAR, and the perforatorium), prior to extending into the PAPM. The redistribution occurred within 10-100 sec after the cessation of motility. This change was consistent with the pattern seen in dead sperm and was not associated with acrosomal exocytosis (data not shown). Similar localization of G_{M1} to the PAPM was also seen in most sperm that were not motile at the beginning of these same experiments, suggesting that the loss of segregation coincided with the sperm becoming non-viable.

Two possibilities exist for the change in pattern seen upon the cessation of motility/death. The first would be a redistribution of G_{M1} from the APM to the PAPM, whereas the second would involve the unmasking or appearance of new G_{M1} on the outer leaflet of the PAPM in conjunction with some degree of redistribution. To distinguish between these possibilities, we repeated these experiments with repetitive washing out of unbound CTB from the medium. This would minimize free CTB available to bind to new molecules of G_{M1} that might become exposed in the PAPM. Quantification of fluorescence intensity in the APM against the PAPM revealed a statistically significant decrease in the APM coincident with a significant increase in signal in the PAPM (Fig. 2A,B,D). These changes, and the pattern of movement seen indicate that redistribution did indeed occur.

Figure 2. Fluorescence intensity of CTB in different regions of the sperm head. Images were taken of live sperm having an APM pattern of fluorescence and then again at the same exposure settings after cessation of motility when they displayed a PAPM pattern (n = 9). Mean fluorescence intensities within the APM and PAPM were recorded before and after (*) the shift in pattern of localization (Part B), using an equal-sized circle within each region (Part A). Fluorescence intensity was also recorded for the whole sperm head before (WA) and after (WP) the shift in localization pattern (Part C). Results are shown as box-whisker plots in Parts B-D, with the boundaries of the boxes representing the 25th and 75th quantiles, the 50th quantile displayed as a line within the box, and the mean as a line extending through the box. Whiskers extend to the 10th and 90th quantiles, and circles represent outliers. Statistically significant differences were found between groups (Part B, $P < 0.004$), and between the whole sperm heads upon shift in pattern (Part C, $P < 0.03$), using Wilcoxon's signed rank test. The percent change in intensity was also determined for the APM, PAPM, and whole sperm head (Part D). In this part, a negative value denotes a decrease in fluorescence intensity, and a positive value an increase in fluorescence intensity.


A
B
fluorescence intensity
0
200
400
600
APM
APM*
PAPM
PAPM*
C
fluorescence intensity
0
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WA
WP
D
% change in fluorescence intensity
0
500
1000
1500
2000
APM
PAPM
WS

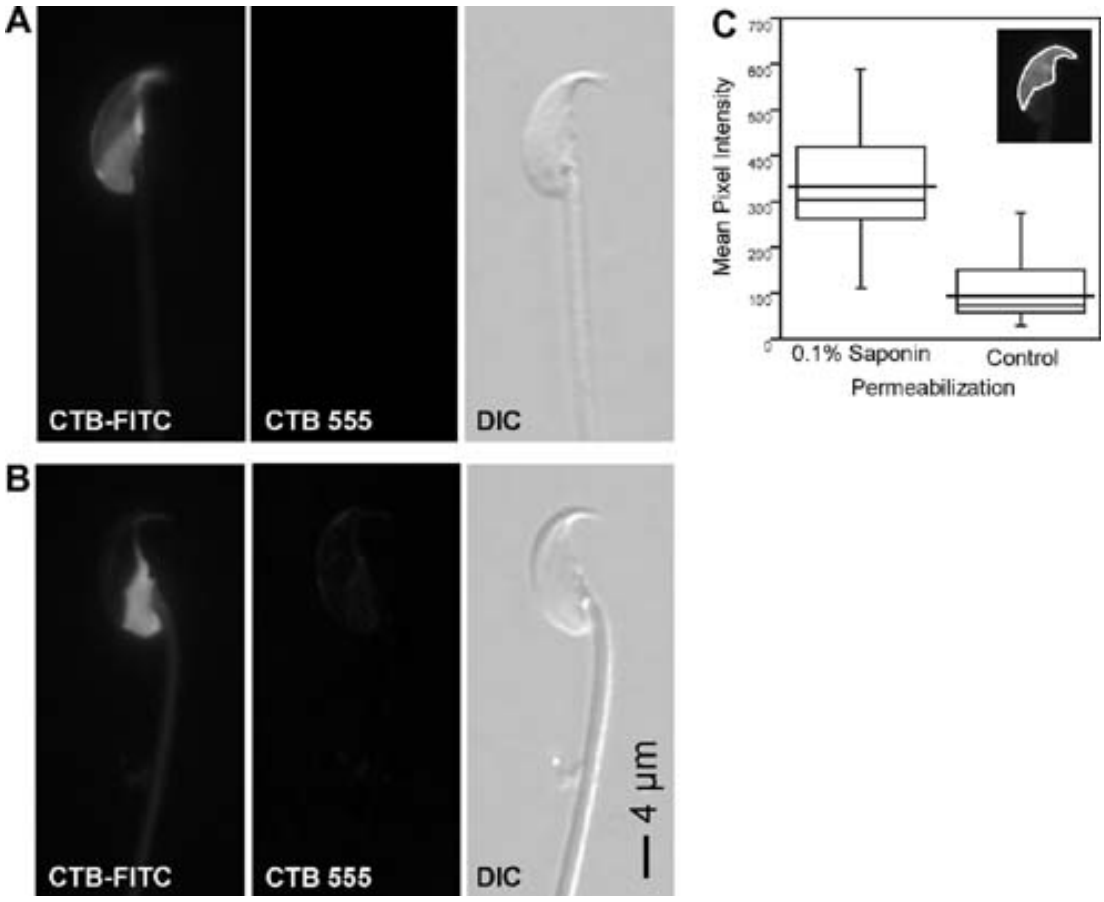
However, the decrease in signal over the APM could not account for the disproportionate increase in fluorescence intensity over the PAPM, even taking into consideration the fact that the PAPM is approximately one-half the size of the APM (Fig. 2B,D). The increase in fluorescence associated with the PAPM also caused an increase in whole head intensity after redistribution (Fig. 2C). To determine if redistribution induced some change in the fluorescence properties of the fluorophore ((Maxfield, 1982), we used both a fluorimeter and ELISA plate reader to quantify total fluorescence intensity of AlexaFluor 488-conjugated CTB in entire incubation tubes before and after the CTB-induced redistribution of G_{M1} (In separate experiments with n 3, these readings were taken both with excess CTB in the medium, and after washing out unbound CTB. In addition, in separate experiments, sperm were allowed to lose motility on their own, or by the addition of Fixative A.). We did not detect a significant change in total fluorescence of the system under any of these conditions (data not shown). Interestingly, experiments involving the quenching of fluorophores conjugated to CTB have suggested that internalization and reappearance of G_{M1} in the APM is also occurring prior to redistribution, suggesting that a combination of mechanisms is responsible for the change in pattern. We are therefore continuing to investigate this phenomenon.

Redistribution caused an increase in G_{M1} on the plasma membrane

To address the increase in total CTB fluorescence as a result of redistribution upon cell death, we investigated whether intra-cellular compartments were contributing to an increase in surface G_{M1}. For these experiments, we employed a general strategy of saturating all surface-accessible G_{M1} with FITC-conjugated CTB (250 µg/ml), and then probing with AlexaFluor 555-conjugated CTB (5 µg/ml) to look for any newly exposed binding sites. First, to verify that saturation could be attained, we incubated

live sperm with the FITC-CTB, and then probed with AlexaFluor 555-CTB; no binding by the latter conjugate was seen (data not shown). To confirm that saturation could be attained after G_{M1} redistribution to the PAPM, we incubated live sperm with the FITC-CTB, and then induced redistribution with 0.004% PF, still in the presence of the FITC conjugate. We have shown previously that this concentration of PF does not interfere with CTB binding and that it induces redistribution (Selvaraj et al., 2006). In this way, all surface-accessible G_{M1} should be bound by FITC-CTB before, during and after redistribution. We then added AlexaFluor 555-conjugated CTB to see if any unbound G_{M1} remained. No new binding was detected, confirming saturation (Fig. 4A). Next, we repeated the first incubation of live sperm with FITC-CTB. Then we added the AlexaFluor 555-conjugated CTB simultaneously with the 0.004% PF. If new G_{M1} were exposed on the surface during redistribution, there should be a competition between the FITC-CTB and AlexaFluor 555-CTB for these new sites. Assuming they have equal binding efficiencies and based on relative concentrations, only approximately 2% of newly exposed G_{M1} should be bound by the AlexaFluor 555-CTB. For technical reasons related to risk of membrane damage and sperm death during washing, we chose this approach over first washing out the unbound FITC-CTB. Nonetheless, when we performed this experiment, a percentage of the sperm corresponding roughly with the initial percentage of motile sperm showed AlexaFluor 555-CTB fluorescence, primarily on the borders of the APM and in the PAPM (Fig. 3B). Another subpopulation of sperm showed only FITC-CTB signal. These sperm were probably not viable during the initial incubation and served as an efficient internal control, showing that the initial surface saturation with the FITC-conjugate was thorough.

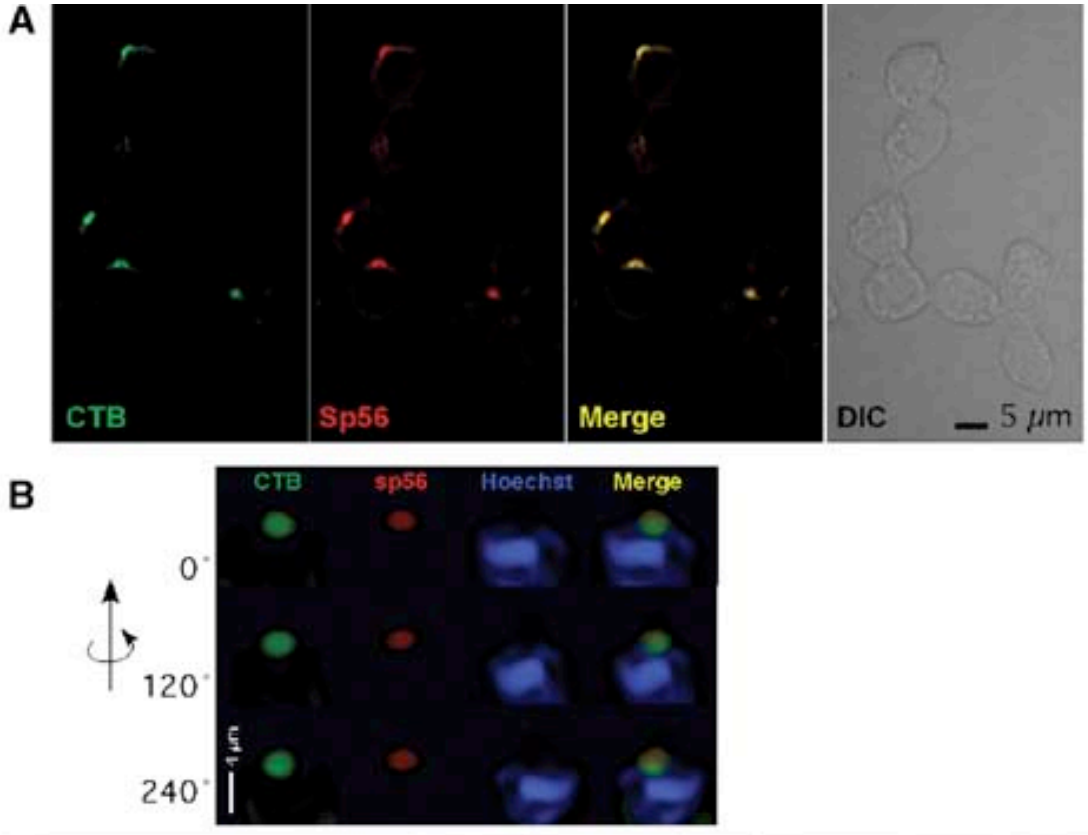
Figure 3. Plasma membrane G_{M1} dynamics in sperm. We saturated all surface-accessible G_{M1} using 250 μg/ml FITC-conjugated CTB, and then added 5 μg/ml AlexaFluor 555-conjugated CTB to see if there was exposure of additional G_{M1} during CTB-induced redistribution to the PAPM. A: Live sperm were incubated with FITC-CTB, fixed using 0.004% PF to induce redistribution, and then AlexaFluor 555-CTB was added. All sperm had an intense FITC-CTB fluorescence and no detectable AlexaFluor 555-CTB fluorescence, showing that FITC-CTB completely saturated G_{M1} in murine sperm. B: Sperm were incubated with FITC-CTB, then simultaneously treated with AlexaFluor 555-CTB and fixed using 0.004% PF to induce redistribution. Some sperm had intense FITC-CTB fluorescence and no detectable AlexaFluor 555-CTB fluorescence [similar to (A); not shown] and others showed AlexaFluor 555-CTB labeling over the APM and PAPM (shown). This finding suggested that additional molecules of G_{M1} appeared in the plasma membrane during redistribution to the PAPM. The percentages of cells showing the two patterns corresponded with the percentage of dead and live cells, respectively. C: Fluorescence intensity measurement over the region of the APM with and without saponin permeabilization in fixed cells. Box-whisker plots show mean pixel intensities measured in the region of the APM (inset: Region of quantification outlined) in saponin permeabilized and control, unpermeabilized cells. The lower and upper ends of the box mark the 25th and 75th quantiles; the median is represented as a horizontal line within the box, and the mean as a horizontal line through the box. Vertical whiskers extend from the ends of the box to the 10th and 90th quantiles. A Student's t-test showed significant differences between the permeabilized and control cells ($P < 0.0001$). This finding provides evidence for an acrosomal pool of G_{M1} accessible to CTB after saponin permeabilization.


A
CTB-FITC
CTB 555
DIC
B
CTB-FITC
CTB 555
DIC
4 µm
C
Mean Pixel Intensity
700
600
500
400
300
200
100
0
0.1% Saponin
Control
Permeabilization

G_{M1} enrichment in the acrosomal membrane in spermatids and sperm

In light of our previous work (Selvaraj et al., 2006) and the above findings that suggested that redistribution of CTB also involved an increase in G_{M1} on the sperm surface, we investigated whether an internal membranous compartment might represent a source of additional G_{M1}. Because of its location immediately underneath the APM, and because it is the only intracellular vesicle in the sperm head, we hypothesized that the acrosome was the likely intracellular pool of G_{M1}. We addressed this possibility using two different approaches. First, we labeled sperm after strong fixation with or without saponin permeabilization and compared mean fluorescence intensity in the APM. Our results showed that there was significantly higher mean fluorescence intensity over the APM in permeabilized compared to nonpermeabilized sperm (Fig. 3C; $P < 0.0001$). Note also that saponin did not reveal additional fluorescence in the PAPM, showing that the additional labeling was not originating from some source in that region of the sperm. These data suggested that G_{M1} was present in the acrosomal vesicle underlying the APM. In mature sperm the APM and acrosome are in extremely close apposition, and differential localization to one membrane or the other cannot be distinguished at the level of light or even transmission electron microscopy. Therefore, in the second approach we examined round spermatids for the presence of G_{M1} in the developing acrosome. In these cells one can more easily distinguish between the acrosomal and plasma membranes because they are separated by cytoplasm. From this experiment, we found that G_{M1} was strongly enriched in the developing acrosome in round spermatids. In cap phase round spermatids, the G_{M1} approximately co-localized with the acrosomal matrix component sp56 in the developing acrosome (Fig. 4A). Deconvolution and reconstruction of serial sections taken at higher magnification revealed that the G_{M1} appeared to surround the acrosomal matrix (Fig. 4B). These findings showed that G_{M1}

Figure 4. Localization of G_{M1} and sp56 in murine male germ cells. A: Parts show confocal images of CTB (green) and sp56 (red) localization in round spermatids connected by intercellular bridges. Merged image (yellow) from the two channels and the corresponding Nomarski DIC image are also shown. CTB approximately co-localized with the acrosomal matrix protein sp56 during development of the acrosome. B: 3D-rendered image compilation of G_{M1} and sp56 localization in a murine round spermatid. Serial z stacks were deconvoluted and reconstructed in three dimensions as described. Each row represents frames from the supplemental movie file and show rotation along the vertical axis. CTB (green) and sp56 (red) are seen localized to regions of the developing acrosomal vesicle. The nucleus was stained using Hoechst (blue). These frames (and also the Supplemental Material movie) show that G_{M1} largely enveloped the sp56 fluorescence of the acrosomal matrix. This suggested that G_{M1} was associated with the acrosomal membranes and was not localized within the acrosomal matrix.


A
CTB
Sp56
Merge
DIC
5 μm
B
CTB
sp56
Hoechst
Merge
0°
120°
240°
1 μm

was enriched in the acrosomal membranes of round spermatids and strongly suggested that G_{M1} could be present in the acrosomal membrane of mature sperm.

Evidence of membrane communication between the APM and OAM

Recently, evidence has begun to accrue that acrosomal exocytosis is a more gradual process than previously thought. This stepwise membrane fusion is similar to the kiss-and-run fusion events seen in neurons and could account for the appearance of proteins of acrosomal origin on the surface of the sperm prior to acrosomal exocytosis (Kim et al 2002), including the apparent increase in G_{M1} fluorescence noted on the apical ridge of capacitated sperm. This seemed even more plausible given our discovery of the acrosomal membrane as a source of G_{M1}. We addressed this question using two approaches. We reasoned that if proteins and lipids were being transferred from the APM to the OAM, the reverse would likely be true. In order to test this hypothesis, we incubated sperm with FITC-CTB for 20 minutes under capacitating and non-capacitating conditions. The extracellular FITC fluorescence was then quenched by adding 0.75% trypan blue, which had no effect on sperm viability. In capacitated sperm, a ridge of FITC fluorescence remained over the apical acrosome (Fig. 5). This fluorescence remained after two additional volumes of trypan blue were added, but disappeared when sperm were permeabilized with 0.1% triton-X 100, suggesting that the remaining FITC fluorescence had been internalized and was not longer assessable to the extracellular environment. Cells treated with .20% tannic acid prior to FITC-CTB incubation to immobilize the plasma membrane failed to retain any FITC fluorescence after trypan blue addition, supporting the hypothesis that membrane communication between the APM and OAM was allowing the intermixing of APM and OAM G_{M1}. Adding tannic acid at the end of the FITC-CTB incubation had no effect on the residual CTB fluorescence over the apical acrosome region.

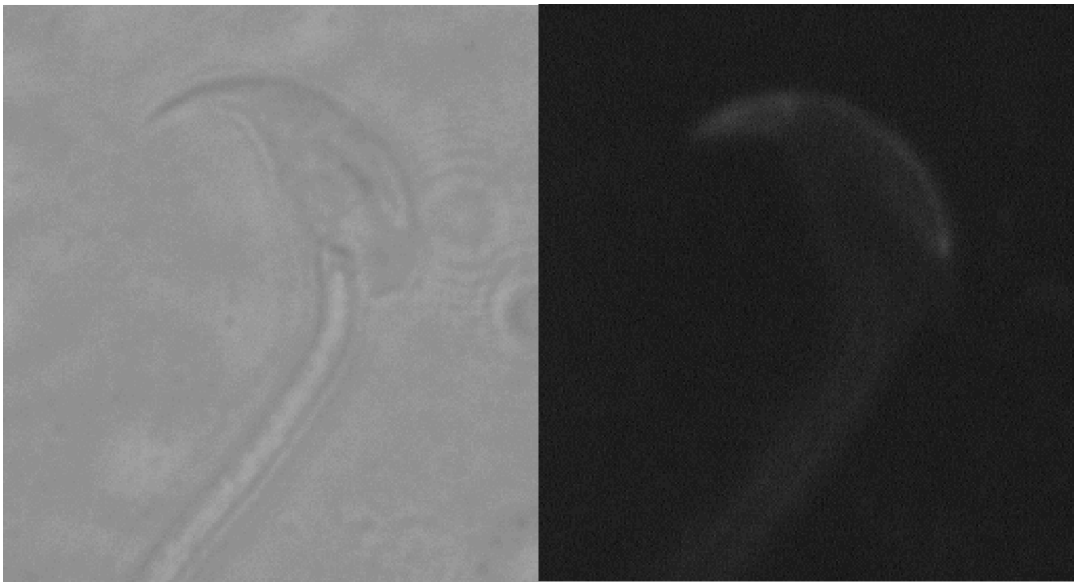


Figure 5. Phase contrast (left) and fluorescence (right) image of a sperm showing remaining apical acrosomal fluorescence after extracellular FITC fluorescence was quenched with trypan blue. Sperm were incubated with FITC-CTB for 20 mintues followed by the addition of 0.75% trypan blue to quench extracellular fluorescence. In the majority of live sperm, a ridge of fluorescence remained over the apical acrosomal ridge (n=8). This ridge of fluorescence disappeared when cells were permeabilized with 0.1% triton-X 100, suggesting the FITC fluorescence had become internalized. No FITC fluorescence was seen after trypan blue addition if cells were treated with the viable membrane fixative tannic acid prior to FITC-CTB addition (data not shown).

Discussion

Several studies describing G_{M1} localization in sperm exist, with widely varied results. Previous studies using fixed cells in the mouse have localized G_{M1} to the PAPM (Trevino, Serrano, Beltran, Felix, & Darszon, 2001). Other labs have utilized human sperm at ambient temperatures and reported a diffuse localization after capacitation (Cross, 2004). It has also been suggested using rat spermatozoa that G_{M1} is present in the PAPM and then moves to the APM after capacitation (Roberts, Wamstad, Ensrud, & Hamilton, 2003). Coalescence of membrane rafts at temperatures below 16 degrees Celsius is well documented and can influence the localization of rafts in fixed cells as well. Our lab has also reported the influence of the choice and relative concentrations of fixatives on changes in fixed sperm membranes relative to live cell localization (Selvaraj et al., 2006). The studies reported here, together with other reports from our lab (Buttke, Nelson, Schlegel, Hunnicutt, & Travis, 2006; Selvaraj et al., 2006), help resolve differences reported in the literature as differences induced by fixative choice and the use of epididymal versus ejaculated sperm (see chapter 3).

The organization of membrane microdomains in mammalian sperm is similar to membrane raft organization in somatic cells in many ways, but striking differences are noted here. The size and stability of the sperm membrane subdomain enriched in G_{M1} is unprecedented in live cells. The diffusion barrier present between the APM and PAPM appears exceptionally stable in live cells. The relative size of the G_{M1} enriched membrane far exceeds the typical nanometer-scale rafts described in somatic cells, and may represent numerous smaller rafts within this subdomain. The same subdomain is responsible for initial interaction with the cumulus complex and zona pellucida of the

oocyte, and may exhibit such extreme size and stability to increase the surface area of the sperm capable of responding to the cumulus-oocyte complex.

Membrane communication between the APM and OAM has been hypothesized to occur due to the appearance of acrosomal contents on the APM during the process of capacitation and prior to acrosomal exocytosis. A putative receptor for the zona pellucida, Sp56, is located within the acrosomal matrix and membrane of non-capacitated cells but gradually moves to the APM as capacitation ensues. The relative increase in CTB fluorescence in sperm both upon cell death as well as prior to acrosomal exocytosis and upon capacitation (Buttke et al., 2006; Jones et al., 2010), suggests that an internal store of G_{M1} existed. The discovery that the acrosomal membrane is highly enriched in G_{M1} suggested a plausible internal source of the extra G_{M1} seen with capacitation in some species and cell death in murine sperm. The inability to quench all FITC-CTB fluorescence suggests that the remaining fluorescence is no longer accessible to the extracellular environment and has been internalized through membrane intermixing between the APM and OAM. This hypothesis is supported by the observation that permeabilization of the sperm with low concentrations of detergent either before or after the addition of the quenching agent resulted in a loss of all fluorescence. Additionally, low concentrations of tannic acid, which fixes the surface of the membrane of cells without affecting the cells' viability and is used in studies of endocytosis and pinocytosis, also prevented any fluorescence from remaining in the apical acrosome when added prior to the FITC-CTB.

Intermixing of the APM and OAM membrane components provides an attractive means by which a sperm could regulate and time the appearance of signaling and receptor molecules on the sperm surface. This may help prevent premature acrosomal

exocytosis, as well as provide a mechanism by which a sperm could regulate membrane composition and raft formation. Delivery of lipids to the plasma membrane of somatic cells occurs during periods of cellular activity through vesicle fusion with the plasma membrane, and can influence the raft composition of the plasma membrane, specifically at times of growth or differentiation, such as neurite outgrowth and synapse formation in neurons (Guirland, Suzuki, Kojima, Lu, & Zheng, 2004; Helms & Zurzolo, 2004; Salaun et al., 2004). A similar mechanism might be utilized by the sperm in punctate fusion events with the OAM, allowing transfer of lipids and proteins from the OAM to the PAPM. Whether or not this process occurs along a concentration gradient or is energy dependent is not yet known. Further study characterizing the amount and dynamics of potential membrane transfer could elucidate how a sperm prevents and then later facilitates full exocytosis of the single sperm vesicle of the acrosome.

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CHAPTER 3

VISUALIZATION OF G_{M1} WITH CHOLERA TOXIN B IN LIVE EPIDIDYMAL VERSUS EJACULATED BULL, MOUSE, AND HUMAN SPERMATOZOA

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As published in (2006) *Biol Reprod 74*(5), 889-95. Nelson contributed technical support, PN Schlegel and GR Hunnicut provided human sperm and experiments. AJ Travis was primary supervisor.

Abstract

The organization of membrane sub-domains in mammalian sperm has recently generated controversy, with several reports describing widely differing localization patterns for the ganglioside, G_{M1}. Using the pentameric B subunit of cholera toxin (CTB), we found G_{M1} to be restricted to the plasma membrane overlying the acrosome in the heads of live murine sperm. Interestingly, CTB had minimal binding to live bovine and human sperm. To investigate whether this difference in G_{M1} localization was due to species differences or differences between collection from the epididymis (mouse) or an ejaculate (bull, human), we examined epididymal bovine and human sperm. We found that G_{M1} localized to the plasma membrane overlying the acrosome in sperm from these species. To determine whether some component of seminal plasma was interfering with the ability of CTB to access G_{M1}, we incubated epididymal mouse sperm with fluid from murine seminal vesicles and epididymal bull sperm with bovine seminal plasma. This treatment largely abolished the ability of the CTB to bind to G_{M1}, producing a fluorescence pattern similar to that reported for the ejaculated human sperm. The most abundant seminal plasma protein, PDC-109, was not responsible for this loss. As demonstration that the seminal plasma was not removing G_{M1}, sperm exposed to seminal plasma were fixed prior to CTB addition, and again displayed fluorescence over the acrosome. These observations reconcile inconsistencies reported for the localization of G_{M1} in sperm of different species, and provide evidence for the segregation of G_{M1} to a stable sub-domain in the plasma membrane overlying the acrosome.

Introduction

The organization of co-localized receptor and effector complexes is an effective way to transduce signaling events into functional changes within a specific area of a cell (Golub et al. 2004). Membrane sub-domains enriched in sterols and sphingolipids such as gangliosides have been postulated to play an important role in a wide variety of cellular functions, by acting as scaffolds or foci for the compartmentalization of signaling molecules to specific regions of membrane (see Simons & Toomre 2000, for review). Such "lipid raft" sub-domains have been found to anchor signaling molecules in somatic cells until stimulation allows for the release and activation of the molecule (Okamoto et al. 1998). Dynamic changes in raft components and localization can also allow for the interaction of previously segregated signaling and effector molecules, as seen in the transduction of downstream events in B and T lymphocytes (Sedwick & Altman 2002; Naal et al. 2003; Harder 2004).

The presence of lipid raft sub-domains in mammalian spermatozoa is of special interest due to the importance of plasma membrane alterations in sperm function. For example, the removal of sterols from the plasma membrane is a required stimulus for the process of capacitation, in which sperm acquire the ability to undergo acrosomal exocytosis (Florman & First 1988) and a hyperactivated pattern of motility (Katz & Yanagimachi 1980). These changes render the sperm fertilization competent. Because of their ability to transduce sterol efflux into functional changes, lipid rafts have been suggested to be involved in a number of processes of capacitation, including sterol efflux and acrosomal exocytosis (Flesch et al. 2001; Travis et al. 2001; Cross 2004; Shadan et al. 2004). Evidence of the presence of membrane sub-

domains in fixed/dried sperm has been provided by several methods, including direct labeling with membrane antibodies (Koehler 1975), fluorescence imaging of lipid-binding probes (Bearer & Friend 1980) and exogenous lipid probes (Wolfe et al. 1998; James et al. 2004), freeze fracture, surface replica, and freeze-etch electron microscopy (Friend & Fawcett 1974; Elias et al. 1978; Bradley et al. 1980; Friend 1982; Suzuki 1988), and atomic force microscopy (Ellis, Shadan et al. 2002). For example, the polyene antibiotic, filipin, has been used to show that the distribution of sterols within the plasma membrane of fixed sperm is heterogeneous. Visualized by freeze fracture electron microscopy or autofluorescence, filipin-sterol complexes delineate an area of extreme sterol enrichment in the plasma membrane overlying the acrosome, with a much lower sterol content found in the post-acrosomal region (Friend 1982; Pelletier & Friend 1983; Lin & Kan 1996; Visconti et al. 1999). Caveolin-1, a sterol-binding protein associated with rafts (Smart et al. 1999), has since been shown to co-localize with the sterol-rich sub-domain overlying the acrosome (Travis et al. 2001). Caveolin-1 has been reported to scaffold signaling complexes and to participate in the movement of sterols across membranes (see Schlegel et al. 1998, for review).

Despite the rapidly-growing literature regarding lipid raft membrane sub-domains in both sperm and other cell types, legitimate controversy has arisen regarding whether lipid rafts are found in nature, or instead represent artifacts induced by the methods used to isolate them. For example, the use of fixatives/cross-linking reagents when trying to visualize lipid rafts can cause "patching artifacts" as different membrane components are brought into proximity. In addition, there is also controversy regarding the use of detergents during biochemical isolation, as they can cause artifactual coalescence of membrane components that might not interact under

physiological conditions (Edidin et al. 1991; Heerklotz 2002; Rouvinski et al. 2003). We have demonstrated that the same region of the plasma membrane that is enriched in sterols and caveolin-1 in fixed cells is enriched in the ganglioside, G_{M1}, in living, motile sperm (Selvaraj et al. 2005). This demonstrates that the membrane sub-domain overlying the acrosome is not an artifact of fixation. In addition, we have been able to use a protocol without detergents to partition sperm membrane sub-domains by means of their relative buoyancy alone (Travis et al. 2001). Together, these data demonstrate the existence of membrane sub-domains in sperm.

Much attention has been focused on G_{M1} because of its suggested association with lipid raft sub-domains and the ease and specificity of its localization by means of fluorescence conjugates of the pentameric subunit B of cholera toxin (CTB). Previous work has also suggested that sperm-egg interactions in several non-mammalian species might be mediated in part by one or more ganglioside(s) (Sato et al. 2002; Yu et al. 2002; Maehashi et al. 2003).

However, our experiments using murine sperm (Selvaraj et al. 2005) have yielded results that contrast with other published results for the mouse (Trevino et al. 2001) and rat (Roberts et al. 2003), which themselves contrast with studies localizing G_{M1} in human (Cross 2004) and boar sperm (Shadan et al. 2004). For example, we demonstrated localization to the plasma membrane overlying the acrosome in epididymal murine sperm, a region consistent in terms of size and stability (Selvaraj et al. 2005), while other rodent studies localized G_{M1} to the post-acrosomal plasma membrane (Trevino et al. 2001; Roberts et al. 2003). Conversely, G_{M1} localization in non-capacitated human and boar sperm was reported as nonexistent or patchy and inconsistent throughout the entirety of the sperm cell (Cross 2004; Shadan et al. 2004).

One likely difference between the studies in rodents was the use of fixation conditions. We demonstrated that upon cessation of motility/cell death in unfixed or lightly fixed cells, G_{M1} moved rapidly from its position overlying the acrosome to the post-acrosomal plasma membrane (Selvaraj et al. 2005). This finding underscores some of the difficulty inherent in visualizing lipid sub-domains. One clear difference between the studies in rodents and those in other species was that the murine sperm were collected from the epididymis, whereas the human and boar sperm were collected from ejaculates. This raised the possibility of sperm source as an alternative to true species differences regarding the localization of G_{M1}. In an attempt to resolve these dissimilar findings and improve understanding of the dynamics of membrane sub-domains in mammalian sperm, we have compared the localization of G_{M1} in epididymal and ejaculated sperm of bull, mouse, and human.

Materials and Methods

Reagents and Sources of Samples

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. CTB conjugated with Alexa-Fluor 488 and anti-rabbit IgG conjugated with Alexa-Fluor 647 were purchased from Molecular Probes (Eugene, OR). Purified PDC-109 (SPF1_BOVIN; UniProt/Swiss-Prot Accession number P02784; also known as BSP A1/A2) was a gift from the labs of Puttaswamy Manjunath and Susan Suarez. Rabbit polyclonal antiserum against PDC-109 was a gift from the lab of Susan Suarez. GVA mount was from Zymed (San Francisco, CA). Semen was collected from proven high fertility bulls at Genex/CRI (Ithaca, NY). Bull epididymides were collected from Cudlin's Meat Market (Newfield, NY) and Wyalusing Livestock Market (Wyalusing,

PA). Male CD-1 mice were obtained from Charles River Laboratories (Kingston, NY). All animal work was conducted under the approval of Cornell University's Institutional Animal Care and Use Committee, in accordance with the Guide for Care and Use of Laboratory Animals. Human epididymal sperm was collected from the Center for Male Reproductive Medicine and Microsurgery at Cornell University's Weill Medical College from men with obstructive disorders undergoing epididymal aspiration for use in assisted reproduction. All procedures were performed as part of treatment of these patients and with institutional review board oversight at Weill Cornell Medical Center.

Collection and Handling of Sperm

Bull: Ejaculated bull semen was immediately diluted at a 1:4 ratio in sperm Hepes-buffered Tyrode-albumin lactate pyruvate (TALP H: 100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 0.4 mM $MgCl_2$, 40 mM HEPES, 0.4 mM EDTA, 10 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM pyruvic acid, 50 μg/ml Gentamycin, 1 mg/ml PVA) (Parrish et al. 1988) and transported to the laboratory at 39˚C. Sperm were washed at 39˚C in TALP H. One ml of diluted semen was brought to 7 ml with TALP H and spun at 170 g's for 8 minutes. The sperm were transferred to a round-bottomed tube to repeat the spin twice more, resulting in a loose pellet of sperm, which was resuspended in a final volume of 5 ml.

For experiments involving additional washes of ejaculated bull sperm, the sperm were washed under four separate media conditions: 1) TALP with 250 mM NaCl, 2) TALP with 500 mM NaCl, 3) TALP pH 9, and 4) TALP pH 11. Motility was assessed and sperm counted. No experiment was performed if motility was <50% immediately prior to fluorescent microscopy or fixation. Sperm ($2x10^6$) were then resuspended in

300µl TALP (TALP: 100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 0.4 mM $MgCl_2$, 10 mM HEPES, 0.4 mM EDTA, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM pyruvic acid, 50 µg/ml Gentamycin, 1 mg/ml PVA) (Parrish et al. 1988).

Cauda epididymides of bulls were transported on ice from the abattoir and then warmed to room temperature for the dissection of the epididymides from the testis and connective tissue. Epididymides were washed three times in PBS before being minced in TALP H. Sperm were allowed to swim out for 15 minutes before being washed in TALP H, resuspended in TALP and incubated at 39°C and 5% CO_2.

Mouse: A modified Whitten's medium (MW; 22 mM HEPES, 1.2 mM $MgCl_2$, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemi-calcium salt, pH 7.35) (Travis et al. 2001) containing 5.5mM glucose was used for all mouse sperm incubations. Mature sperm were collected from the cauda epididymides by a swim-out procedure as described previously (Travis et al. 2001) and washed at 37˚C. All incubations of mouse sperm were conducted at 37˚C in MW.

Human: Ejaculated human sperm were collected from healthy male donors, allowed to liquefy for 30 minutes at 37°C, and then diluted 1:4 in MW. The sperm were washed by centrifugation (200 *g*) for 10 min at 37˚C into MW. All incubations of human sperm were carried out at 37°C.

Microsurgical epididymal sperm aspiration was carried out as previously described (Schlegel, Berkeley et al. 1994). Briefly, the epididymides of two patients with documented normal spermatogenesis and obstruction were explored, and the

epididymal segment with optimal sperm motility was identified and aspirated using a micropuncture technique to avoid contamination with red blood cells. The presence of sperm and their motility were confirmed by evaluation during the operative procedure.

Fluorescence Localization of G_{M1} in Live Sperm

All steps of localization experiments were carried out under dim lighting in a light-protected humidity chamber. Sperm ($2x10^6$) were incubated in 300µl MW (mouse, human) or 300µl TALP (bull) containing 10 µg/ml CTB for 10 minutes. For some experiments, different concentrations of epididymal bull sperm were pre-incubated with purified PDC-109 (0.4mg/ml) for 30 minutes prior to CTB addition. For the mouse and bull, a 10 µl aliquot was transferred to a pre-warmed slide and a coverslip was placed over the slide and viewed under a Nikon Eclipse TE 2000-U microscope (Nikon, Melville, NY) equipped with a Photometrics Coolsnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany), and Openlab 3.1 (Improvision, Lexington, MA) automation and imaging software. Human sperm were visualized with an Olympus BX60 epifluorescent microscope equipped with a 37˚C stage warmer and a Peltier-cooled, CCD digital camera controlled by QCapture 2.68.6 software (Quantitative Image Corporation, Burnaby, BC, Canada).

Fluorescence Localization of G_{M1} in Fixed Cells

Bull sperm motility was assessed at the start and completion of washing, and after 1.5 and 3 hours incubation. Mouse and bull samples were removed from the incubation tubes described above and settled on coverslips and the human sperm were placed onto Cel-Line HTC SuperCured 10-spot slides (Cel-Line Association Inc., Newfield, NJ) for 15 minutes to allow the sperm to attach before the supernatant was aspirated. Mouse and human sperm were fixed with 4% paraformaldehyde (PF), 0.1%

glutaraldehyde, and 5 mM $CaCl_2$ in PBS, whereas bull sperm were fixed with 1% PF and 12.5 mM $CaCl_2$ in PBS. The sperm were then washed three times with PBS and incubated for 10 minutes in CTB (10 μg/ml) for G_{M1} localization.

Mouse Seminal Vesicle Fluid Collection and Incubation

The seminal vesicles (SV) were isolated from surrounding blood vessels and the coagulating glands by sharp dissection. Care was taken to avoid contamination with either blood or coagulating gland secretions, as these rapidly catalyze the precipitation of proteins within the SV fluid (Schon et al. 1982). The glands were removed individually and the fluid contents were allowed to drip out and/or were manually expressed into microcentrifuge tubes. Using a large orifice pipette tip, 10 μl of SV fluid was aspirated and the tip and contents were placed in a humidity chamber at 37°C to await sperm addition. Sperm ($4x10^6$) were added to the SV fluid in the pipette tip, the tip was immersed in a 600 μl drop of PBS, and sperm were allowed to swim through the SV fluid, out of the tip into the PBS for 15 minutes. The tip and associated SV fluid were then carefully removed and CTB (10 μg/ml final concentration) was added to the coverslip and allowed to incubate for 10 minutes. The supernatant was aspirated before the coverslip was placed on a pre-warmed slide and viewed for G_{M1} localization.

Protein assays were conducted on seminal vesicle fluid, and the concentrations (ranging from 167 to 350 mg/ml) were in accord with published levels**,** which range from 250 to 350 mg/ml (Mann & Lutwak-Mann 1981). As a control for exposure to an equivalent amount of protein as that found in the SV fluid, sperm ($4x10^6$) were incubated in 600 μl of PBS with casein that was varied to match the total amount of

protein contained in the SV fluid (ranging from 1.67 to 3.5 mg) for 15 minutes, prior to incubation with CTB.

Bull Seminal Plasma Isolation and Incubation

Ejaculated bull sperm was diluted 1:3 in TALP H and transported to the laboratory at 39°C. This volume was then spun at 800 g's for 10 minutes to pellet the sperm fraction. The supernatant was carefully aspirated without disturbing the sperm pellet and snap frozen for later use.

Aliquots of frozen, dilute seminal plasma were thawed at 39°C and examined with light microscopy to verify the absence of sperm before incubation. Epididymal bull sperm (4×10^6) were incubated in 600 μl dilute seminal plasma) for 10 minutes before fluorescence localization as described for live epididymal bovine sperm.

Immunofluorescence

Epididymal bull sperm incubated with and without purified PDC-109 were processed as previously described (Desnoyers & Manjunath 1992) with minor modifications. Briefly, aliquots of the sperm suspensions were added to slides on a 37°C warming stage and allowed to air dry. Slides were blocked in 0.1% BSA overnight before an additional overnight incubation with anti-PDC-109 (1:500) followed by three washes with PBS and incubation with secondary antibody. Slides were washed three times in PBS before being mounted with GVA and visualized by epifluorescence.

Results

Ejaculates from some bulls displayed a low percentage of sperm with faint CTB binding over the acrosome, but the vast majority of motile ejaculated bull spermatozoa failed to bind CTB. However, immotile bull sperm uniformly displayed a post-acrosomal pattern of fluorescence (Fig. 1A-B). These findings were remarkable in comparison with motile epididymal murine sperm, which show an acrosomal pattern of CTB in all motile cells (Fig. 1C-D). We have shown previously that upon cessation of motility, epididymal murine sperm switch from having a pattern of CTB localization overlying the acrosome to a post-acrosomal pattern ((Selvaraj et al. 2005); shown here in Fig. 1E-F).

During the process of ejaculation, sperm are exposed to several accessory sex gland secretions that interact with a sperm's plasma membrane (Desnoyers & Manjunath 1992; Manjunath et al. 1994) and which could potentially interfere with the ability of exogenous reagents to bind to sites on this membrane. The differences in reported results between species, and between our findings in the mouse and the findings shown in Fig. 1 in the bull, could therefore have been due to true species differences or a difference between sperm collected from the epididymis versus those from an ejaculate. To distinguish between these possibilities, we next localized G_{M1} in epididymal bull spermatozoa. All morphologically normal epididymal bull sperm that were motile (Fig. 2A) displayed a pattern of G_{M1} localization identical to that seen in epididymal mouse sperm (Fig. 1C-D), with fluorescence restricted to an area of the plasma membrane overlying the acrosome and also throughout the flagellum. Interestingly, live epididymal spermatozoa exhibiting abnormal morphology also displayed aberrant patterns of G_{M1} localization (Fig. 2B). For example, sperm with

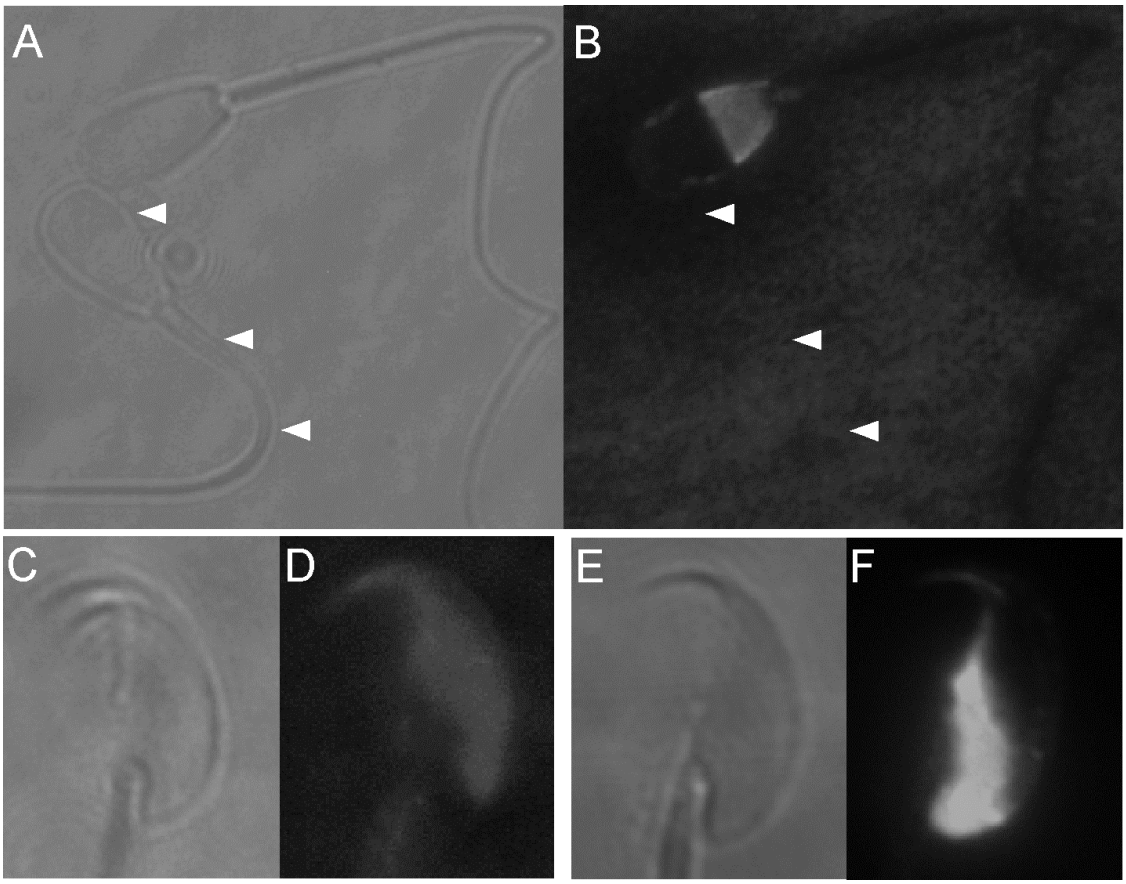

Figure 1. A comparison of motile and immotile ejaculated bull sperm incubated with CTB (n=9 experiments; over 100 cells examined per treatment for this and subsequent experiments). A) Transmitted and B) fluorescent images of ejaculated bull sperm. Note the lack of signal in the motile cell. Arrows indicate the location of the live cell present next to an immotile cell displaying a post-acrosomal pattern of fluorescence. Transmitted and fluorescent images of motile (C-D) and immotile (E-F) epididymal murine spermatozoa incubated with CTB.

proximal droplets were frequently observed to have abnormal CTB binding over the area of the proximal droplet, connecting piece, and caudal portion of the post-acrosomal sub-domain of the head.

Based on the results of G_{M1} localization in epididymal bull sperm, we hypothesized that exposure of murine epididymal sperm to accessory sex gland fluids would also result in a loss of CTB binding and/or fluorescence in the plasma membrane overlying the acrosome. The majority of bull and mouse ejaculate volume originates from the SV (Seidel & Foote 1970; Mann & Lutwak-Mann 1981). We therefore allowed epididymal mouse sperm to swim through SV secretions and then observed the pattern of CTB fluorescence. As predicted, exposure to SV fluid significantly reduced the ability of CTB to bind G_{M1} as compared to normal epididymal murine sperm (Fig. 3; $p<0.05$, n=3). CTB fluorescence was undetectable in the heads of the majority of cells (49%; Fig. 3A, D), with remaining cells displaying a patchy, mottled pattern of fluorescence (47%; Fig. 3B). To demonstrate that non-specific interactions with an equivalent amount of protein would not cause this decrease in binding, motile sperm were incubated in base medium alone (data not shown) or with casein (Fig 3C). In both cases, motile sperm displayed an acrosomal pattern of fluorescence. In both of those treatment conditions, the vast majority of immotile cells displayed a post-acrosomal pattern (n=4 experiments; data not shown). Together, these results suggested an interaction of the SV fluid with the sperm plasma membrane overlying the acrosome that sterically and/or specifically prevented the binding and/or fluorescence of CTB to G_{M1}. Similarly, epididymal bull sperm exposed to seminal plasma isolated from an ejaculate lost CTB binding over the acrosome and appeared identically as those collected from an ejaculate (n=3 experiments; data not shown).

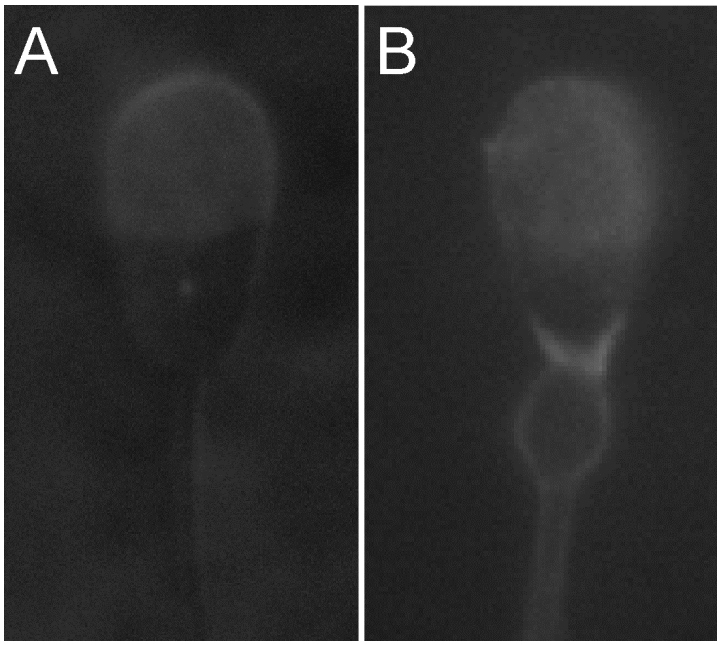

Figure 2. Epididymal bull sperm incubated with CTB (n=3 experiments representing 12 individual bulls). Survey image of motile epididymal sperm displaying acrosomal patterns of fluorescence (A). Fluorescent image of a motile cell displaying an acrosomal pattern of fluorescence (B). Motile cell possessing a proximal cytoplasmic droplet and displaying abnormal CTB fluorescence (C).

The similarities between the pattern of G_{M1} localization reported for human sperm (Cross 2004) and our results for epididymal mouse sperm exposed to SV secretions suggested that the localization in epididymal human sperm would also be in the plasma membrane overlying the acrosome. We found that CTB binding in ejaculated human sperm matched data previously reported for the human (Cross 2004), with little to no fluorescence in motile cells (data not shown). We therefore examined G_{M1} localization in epididymal human sperm. One caveat regarding these experiments is that normal human epididymal sperm are difficult to procure. Typically, epididymal aspirations are performed in men who have obstructive pathologies, and the sperm collected tend to be abnormal. Accordingly, the epididymal human sperm we examined exhibited a very high percentage of abnormal morphologies, particularly large proximal droplets and irregular head shapes. However, the small population of motile cells with normal morphology seen did display an acrosomal pattern of fluorescence (Fig. 4A-B). As found in the bull, a high percentage of cells possessing abnormal morphologies also exhibited atypical patterns of G_{M1} localization (Fig. 4C-F).

In all three species examined, we observed a distinct and reproducible pattern of G_{M1} localization to the plasma membrane overlying the acrosome in motile epididymal sperm. Exposure of sperm of these three species to accessory sex gland secretion either through normal ejaculation (bull or human) or in vitro exposure (mouse and bull) resulted in a loss of CTB signal. Two possible interpretations existed. One was

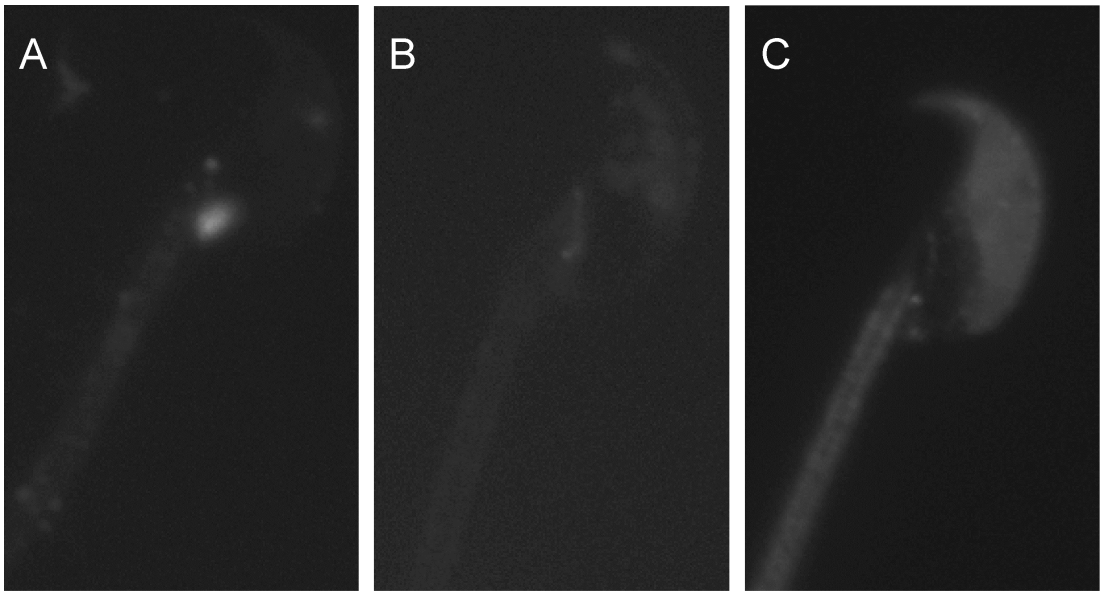

Figure 3. Effect of exposure to seminal vesicle fluid on CTB binding in murine spermatozoa (n=3 experiments). Sperm incubated with SV fluid prior to incubation with CTB displayed little (A) or dim and mottled (B) fluorescence when compared to sperm incubated with an equivalent amount of casein prior to CTB incubation (C). Normal motile epididymal mouse sperm display 100% acrosomal fluorescence prior to SV fluid exposure (n=3).

that exposure to these secretions inhibited binding of CTB to the G_{M1}, while the other was that exposure to seminal plasma removed G_{M1} from the plasma membrane. To rule out qualitatively the large-scale loss of GM1, we fixed ejaculated bull sperm and epididymal mouse sperm exposed to SV fluid prior to incubation with CTB. Sperm exposed to accessory sex gland secretions and subsequently fixed had a GM1 localization pattern that mirrored the pattern and intensity of live epididymal sperm and the pattern and intensity of CTB signal in epididymal sperm fixed without exposure to SV fluid (Fig. 5). This unmasking of G_{M1} with fixation suggested that the majority of G_{M1} remained in the sperm plasma membrane even after exposure to accessory sex gland secretions.

The majority of accessory sex gland secretions in the bull originate from the seminal vesicles, with the major constituent being PDC-109, which binds phospholipids in the sperm plasma membrane (Desnoyers & Manjunath 1992). To determine whether PDC-109 binding to sperm was responsible for the loss of CTB binding to G_{M1}, we incubated epididymal bull sperm with purified PDC-109 prior to incubation with CTB. We found no loss of CTB binding, with labeling of the plasma membrane overlying the acrosome as in untreated epididymal sperm (Fig. 6A; n=3 experiments) As a control to demonstrate that the purified PDC-109 was binding these sperm, we performed indirect immunofluorescence (Fig. 6B-C).

In a further attempt to remove and qualify the inhibition of CTB binding G_{M1} in ejaculated sperm, ejaculated bull sperm was washed in TALP modified with either high salt (NaCl 250 mM and 500 mM) or high pH (9 and 11). Although binding was recovered in a low percent of sperm washed in 500 mM NaCl (13.5%), a treatment

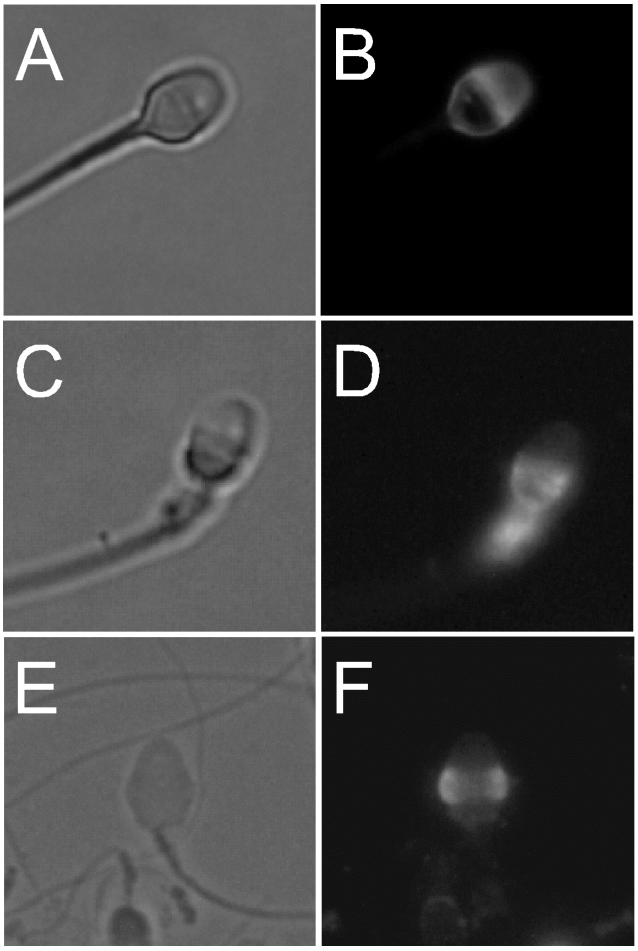

Figure 4. G_{M1} localization in epididymal human sperm (n=2 experiments, representing two individuals). Transmitted (A) and fluorescent images (B) of motile epididymal human sperm, showing normal morphology and an acrosomal pattern of fluorescence with CTB. Transmitted (C) and fluorescent images (D) of immotile human sperm displaying signal over the post-acrosomal region and a proximal droplet. Other abnormal morphologies observed in brightfield, such as this misshapen head (E), were often accompanied by abnormal patterns of CTB binding and fluorescence (F).

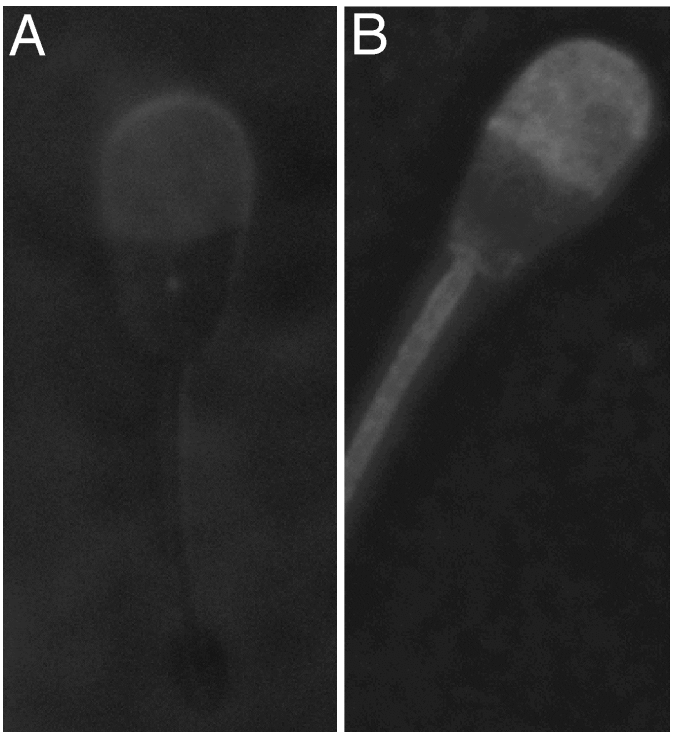

Figure 5. G_{M1} was not removed from sperm upon exposure to accessory sex gland secretions. G_{M1} localization in live epididymal bull sperm (A) or ejaculated bull sperm fixed with 1% paraformaldehyde, 12.5mM $CaCl_2$ before incubation with CTB (B; n=6 experiments).

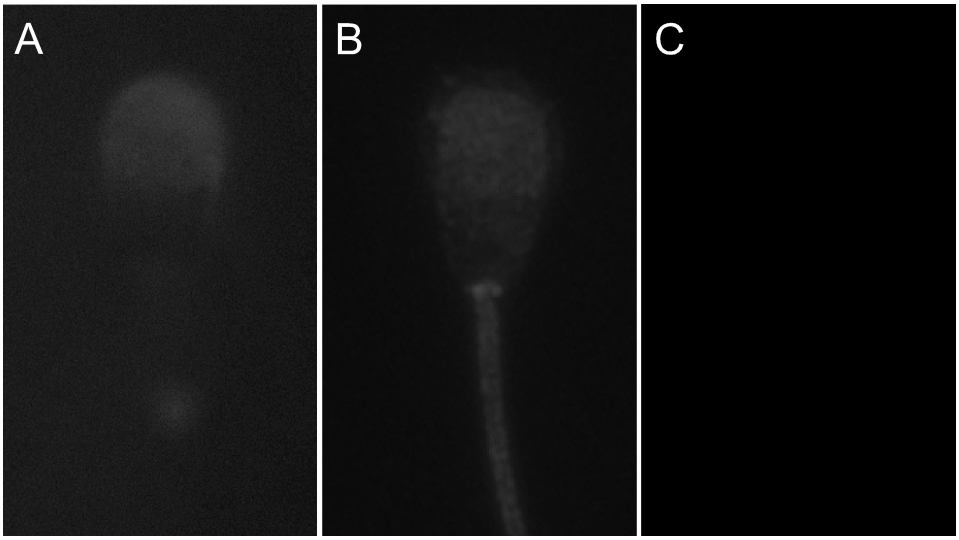

Figure 6. Incubation of epididymal bull sperm with purified PDC-109 did not prevent CTB binding to G_{M1}. Fluorescent image of CTB bound to epididymal bull sperm after incubation with purified PDC-109 (A; n=3 experiments). Indirect immunofluorescence localization of PDC-109 in epididymal bull sperm after this incubation, showing that the purified PDC-109 did bind to the plasma membrane overlying the acrosome (B). Incubation of sperm with the secondary antibody alone served as a control for specificity of binding (C).

which also stopped motility, none of the conditions tested significantly altered the fluorescence pattern in ejaculated bull sperm from that of ejaculates washed in normal TALP H (pH 7.35, NaCl 100mM) (n=3, data not shown).

Discussion

Lipid rafts have been postulated to play a role in signaling and effector complex compartmentalization in sperm plasma membranes. Because of this, several studies have attempted to observe membrane sub-domains in live and fixed sperm, but have reported widely differing results. The results obtained in this study suggest that exposure to secretions from accessory sex glands might mask the true localization of specific lipids—and therefore membrane sub-domains—in mammalian sperm, accounting for some of the discrepancies within the literature.

Our previous work has described the segregation of the ganglioside G_{M1} to the plasma membrane overlying the acrosome in live epididymal murine spermatozoa (Selvaraj et al. 2005). This pattern was identical to the pattern of sterol localization seen in fixed sperm with filipin (Friend 1982; Lin & Kan 1996; Visconti et al. 1999) and of the lipid-raft-associated protein, caveolin-1 (Travis et al. 2001). The segregation of a sphingolipid (G_{M1}), sterols, and caveolin-1 to this region suggests the presence of a lipid raft sub-domain extreme in terms of size and stability (Selvaraj et al. 2005). The biochemical partitioning of caveolin-1 to detergent-resistant membranes, as well as to fractions with light buoyant-density separated without the use of detergents (Travis et al. 2001), supports the raft-like nature of such a sub-domain. Use of an exogenous lipid probe that partitions to liquid-ordered domains has recently confirmed the "raft" nature of this micron scale sub-domain (Sleight et al. 2005).

Other studies of CTB binding in epididymal rodent sperm had previously suggested a different pattern of localization, with G_{M1} being restricted to the plasma membrane of the post-acrosomal sub-domain in rat (Roberts et al. 2003) and mouse (Trevino et al. 2001). Our recent discovery that CTB induces a redistribution of G_{M1} from the plasma membrane overlying the acrosome to the post-acrosomal plasma membrane upon cell death and in lightly fixed sperm (Selvaraj et al. 2005) can account for the differences observed in rodent studies. However, the present report is the first to demonstrate that interaction with seminal plasma can account for many of the other reported differences among species.

For example, fluorescence localization of G_{M1} in ejaculated human spermatozoa was reported to show no large-scale segregation of G_{M1} (Cross 2004). Similar studies of ejaculated boar sperm reported a lack of G_{M1} localization in the majority of sperm heads, although this percentage decreased with time and incubation with reagents known to mediate sterol efflux (Shadan et al. 2004). The trend toward increased acrosomal fluorescence observed in that study was interpreted as sterol efflux-induced raft organization, which would be consistent with previous models suggesting that lipid rafts form during capacitation (Flesch et al. 2001). Both our published results and those herein contrast with such a model, and alternately suggest that lipid sub-domain segregation exists on a micron scale in epididymal sperm prior to exposure to capacitating stimuli. Our results therefore help reconcile these conflicting reports, suggesting either a specific competitive inhibitor of binding to G_{M1} or a non-specific steric masking of G_{M1} in the plasma membrane overlying the acrosome by substances within accessory sex gland secretions.

The binding of proteins secreted by the accessory sex glands to specific molecules of the sperm plasma membrane has been well characterized in several species, including the mouse and bull (Manjunath and Sairam 1987; Miller et al. 1990; Desnoyers & Manjunath 1992; Manjunath et al. 1994; Greube et al. 2001; Ignotz et al. 2001; Luo et al. 2001). Bovine seminal plasma (BSP) proteins such as PDC-109 (SPF1_BOVIN), BSP A3 (SPF3_BOVIN), and BSP-30kDa (SPF4_BOVIN) (UniProt/Swiss-Prot Accession numbers P02784, P04557, and P81019, respectively) preferentially bind to phospholipids enriched in the plasma membrane overlying the acrosome and promote membrane stabilization and subsequent destabilization upon their removal during capacitation (Desnoyers & Manjunath 1992; Manjunath et al. 1994; Therien et al. 1998; Manjunath & Therien 2002). PDC-109 is the most abundant of these proteins, so we investigated whether its binding to plasma membrane phospholipids would inhibit CTB- G_{M1} binding. This was not the case, suggesting either another protein or seminal plasma component was responsible for this loss.

One additional finding of this work bears discussion for its possible clinical relevance. The aberrant patterns observed in human and bull sperm with proximal droplets and other abnormalities provide a correlation between morphological defects associated with reduced fertility and abnormal distribution of plasma membrane lipids. Interestingly, sperm with morphological defects in one region, such as proximal droplets, also showed abnormal G_{M1} distribution in surrounding regions that appeared morphologically normal at the level of light microscopy. This suggests such defects might be more widespread than are immediately obvious at the level of light microscopy. Large proximal droplets have been associated with reduced fertility (Thundathil et al. 2001; see Cooper 2005, for review), although an exact cause for this impairment has not been described. It is intriguing to speculate that appropriate lipid

compartmentalization and function might provide a molecular underpinning for such defects. The localization of lipids such as G_{M1} may therefore prove to be of value as a screening tool in evaluating male fertility.

The data presented in this chapter provide evidence for the segregation of G_{M1} to the plasma membrane overlying the acrosome in three different families of mammals. These data suggest that the formation of large membrane sub-domains in mammalian sperm has been conserved evolutionarily, and that these compartmentalized domains might have important roles in sperm function. The co-localization of G_{M1}, sterols, and caveolin-1 to this lipid raft sub-domain suggests possible mechanisms by which the process of sterol efflux might be transduced into the functional changes that allow a sperm to fertilize an egg. Because of this, studies into the dynamic responses of G_{M1} and this sub-domain to stimuli associated with sperm capacitation, the acrosome reaction, and fertilization have begun.

ACKNOWLEDGEMENTS

We would like to thank Puttaswamy Manjunath (Department of Medicine, University of Montreal and Guy-Bernier Research Center, Maisonneuve-Rosemont Hospital, Montreal, Quebec, Canada) for the generous gift of purified PDC-109, and Susan Suarez (College of Veterinary Medicine, Cornell University, Ithaca, NY) for the generous gifts of purified PDC-109 and PDC-109 antibody.

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CHAPTER 4

G_{M1} DYNAMICS AS A MARKER FOR MEMBRANE CHANGES ASSOCIATED WITH THE PROCESS OF CAPACITATION IN MURINE AND BOVINE SPERMATOZOA[1]

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As published in (2007) *J Androl 28*(4), 588-99.
*These authors contributed equally to this work. V. Selvaraj completed experiments for patterns of G_{M1} in the mouse and the SEM of flagellar GM1 while D. E. Buttke completed patterns of G_{M1} in the bull and G_{M1} during acrosomal exocytosis in the mouse and bull. A Asano assisted in figure 4; J McElwee, C Wolff, J Nelson A Klaus provided technical assistance; G Hunnicutt assisted in scientific design, and A Travis was primary supervisor.

Abstract

We previously showed that in live murine and bovine sperm heads, the ganglioside G_{M1} localizes to the sterol-rich plasma membrane overlying the acrosome (APM). Labeling G_{M1} using the pentameric cholera toxin subunit B (CTB) induced a dramatic redistribution of signal from the APM to the sterol-poor postacrosomal plasma membrane (PAPM) upon sperm death. We now show a similar phenomenon in the flagellum where CTB induces G_{M1} redistribution to sterol-poor membrane subdomains of the annulus and flagellar zipper. Because sterol efflux from the plasma membrane is required for capacitation, we examined whether G_{M1} localization might be useful to detect membrane changes associated with capacitation and/or acrosomal exocytosis. First, incubation of murine and bovine sperm with their respective stimuli for capacitation did not change G_{M1} distribution in live cells. However, incubation of sperm of both species with specific stimuli for capacitation, followed by the use of specific fixation conditions, induced reproducible, stimulus-specific patterns of G_{M1} distribution. By assessing changes in G_{M1} distribution in response to progesterone-induced AE, we show that these patterns reflect the response of murine sperm populations to capacitating stimuli. These data suggest that G_{M1} localization can be used as a diagnostic tool for evaluating sperm response to stimuli for capacitation and/or AE. Such information could be useful when deciding between technologies of assisted reproduction or when screening for male fertility. Furthermore, stimulus-specific changes in G_{M1} distribution showed that sperm could respond to NaHCO3 or mediators of sterol efflux independently, thereby refining existing models of capacitation.

Introduction

"Capacitation" describes the maturational changes a sperm must undergo in the female reproductive tract to gain competence to fertilize an egg (Chang, 1951; Austin, 1952). The external stimuli required for capacitation can differ among species based on conditions encountered in the respective female tracts. For many species, these stimuli include efflux of sterols from the sperm plasma membrane (Davis, 1974; Davis et al, 1979) and the presence of bicarbonate and calcium ions (Neill & Olds-Clarke, 1987; DasGupta et al, 1993; Visconti et al, 1995a) and glucose (Travis et al, 2001a; Urner et al, 2001). Notably, bovine sperm require the presence of heparin (Parrish et al, 1988) and the absence of glycolyzable substrates (Parrish et al, 1989; Galantino-Homer et al, 2004) for capacitation.

In vitro capacitation of sperm using such stimuli effects changes in membrane properties that have been reported to lead either directly or indirectly to several downstream events. These include plasma membrane hyperpolarization (Zeng et al, 1995; Arnoult et al, 1999), cyclic adenosine monophosphate-dependent protein kinase A activation and protein tyrosine phosphorylation (Visconti et al, 1995a; Visconti et al, 1995b), loss of plasma membrane bilayer phospholipid asymmetry and lipid order (Harrison et al, 1996; Gadella & Harrison, 2000; Flesch et al, 2001; Gadella & Harrison, 2002; Cross, 2003), phosphatidyl inositol signaling-mediated cytoskeletal remodeling (Brener et al, 2003; Breitbart et al, 2005), and calcium influx/release from internal stores (Ho & Suarez, 2001a, 2001b; Carlson et al, 2003; Herrick et al, 2005). However, it remains unclear how the changes at the level of the membrane and the downstream signaling events are transduced into hyperactivated motility in the tail and

priming of the membranes of the sperm head for acrosomal exocytosis (AE). Although it has been shown that only a subpopulation of sperm responds to the stimuli for capacitation through protein tyrosine phosphorylation (Urner et al, 2001), there is lack of reliable and easy means to evaluate the capacitation status of sperm in response to a given stimulus/stimuli, be it within a population or a comparison between populations.

Currently, the most widely used assay for capacitation status involves patterns of fluorescence intensity in the sperm head using the fluorescent antibiotic chlortetracycline (Saling & Storey, 1979). More recently, studies on sperm membrane lipid organization have led to assays using merocyanine 540 to detect changes in packing order of lipids on the outer leaflet of the plasma membrane (Williamson et al, 1983) that are believed to change with capacitation (Rathi et al, 2001). In addition, annexin V has been used to bind and detect phosphatidyl serine on the outer leaflet of the plasma membrane indicating activation of phospholipid scramblase activity (Flesch et al, 2001). However, concerns about the effectiveness of merocyanine 540 in detecting capacitated vs abnormal/damaged sperm have been raised (Muratori et al, 2004), and phospholipid scramblase-mediated phosphatidyl serine exposure in capacitated sperm does not appear to be conserved in all species (Baumber & Meyers, 2006). Further complicating the study of changes to the state of the plasma membrane of the sperm head is the organization of this membrane into discrete micron-scale subdomains based on sterol and sphingolipid composition (Friend & Fawcett, 1974; Selvaraj et al, 2006). In the heads of fixed sperm from several species, the plasma membrane overlying the acrosome (APM) was found to be enriched in sterols and distinctly segregated from the postacrosomal plasma membrane (PAPM) that was found to be relatively sterol poor (Friend, 1982, 1989; Pelletier & Friend, 1983; Lin & Kan, 1996). In addition, within the APM are at least 2 distinct areas of membrane—

one over the apical acrosome (AA) and a larger one over the equatorial segment (ES) (Friend, 1989; Lin & Kan, 1996; Selvaraj et al, 2006).

Membrane rafts are defined as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Groups of small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006). However, studies on membrane rafts have generated significant controversy regarding the existence and dynamics of these subdomains in cells—specifically, that artifacts appearing as rafts might be induced by the use of detergents or crosslinking reagents/fixatives in attempts to visualize these subdomains (Munro, 2003).

To visualize potential membrane subdomains in live sperm in the absence of any fixative, we used the B subunit of cholera toxin (CTB) to bind the ganglioside G_{M1}. We found that this sphingolipid does indeed segregate to the APM, as did sterols and the sterol-binding protein caveolin-1 in fixed sperm (Travis et al, 2001b). Other investigators have reported the use of fluorescent lipid probes to suggest that the APM subdomain behaves in a liquid-ordered fashion consistent with a raft (Sleight et al, 2005). The size and stability of the APM and PAPM subdomains in mammalian sperm are quite extreme in comparison with their counterparts in somatic cells, making it possible that the APM of live sperm represents a "super raft" of stably segregated smaller sub-subdomains. Fitting the proposed theory behind larger raft platforms, we found that the lipid segregation in sperm is maintained at least in part by disulfide-bonded proteins (Selvaraj et al, 2006). This is consistent, albeit at a larger scale, with a membrane compartmentation model of segregation (Kusumi et al, 2004). We found that this segregation to the APM was highly conserved across mammals, being present

in murine, bovine, and human sperm, and that discrepancies in the literature between species were at least in part due to confounding effects of seminal plasma (Buttke et al, 2006). The organization of these membrane subdomains in sperm continues to be of great interest because of the pathways that potentially might be targeted to the APM super raft, which could function in capacitation, binding to the zona pellucida, and/or AE.

Although we were able to demonstrate distinct segregation of endogenous lipids in live sperm, the pitfalls inherent to localizing lipids in a biological system did reveal themselves in our studies. For example, we observed the interesting phenomenon that within seconds of a sperm's death (inferred by cessation of motility), CTB bound to G_{M1} helped induce a dramatic redistribution to the PAPM (Selvaraj et al, 2006). In the present study, we show a similar redistribution phenomenon seen in the sperm tail while exploring variations in sperm G_{M1} dynamics in response to stimuli for capacitation and AE. Our results not only demonstrate changes in individual cells but also shed light on the nature of functional subpopulations of sperm and the temporal dynamics of capacitation pathways.

Materials and Methods

Reagents and Animals

All reagents were purchased from Sigma (St Louis, Mo) unless otherwise noted. CTB conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, Calif) was used. Male CD-1 mice were purchased from Charles River Laboratories (Kingston, NY). Bovine semen was collected from Holstein bulls of known high fertility at Genex Corporation

(Ithaca, NY). All animal procedures were performed under the guidelines of the Institutional Animal Care and Use Committee at Cornell University.

Preparation of Media

For murine sperm, a modified Whitten medium (MW; 22 mM HEPES, 1.2 mM MgCl2, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemicalcium salt, pH 7.35 [Travis et al, 2001a]) was used for all incubations. Glucose (5.5 mM), NaHCO3 (10 mM), and 2-hydroxypropyl-ß-cyclodextrin (2-OHCD; 3 mM) were supplemented as needed. The 2-OHCD supports sperm capacitation and in vitro fertilization (IVF) by functioning as a sterol acceptor and is preferred over the more potent methyl-ß-cyclodextrin (Visconti et al, 1999).

For diluting and transporting bovine semen, a HEPES-buffered Tyrode-albumin lactate pyruvate medium (TALP-H; 100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH2PO4, 21.6 mM sodium lactate, 0.4 mM MgCl2, 40 mM HEPES, 0.4 mM ethylenediamine tetraacetic acid (EDTA), 10 mM NaHCO3, 2 mM CaCl2, 1 mM pyruvic acid, 1 mg/mL polyvinyl alcohol (PVA; Parrish et al, 1988) was used. For washing and incubation of bovine sperm, TALP medium (100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH2PO4, 21.6 mM sodium lactate, 0.4 mM MgCl2, 10 mM HEPES, 0.4 mM EDTA, 25 mM NaHCO3, 2 mM CaCl2, 1 mM pyruvic acid, 1 mg/mL PVA (Parrish et al, 1988) was used. Bovine serum albumin (BSA; 6 mg/mL) and heparin (20 µg/mL) were supplemented as needed to facilitate capacitation (Galantino-Homer et al, 1997).

Sperm Collection and Handling

Murine sperm were collected from the cauda epididymides of male CD-1 mice by a swim-out procedure as described previously (Travis et al, 2001b). All steps of

collection and washing were performed at 37°C using MW medium, and large-orifice transfer pipettes or large-orifice pipette tips were used for handling sperm to minimize membrane damage. Bull semen collected from proven high-fertility bulls was immediately diluted (1:4) using TALP-H and transported to the laboratory at 39°C. All steps of washing and sperm handling were performed at 39°C as described previously (Buttke et al, 2006). After the initial washes but prior to experimental incubations, motility assessment was carried out for both mouse and bull sperm, and samples showing less than 60% motility for murine sperm and less than 80% motility for bovine sperm were not used.

Sperm Capacitation and Induction of AE

For murine sperm, incubation with different stimuli for capacitation was carried out with 2 x 10^6 sperm in 300 µL of medium with glucose under 1 of 4 conditions: (a) MW base medium, (b) MW supplemented with 10 mM NaHCO3, (c) MW supplemented with 3 mM 2-OHCD, and (d) MW with both 10 mM NaHCO3 and 3 mM 2-OHCD for 45 minutes (or 60 minutes for all conditions when inducing AE). The pH of medium for all incubation conditions was adjusted to 7.35. The medium in incubation condition ("d") has been shown to be sufficient to support IVF (Travis et al, 2004) and capacitation-induced tyrosine phosphorylation (Travis et al, 2001a) in murine sperm. Progesterone was added to a final concentration of 20 µM to induce AE in capacitated murine sperm (Roldan et al, 1994; Murase and Roldan, 1996; Kobori et al, 2000) (A 2 mM working stock was prepared in MW immediately before use from a 20 mM stock of progesterone in dimethylsulfoxide (DMSO); 0.2% vol/vol final DMSO concentration.). The dead spaces of tubes used for all incubations were filled with nitrogen to avoid the generation of bicarbonate anions in the aqueous media in

conditions "a" and "c." This had no effect on protein tyrosine phosphorylation events associated with capacitation (data not shown).

For bovine sperm, incubation with different stimuli for capacitation was carried out with 2 x 10^6 sperm in 300 µL of medium under 1 of the 4 conditions: (a) TALP base medium, (b) TALP supplemented with BSA (6 mg/mL), (c) TALP supplemented with heparin (20 µg/mL), (d) TALP with both BSA and heparin at the same concentrations for 90 minutes at 39°C. Lysophosphatidyl choline (100 µg/mL final concentration) was used for the induction of AE (Parrish et al, 1989). For live sperm of both species, the localization pattern of G_{M1} was visualized using CTB after incubation under one of the conditions described and/or after the induction of AE.

Fluorescence Localization of G_{M1} in Mature Sperm

All steps of localization experiments using either live or fixed sperm were carried out under dim lighting at 37°C in a humidity chamber. In all cases, the localization of G_{M1} was visualized with CTB. For localization of G_{M1} in fixed samples, sperm were allowed to adhere to coverslips for 20 minutes (at the end of incubations for capacitation experiments) and then fixed for 10 minutes with either 0.004% paraformaldehyde (PF) in phosphate buffered saline (PBS) for murine sperm or 1% PF with 12.5 mM CaCl2 in PBS for bovine sperm. The sperm were then washed with PBS and incubated for 10 minutes with CTB (5 µg/mL). The sperm were washed again and mounted using a GVA mountant (Invitrogen). For all conditions in experiments evaluating pattern change associated with AE, murine sperm were fixed while in suspension by adding an equal volume of 0.008% PF, were incubated with CTB as above, and aliquots were then placed directly on slides for microscopy.

Microscopy and Image Collection

Cells were viewed with a Nikon Eclipse TE 2000-U microscope (Nikon, Melville, NY) equipped with a Photometrics Coolsnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany) and Openlab 3.1 (Improvision, Lexington, Mass) automation and imaging software. Assignments of sperm to G_{M1} localization patterns were performed in a blind fashion regarding incubation condition. To compare shifts in population tendencies, the numbers of sperm having a given pattern were converted to percentages prior to statistical evaluation. In all cases, 100 or more cells were counted for each test condition, and every sperm in a given field was counted to avoid potential bias. Sperm with morphologic abnormalities showing aberrant G_{M1} localization patterns (as described in Buttke et al, 2006) were not included in the count.

Scanning Electron Microscopy

SEM was performed using 2 techniques to visualize surface topography. In the first method, sperm were fixed for 2 hours with 2.5% glutaraldehyde in 100 mM sodium cacodylate and 1% tannic acid at pH 7.4 in a culture tube. After washing by centrifugation and resuspension, they were again fixed with 2% osmium tetroxide and 2% sodium cacodylate at 4°C overnight. The cells were washed and dehydrated in ethanol with a 20-minute incubation in 2% uranyl acetate at 70% ethanol. Once in absolute ethanol, they were critical point dried, coated, and viewed using a Hitachi S4500 scanning electron microscope (Hitachi, Pleasanton, Calif). Digital micrographs were collected using a Princeton Gamma Tech digital beam acquisition program (Imix, Princeton, NJ).

In the second method, sperm were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate (pH 7.4) overnight at 4°C. A 20 µL aliquot of sperm suspension was then

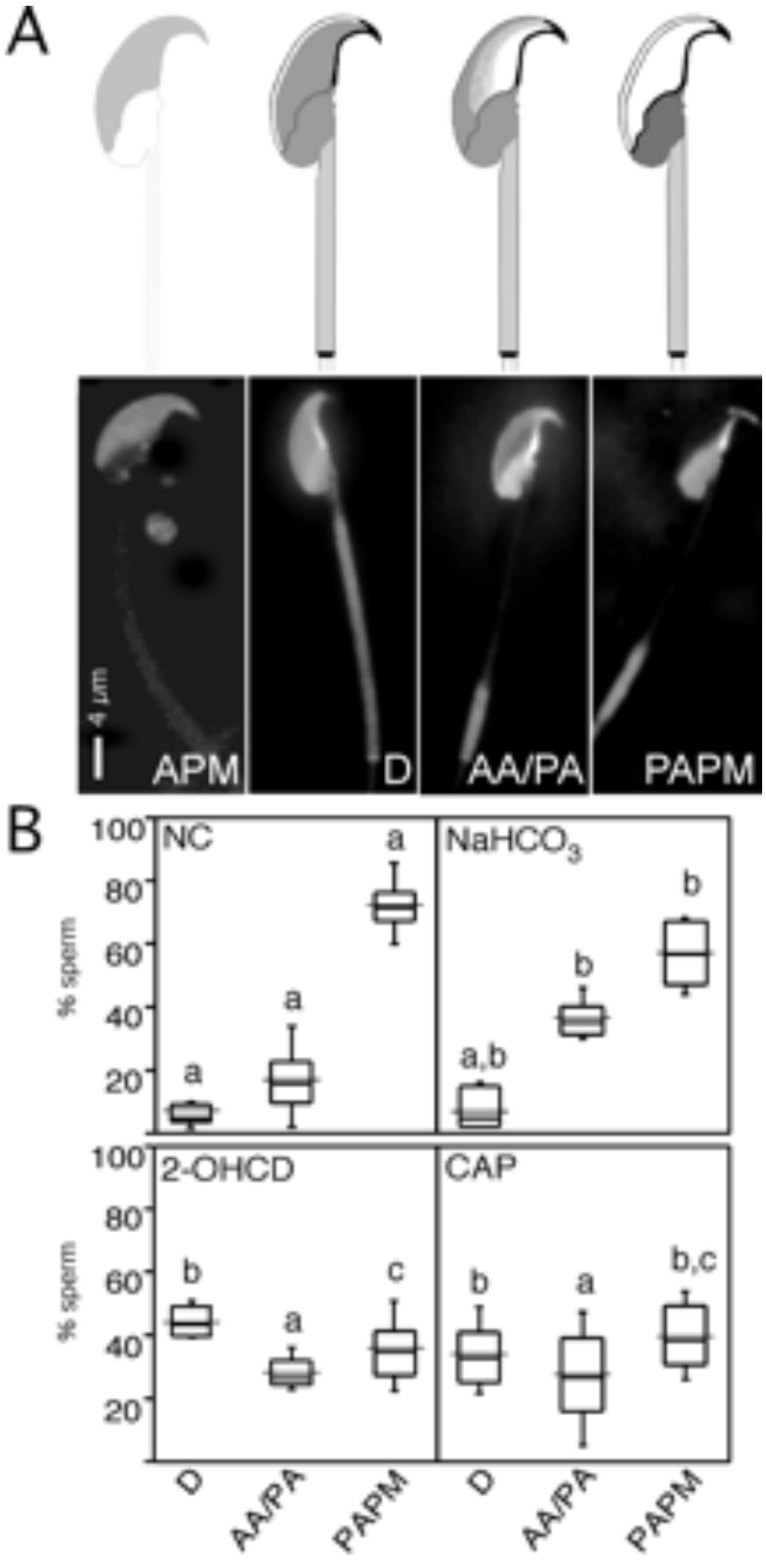
washed in 100 mM sodium cacodylate buffer by centrifugation and resuspension. Aliquots were placed on poly-L-lysine–coated 12-mm round coverslips and allowed to incubate for 45 minutes at 4°C. The coverslips were rinsed by gentle dipping 3 times in cacodylate buffer and then dehydrated in ethanol followed by 2 changes in 100% ethanol for 20 minutes each. Coverslips were critical point dried, sputter-coated with gold/palladium (Au/Pd) and imaged on a Hitachi S4700 cold field-emission scanning electron microscope operating at 10-kV accelerating voltage and 7-μA emission current. Digital images were captured at 2500 x 1900 pixel resolution.

Results

Effect of stimuli for capacitation on CTB-induced G_{M1} patterns

We previously showed in live murine sperm that CTB bound to G_{M1} exclusively in the APM. However, almost immediately upon sperm death, the crosslinking produced by the pentameric CTB induced a redistribution of G_{M1} to the PAPM. We also showed that weak fixatives (eg, 0.004%–1% PF) did not prevent this redistribution but that strong fixation (eg, 4% PF with 0.1% glutaraldehyde) could immobilize G_{M1} to where it had been in live murine sperm (Selvaraj et al, 2006). In the present study, we investigated whether membrane changes brought about by different stimuli for capacitation (alone or in combination) could affect the distribution of G_{M1} in murine and bovine sperm. In live sperm, as we demonstrated for sterol efflux (Selvaraj et al, 2006), bicarbonate had no effect on the localization of G_{M1} (data not shown). However, we found interesting variations in patterns of G_{M1} localization in response to different stimuli for capacitation followed by incubation under species-specific fixation conditions.

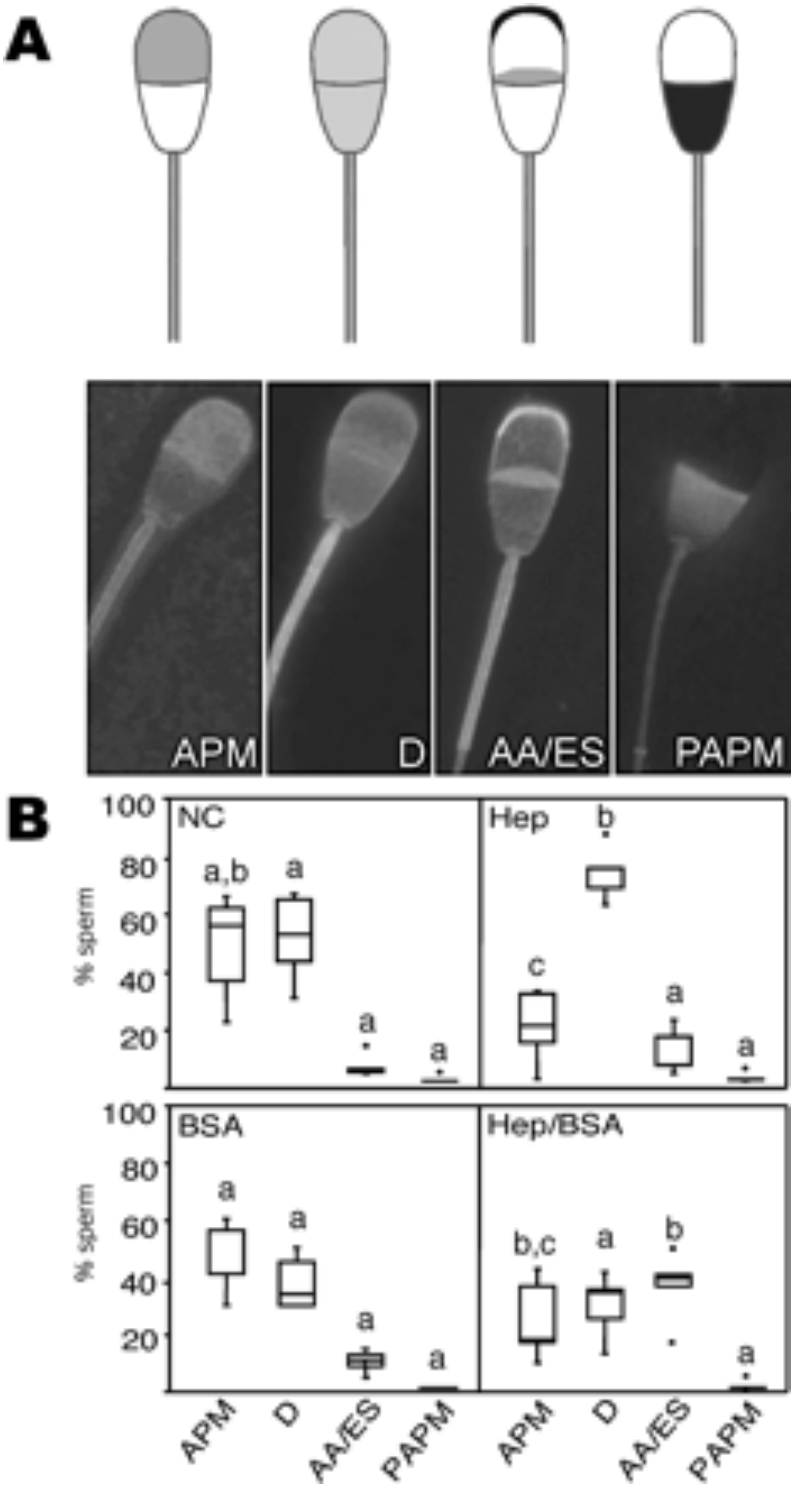
Figure 1. Patterns of G_{M1} localization seen in epididymal murine sperm after incubation under different conditions, either live or followed by fixation. (A) Fluorescence images and schematic diagrams (drawn as negative images) showing the range of patterns seen in live sperm and sperm fixed with 0.004% PF in PBS. Pattern APM denotes signal over the APM and was seen almost exclusively in live sperm. Pattern D denotes diffuse localization. Pattern AA/PA denotes signal over the apical acrosome (AA) and in the PAPM. Pattern PAPM denotes postacrosomal signal. Notably, 2 thin lines of fluorescence labeling, bordering a central unlabeled area of membrane, were sometimes seen over the AA in the D pattern and, less frequently, in the PAPM pattern, suggestive of a smaller "sub-subdomain" in the AA area. The images also represent G_{M1} labeling patterns seen in the midpiece, annulus, and principal piece, which did not depend on specific treatment conditions, and are described further below. (B) Box-whisker plots showing percentages of the different G_{M1} patterns in sperm incubated under a noncapacitating condition (NC) or in the presence of bicarbonate (NaHCO3), cyclodextrin (CD), or both bicarbonate and cyclodextrin (CAP) for 45 minutes. The lower and upper ends of the box mark the 25th and 75th quantiles; the median is represented as a horizontal line within the box and the mean as a horizontal line through the box. Vertical whiskers extend from the ends of the box to the 10th and 90th quantiles. A Kruskal-Wallis rank sum analysis showed significant differences between the different conditions ($P < .05$). Pairwise comparisons made with individual Wilcoxon tests for each pattern between the different conditions are indicated by the letters above the whiskers ($P < .025$). These results show that the AA/PA pattern increased significantly in the presence of NaHCO3, that the D pattern increased significantly in the presence of 2-OHCD and CAP conditions, and that the increases in those patterns were accompanied by significant decreases in sperm showing the PAPM pattern.


A
4 µm
APM
D
AA/PA
PAPM
B
NC
NaHCO3
2-OHCD
CAP
% sperm
100
80
60
40
20
a
a
a
a,b
b
b
b
c
b
a
b,c
D
AA/PA
PAPM
D
AA/PA
PAPM

For murine sperm we used 0.004% PF in PBS, which has been reported to be sufficient to immobilize sperm but not permeabilize their membranes (Harrison and Vickers, 1990). After fixation, noncapacitated murine sperm showed G_{M1} over the PAPM as previously observed (Selvaraj et al, 2006) but, in the presence of capacitating stimuli, new patterns of G_{M1} distribution were seen (Figure 1A). These patterns were highly reproducible, with specific patterns occurring in response to specific stimuli. Under all conditions, labeling over the APM similar to that seen in live sperm was only seen in rare cells after weak fixation. In the presence of NaHCO3, a significant percentage of cells (36.1% ± 2.5%) showed an incomplete redistribution to the PAPM with residual G_{M1} labeling over the AA (the AA/PA pattern; Figure 1B) when compared with noncapacitated sperm. In the presence of 2-OHCD, a significant percentage of cells (42.0% ± 3.4%) had G_{M1} diffusely distributed over the entire APM in addition to the PAPM (the D pattern; Figure 1B) when compared with noncapacitated sperm. The presence of both NaHCO3 and 2-OHCD caused no additional increase in the percentages of sperm showing either the AA/PA or the D patterns (Figure 1B).

In bovine sperm, we previously showed CTB did not bind to ejaculated live sperm due to masking by seminal plasma components; however, G_{M1} in the APM could be visualized using CTB in both epididymal sperm and fixed ejaculated sperm (Buttke et al, 2006). In the present study, we found that the use of a weak fixative, 0.004% PF in PBS, predominantly induced a PAPM pattern in noncapacitated bovine ejaculated sperm. This localization did not show any change in response to capacitating stimuli

Figure 2. Patterns of G_{M1} localization seen in ejaculated bovine sperm after incubation under different conditions followed by fixation. (A) Fluorescence images and schematic diagrams (drawn as negative images) showing the range of patterns seen in bovine sperm fixed using 1% PF with 12.5 mM CaCl2 in PBS. Pattern APM denotes signal over the APM. Pattern D denotes diffuse localization. Pattern AA/ES denotes signal over the AA and the ES. Pattern PAPM denotes postacrosomal signal. (B) Box-whisker plots showing percentages of the different G_{M1} patterns in sperm incubated under a noncapacitating condition (NC) or in the presence of heparin (Hep), BSA (BSA), or both heparin and BSA (Hep/BSA) for 90 minutes. The lower and upper ends of the box mark the 25th and 75th quartiles; the median is represented as a horizontal line within the box and the mean as a horizontal line through the box. Vertical whiskers extend from the ends of the box to the 10th and 90th quartiles. Outliers are represented as dots along the axis of the box. A Kruskal-Wallis rank sum analysis showed significant differences between the different conditions ($P < .05$). Pairwise comparisons made with individual Wilcoxon tests for each pattern between the different conditions are indicated by the letters above the whiskers ($P < .025$). These results show that the D pattern increased significantly in the presence of heparin, that the APM pattern decreased significantly in the Hep and Hep/BSA conditions, and that the AA/ES pattern increased significantly in the Hep/BSA treatment.


A
APM
D
AA/ES
PAPM
B
NC
Hep
BSA
Hep/BSA
% sperm
100
80
60
40
20
a,b
a
a
a
c
b
a
a
a
a
a
a
b,c
a
b
a
APM
D
AA/ES
PAPM
APM
D
AA/ES
PAPM

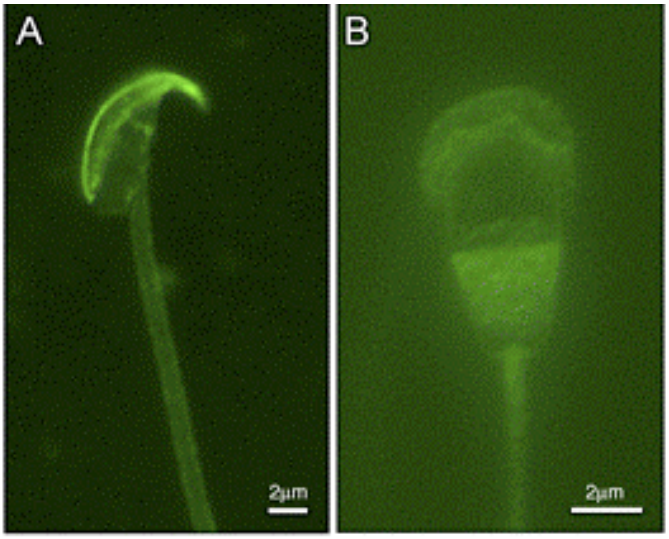
(data not shown). However, when bovine sperm were fixed with a slightly stronger fixative, 1% PF with 12.5 mM CaCl2 in PBS (Buttke et al, 2006), they displayed different patterns of G_{M1} distribution in response to these stimuli. As in the mouse, the bovine sperm showed highly reproducible patterns of G_{M1} localization based on the specific stimulus used for capacitation (Figure 2A). Unlike murine sperm, strong fixation such as 4% PF with 0.1% glutaraldehyde induced significant membrane damage in bovine sperm (data not shown).

Also unlike murine sperm, a very small percentage of bovine sperm showed the PAPM pattern and a large percentage of sperm showed the APM pattern under all conditions (Figure 2B). Incubation with heparin was associated with a significant increase in a diffuse localization, pattern D (70.9% ± 3/7%; Figure 2B). In the presence of BSA, there were no significant changes in patterns vs incubating sperm under noncapacitating conditions, but when both BSA and heparin were present in the medium, there was a significant increase in a pattern showing localization in both the AA and ES (AA/ES pattern; 39.1% ± 9.6%; Figure 2B). These patterns in bovine sperm were similar but did not correspond directly to the patterns seen in murine sperm. However, the G_{M1} redistribution in response to weak fixation (0.004% PF) in noncapacitated sperm from both species was from the sterol-rich APM to the sterol-poor PAPM (Buttke et al, 2006; Selvaraj et al, 2006).

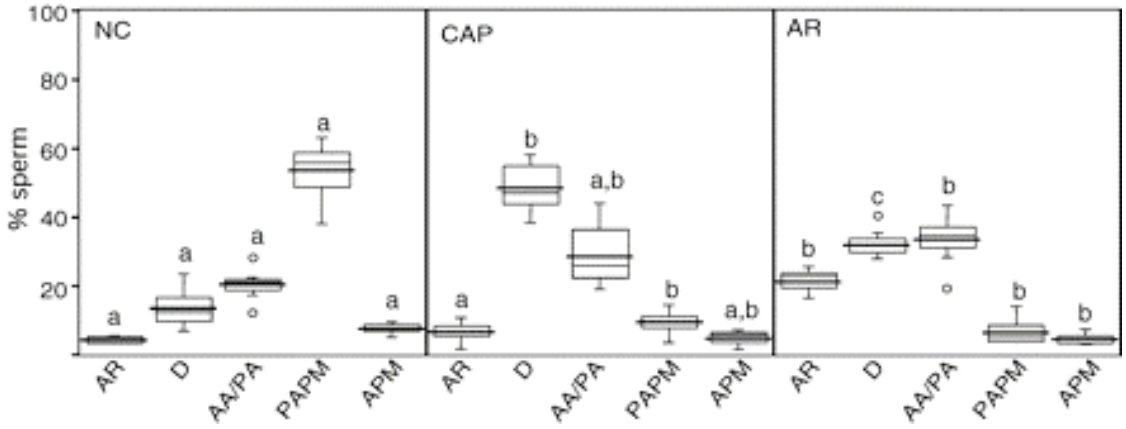
G_{M1} Localization in Acrosome-Reacted Sperm

Distribution of G_{M1} in live epididymal murine and bovine sperm remained unchanged even after incubation with stimuli for capacitation. However, induction of AE using progesterone in capacitated murine sperm and lysophosphatidyl choline in capacitated

Figure 3. Localization pattern of G_{M1} in acrosome reacted murine and bovine sperm. (A) Mouse sperm were capacitated in MW medium with NaHCO3 (10 mM) and 2-OHCD (3 mM) for 45 minutes, and acrosomal exocytosis (AE) was induced by treatment with progesterone (20 μM) for 5 minutes. Sperm were then fixed with 0.004% PF in PBS, and G_{M1} labeling using CTB was carried out as described. (B) Bovine sperm were capacitated in TALP with BSA (6 mg/mL) and heparin (20 μg/mL), and AE was induced by treatment with lysophosphatidyl choline (100 μg/mL) for 15 minutes. Sperm were then fixed with 1% PF with 12.5 mM CaCl2 in PBS, and G_{M1} labeling using CTB was carried out as described. (C) Box-whisker plots showing percentages of the different G_{M1} localization patterns in murine sperm incubated for 60 minutes under a noncapacitating condition (NC), or both bicarbonate and cyclodextrin (CAP), or after the induction of AE at the end of incubation. The lower and upper ends of the box mark the 25th and 75th quantiles; the median is represented as a horizontal line within the box and the mean as a horizontal line through the box. Abbreviations for patterns are same as in Figure 1A. "AR" refers to the acrosome-reacted pattern shown in panel A. A Kruskal-Wallis rank sum analysis showed significant differences between the different conditions ($P < .05$). Pairwise comparisons made with individual Wilcoxon tests for each pattern between the different conditions are indicated by the letters above the whiskers ($P < .025$). These results show that both the D and AA/PA patterns increased significantly under CAP conditions and that there was a corresponding decrease in the PAPM pattern. Moreover, there was a statistically significant increase in the AR pattern upon incubation of capacitated sperm with progesterone. This increase was accompanied by a corresponding decrease in the D pattern under these incubation conditions and no significant decline in the AA/PA pattern, showing that the sperm having the AR pattern came from the D subpopulation.


A
2µm
B
2µm

C


NC
CAP
AR
% sperm
100
80
60
40
20
a
a
a
a
a
a
b
a,b
b
a,b
b
c
b
b
b
AR
D
AA/PA
PAPM
APM

bovine sperm showed an increase in a pattern distinct from those seen after exposure to stimuli for capacitation followed by fixation as described above. After capacitation, a subset of murine and bovine sperm induced to undergo AE demonstrated a pattern characterized by a hollow AA suggestive of loss of both acrosomal contents and membranes from the AA (the AR pattern; Figure 3A and B). In both species, this pattern was characterized by the AA showing somewhat ragged borders consistent with the nature of vesiculations associated with this exocytotic process. In bovine but not murine sperm, this G_{M1} pattern in the AA was associated with a weak PAPM signal. In murine sperm, there was a significant increase in the AR pattern after the induction of AE (21.8% ± 1.3%; Figure 3C) when compared with sperm incubated under noncapacitating and capacitating conditions. This increase in AR pattern was associated with a significant decrease in the D pattern after the induction of AE when compared with sperm incubated under capacitating conditions (D pattern in capacitated sperm [48.9% ± 2.8%] and after AE [32.5% ± 1.6%]).

G_{M1} Localization in the Midpiece and Principal Piece

G_{M1} was also seen in the flagella of both murine and bovine sperm. In live sperm, the CTB signal was faint and appeared diffuse (data not shown). After fixation with 0.004% PF for murine sperm and 1% PF for bovine sperm, the localization of G_{M1} in the midpiece did not conform to a specific pattern. In murine sperm, it appeared either distributed evenly across the entire midpiece or was somewhat more prominent in the distal half to third of the midpiece (Fig 1A), and in bovine sperm the midpiece labeled more uniformly (Figure 2A). In murine sperm, G_{M1} became greatly enriched in the plasma membrane at the region of the annulus, where the mitochondrial sheath of the midpiece abuts the fibrous sheath of the principal piece (Friend and Fawcett, 1974)

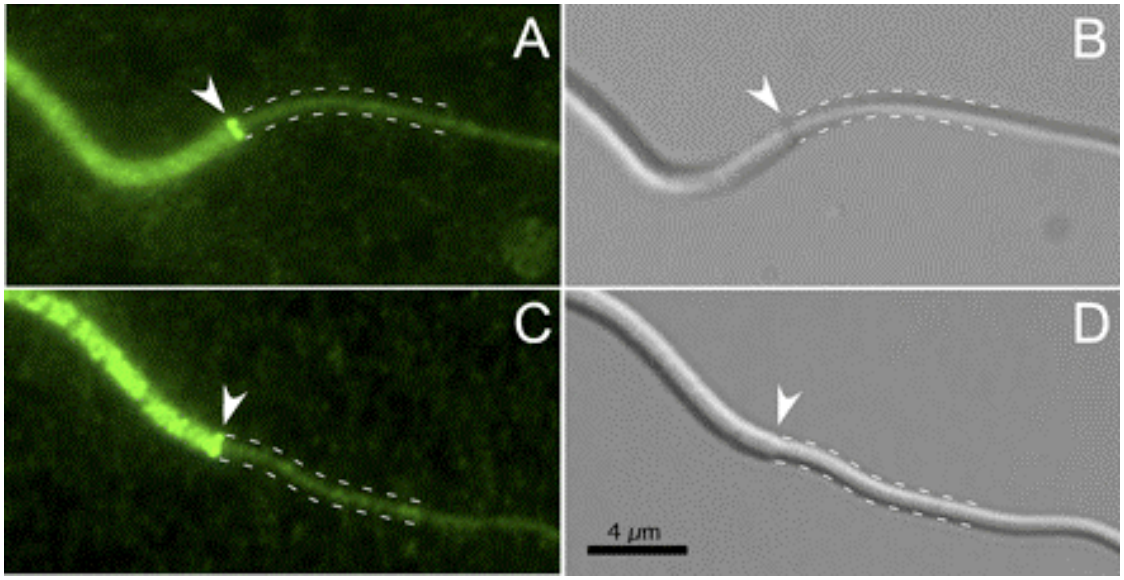


Figure 4. Localization of G_{M1} at the annulus and principal piece in murine sperm fixed with 0.004% PF. Noncapacitated sperm were fixed, and labeling for G_{M1} was carried out as described. The region of the annulus (arrowheads) showed intense G_{M1} labeling. A linear track of CTB fluorescence in the principal piece suggested G_{M1} localization over the flagellar zipper starting at the annulus and running down the length of the principal piece (Panels A and C). Notably, panels A and C have had their brightness and contrast adjusted to highlight the fluorescence over the extremely thin subdomain of the flagellar zipper, although this does result in an effective "overexposure" of the midpiece and annulus. Corresponding Nomarski Differential Interference Contrast images are shown in panels B and D. Dotted lines in both figures outline the proximal part of the principal piece showing that G_{M1} labeling was confined to a linear track narrower than the width of the principal piece.

(Figures 4 and 1A). In the principal piece, G_{M1} fluorescence was seen as a fine line coursing caudally from the annulus (Figure 4). To our knowledge, there is only 1 linear feature that runs the length of the plasma membrane in the principal piece. This is a distinct membrane subdomain known as the flagellar zipper that has been identified primarily through the use of freeze fracture techniques in several species (Friend and Fawcett, 1974; Lin and Kan, 1996). We now show by SEM that the flagellar zipper is a morphologically distinct membrane subdomain within the flagellar plasma membrane (Figure 5A). In an SEM micrograph of a demembranated sperm (Figure 5B), structures underlying or comprising internal components of the annulus and the flagellar zipper can be seen.

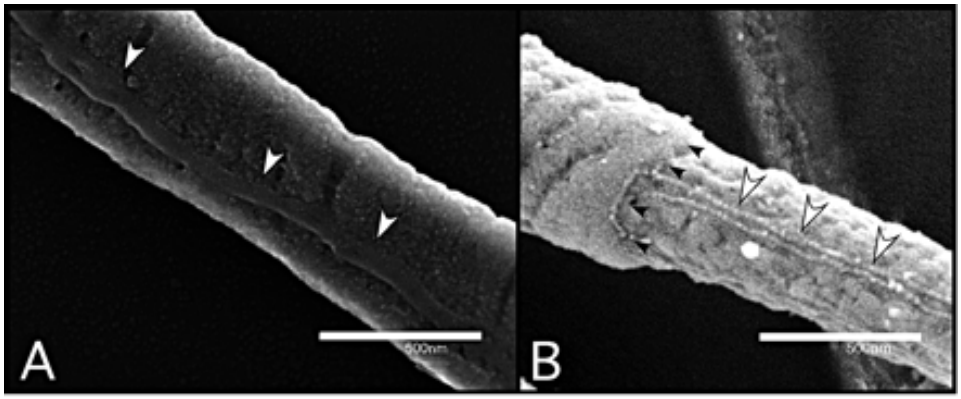


Figure 5. Scanning electron micrographs of murine sperm showing the annulus and flagellar zipper. (A) SEM of a sperm fixed with glutaraldehyde showing a region of the proximal principal piece. Although membranous structures were not specifically stabilized with osmium tetroxide after fixation, it appears that the membrane overlying the surface of the flagellar zipper (white arrowheads) is distinct as a narrow subdomain running longitudinally down the principal piece. (B) For comparison is SEM of a fixed sperm that was completely demembranated during handling. This panel shows the annulus (small black arrowheads), and characteristic substructure, which lends the flagellar zipper its name (white arrowheads).

Discussion

G_{M1} patterns reflect capacitation-associated membrane changes

Efflux of sterols from the sperm plasma membrane (Davis, 1974; Davis et al, 1979) and the presence of bicarbonate and calcium ions (Neill & Olds-Clarke, 1987; DasGupta et al, 1993; Visconti et al, 1995a) have long been known to play a critical role in capacitation. Two models have arisen describing the order of membrane events during capacitation, perhaps reflecting species differences. The first, based on work in the mouse, suggests that sterol efflux is an initial event, stimulating bicarbonate and calcium uptake (Travis & Kopf, 2002; Demarco et al, 2003). Alternatively, based primarily on work in the boar, bicarbonate and calcium uptake have been suggested to trigger an increase in intracellular cAMP and PKA activity, stimulating the activity of phospholipid scramblases (Harrison et al, 1996; Gadella & Harrison, 2000). The resultant increase in lipid disorder was suggested to facilitate raft formation and sterol efflux (Flesch et al, 2001).

Although G_{M1} redistribution upon cell death appears to be induced by CTB, variations in the pattern of G_{M1} revealed several important points of information about the nature of membrane changes during capacitation, both within single cells and at the level of sperm populations. Supporting the hypothesis that different populations of sperm exist within a single ejaculate or collection, only a subset of both murine and bovine sperm (approximately 40%) responded fully to stimuli for capacitation. This figure corresponded to the approximate percentage of sperm showing protein tyrosine phosphorylation in response to incubation under capacitating conditions (Urner et al, 2001).

Interestingly, in murine sperm we observed that both 2-OHCD and NaHCO3 could independently alter membrane properties, showing that unlike either existing model for the chronology of response to stimuli for capacitation, both stimuli could induce membrane changes as an initial event. Here the D pattern was indicative of sperm that responded to sterol efflux (2-OHCD), and the AA/PA pattern indicated sperm that responded to NaHCO3. If the 2 patterns were induced together in the same sperm, they would overlap to provide a D pattern. Because there was no net increase in the percentage of sperm showing the D pattern when both stimuli were included in the medium, this result indicates that the sperm that responded to the presence of NaHCO3 were the same population as those that responded to sterol efflux.

These results suggested that the D pattern represented capacitated sperm. Capacitation can be assessed by means of different end points and is most rigorously defined by the ability to fertilize an egg. However, use of that end point would not allow visualization of the membrane G_{M1} pattern. Therefore, we used the acquisition of the ability to undergo AE as a marker for capacitation. We saw an increase in the AR pattern after AE and an almost identically sized decrease in the D pattern in this treatment. These results showed that it was indeed the population of sperm having the D pattern that responded to progesterone, which would be consistent with the D pattern being a marker for capacitated sperm. This finding confirmed our hypothesis that CTB labeling of G_{M1} could function as an indicator of membrane changes associated with capacitation, being able to identify populations of sperm responding to specific capacitating stimuli.

In bovine sperm, there were no significant changes in patterns compared with noncapacitated sperm even after exposure to BSA, which is commonly used to

mediate sterol efflux. However, the presence of heparin alone did induce an increase in the D pattern in bovine sperm, suggesting that this stimulus could independently exert effects on bovine sperm membranes. The AA/ES pattern emerged only when both heparin and BSA were present in the medium, revealing that bovine sperm require both stimuli to show the full extent of membrane changes suggested by these G_{M1} localization patterns. Appropriately, similar patterns were not observed with bovine epididymal sperm (data not shown), suggesting that epididymal sperm of this species did not effectively respond to capacitating stimuli. This finding supports existing literature showing that epididymal bovine sperm incubated under capacitating conditions fail to undergo zona pellucida protein-mediated AE (Florman & First, 1988).

There have been several studies demonstrating changes in membrane distribution or mobility of membrane raft-associated proteins and/or lipids in the sperm head with capacitation (Cowan et al, 2001; Roberts et al, 2003; Cross, 2004; Shadan et al, 2004; Belmonte et al, 2005; van Gestel et al, 2005). Some of these studies have also examined G_{M1} localization in sperm from different species, with varying results. In the mouse, it was suggested that G_{M1} localizes to the PAPM and that this localization does not change with capacitation (Trevino et al, 2001); another study localized G_{M1} to the midpiece and moving to the head during capacitation (Shadan et al, 2004). Both these studies were done at 16°C, and phase transitions between this and physiologic temperatures (Wolf et al, 1990) might account for some disparity with our results. In rat sperm, it was suggested that G_{M1} localizes to the PAPM and then moves to the APM during capacitation (Roberts et al, 2003). In both this and the study in murine sperm showing no movement, it is clear that the initial localization to the PAPM was an effect of fixation condition (Selvaraj et al, 2006). In human sperm, it was reported

that G_{M1} has a diffuse localization pattern and then assembles in the APM (Cross, 2004). Also as discussed, this was likely due to exposure of sperm to seminal plasma (Buttke et al, 2006).

Possible Explanation for the Redistribution Phenomenon

Based on our observations and current models of sperm membrane properties, we have arrived at one possibility that could explain why CTB induced G_{M1} redistribution from the APM to the PAPM. We suggest that once crosslinked by CTB, G_{M1} moves from sterol-rich, liquid-ordered membrane regions to sterol-poor, less-ordered areas on the sperm. In both live and fixed sperm, it has been suggested that in noncapacitated sperm, the sterol-rich APM is a liquid-ordered subdomain whereas the sterol-poor PAPM is liquid disordered (Sleight et al, 2005).

Therefore, in sperm incubated under noncapacitating conditions, G_{M1} redistributed to the PAPM, being excluded from or forced out of the sterol-rich, more-ordered APM. Upon incubation of murine sperm with NaHCO3 in the presence of calcium (stimulators of sperm phospholipid scramblases [Gadella & Harrison, 2000]), some G_{M1} redistributed to the PAPM, but some also remained in the AA. The aminophospholipid transporter (SAPLT) with homology to a flippase localizes to this region (Wang et al, 2004). Studies comparing sperm from wild-type vs SAPLT-null mice suggest that phospholipid scramblase activity in this region depends on the activity of the flippase as well as the presence of NaHCO3 (Wang et al, 2004). Therefore, the presence of NaHCO3 could be inducing liquid disorder at the region of the AA giving rise to the AA/PA pattern. Sterol efflux from murine sperm has been shown to occur throughout the APM (Visconti et al, 1999), increasing lipid disorder throughout this subdomain (Cross, 2003; Sleight et al, 2005). Accordingly, there was a

diffuse pattern of G_{M1} localization throughout the head (APM and PAPM) of sperm incubated with 2-OHCD. The presence of both NaHCO3 and 2-OHCD induced no additional increase in the percentage of sperm, suggesting again that these populations of sperm were the same.

Also supporting the notion that crosslinked G_{M1} redistributed to membrane subdomains of reduced-order/lower-sterol abundance was the redistribution seen in the flagellum of murine sperm upon death or fixation with 0.004% PF. Originally diffuse throughout the flagellum, G_{M1} became concentrated at the annulus and the flagellar zipper of the principal piece, structures shown by freeze fracture to be devoid of sterols (Pelletier & Friend, 1983; Lin & Kan, 1996).

Our data on the stimulus-specific patterns of changes in G_{M1} localization suggest strongly that temporally sperm can respond to NaHCO3 or mediators of sterol efflux independently of one another. This finding provides a refinement to existing models of capacitation. Yet, in addition to these points of basic science, our findings also suggest a clinical application for G_{M1} as a marker for detecting capacitation-associated membrane changes in murine and bovine sperm. Changes in localization patterns of G_{M1} in response to specific stimuli for capacitation could provide a diagnostic tool for predicting a male's reproductive fitness based on the proportion of sperm that are capable of responding to such stimuli. This information could be used in agricultural industries to make broad classifications regarding a male's fertility or could be used to help guide clinicians when choosing between techniques such as in vitro fertilization or intracytoplasmic sperm injection. Furthermore, because this potential assay is based upon functional membrane responses, it might also be useful when evaluating or comparing media or conditions used to handle sperm in vitro or to cryopreserve them.

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CHAPTER 5

THE VOLTAGE-OPERATED $CA_V2.3$ CALCIUM CHANNEL IS REQUIRED FOR ACROSOMAL EXOCYTOSIS IN MAMMALIAN SPERM AND ITS ACTIVITY IS MODULATED BY THE GANGLIOSIDE G_{M1}

Abstract

We have previously shown G_{M1} to be stably segregated to the plasma membrane overlying the acrosome (APM) in live murine, bovine, and human sperm (Buttke, Nelson, Schlegel, Hunnicutt, & Travis, 2006; Selvaraj et al., 2006) and to be highly enriched in the acrosomal membranes in both developing male germ cells as well as in mature sperm (Asano et al., 2009). This localization, as well as other reports in several other mammalian species (Bou Khalil et al., 2006; Cross, 2004; Tanphaichitr et al., 2007; van Gestel et al., 2005), indicates the presence of membrane rafts in sperm, and a localization of those rafts that might suggest a role in acrosomal exocytosis (AE). We present physiological evidence that clustering of G_{M1}, induced either through the use of the pentameric B subunit of cholera toxin (CTB) or through the addition of exogenous G_{M1}, can induce AE in capacitated mouse sperm through activation of the $Ca_V2.3$ channel. AE mediated by G_{M1} is dependent upon extracellular calcium, is sensitive to pertussis toxin, and is inhibited by nickel and $Ca_V2.3$ inhibitors including SNX-482. The functional interaction of G_{M1} with $Ca_V2.3$ appears to occur in the same pathway to AE as that induced by solubilized zona pellucida-proteins, since their induction of AE was also inhibited by SNX-482. In support of this, mice lacking the α1E subunit of the $Ca_V2.3$ calcium channel failed to undergo AE in response to solubilized zona pellucida, or changes in the G_{M1} microenvironment. These mice also have decreased litter size and impaired IVF success rates. Our results provide the first evidence of function for G_{M1} in sperm, characterize more completely the reproductive phenotype of α1E-deficient mice, point to this channel subunit as being responsible for voltage-gated calcium influx early in AE, and provide an explanation for why G_{M1} localization is so strictly segregated in diverse mammalian species. Together, these results lead us to formulate a new model for the initiation of AE.

Introduction

The lipid microenvironment is a powerful regulator of calcium flux and homeostasis in mammalian cells (see Golub, Wacha, & Caroni, 2004; Rajendran & Simons, 2005, for review). The organization of signaling molecules into functional, pre-assembled complexes through the use of membrane rafts is one way in which a membrane can regulate calcium homeostasis and signaling. Rafts are dynamic, nanometer-sized microdomains enriched in sterols and sphingolipids that have been found to influence the activity of a variety of ion channels, including nucleotide-gated channels (Brady et al., 2004), voltage-operated calcium channels (VOCC; Taverna et al., 2004), and epithelial sodium channels (Shlyonsky, Mies, & Sariban-Sohraby, 2003) in a reversible manner.

Of the raft-associated sphingolipids, the role of the ganglioside G_{M1} has been the most studied. This is largely due to the fact that the B subunit of cholera toxin (CTB) binds to G_{M1} with a remarkable specificity and sensitivity (Cuatrecasas, 1973; Fishman, Pacuszka, & Orlandi, 1993; Lauer, Goldstein, Nolan, & Nolan, 2002), making possible a variety of experimental techniques. G_{M1} has been shown to segregate preferentially to membrane rafts where even a single G_{M1} molecule can exert local effects on the membrane (Goins, Masserini, Barisas, & Freire, 1986; Roy & Mukhopadhyay, 2002). At the cell surface, G_{M1}'s extracellular sugars can act as a receptor, such as G_{M1}'s role as a co-receptor for FGF2 (Rusnati et al., 2002), and as a receptor for murine polyoma and SV40 virus (Tsai et al., 2003), or G_{M3}-carbohydrate mediated sperm-egg adhesion in trout (Yu et al., 2002). G_{M1} has been suggested to promote the open probability of ion channels or the conformation of plasma

membrane calcium ATPases (PMCA), sarcoplasmic-endoplasmic reticulum calcium ATPases (SERCA), or sodium/calcium exchangers (Fang, Xie, Ledeen, & Wu, 2002; Ravichandra & Joshi, 1999; Zhao, Fan, Yang, & Zhang, 2004).

Many of these same calcium transporters and channels have been identified in mammalian sperm (see Darszon et al., 2005, for review). However, despite advances in understanding the complex process of regulated calcium influx during acrosomal exocytosis (AE), understanding of the nature and regulation of upstream channels involved in AE has remained elusive. This is due to many factors including the high redundancy of signaling pathways in sperm that elicit similar functions. The increase in intracellular calcium in the sperm head during AE occurs in discrete steps, including an initial pH-sensitive current potentially attributed to the sperm-specific Ca^{2+} channel CatSper, which raises resting calcium concentrations upon sterol efflux (Florman, 1994; Xia & Ren, 2009a); a second, previously un-identified voltage-operated calcium channel allowing transient calcium elevation (Arnoult, Cardullo, Lemos, & Florman, 1996; Arnoult et al., 1999); followed closely by a final sustained elevation in intracellular calcium (Arnoult, Zeng, & Florman, 1996). These changes in intracellular calcium then allow SNARE-mediated fusion of the plasma membrane overlying the acrosome (APM) and the outer acrosomal membrane (OAM) (De Blas, Roggero, Tomes, & Mayorga, 2005; Michaut et al., 2001; Yunes, Michaut, Tomes, & Mayorga, 2000; Yunes et al., 2002).

Although several subtypes of voltage-operated calcium channels have been described in sperm, their *in vivo* activity has been difficult to characterize. Patch-clamp recordings from developing male germ cells detected low voltage activated (LVA), T-type currents, in agreement with pharmacological characterization (Arnoult, Villaz,

Florman, & 6, 1998). However, male mice lacking T-type calcium channels CaV3.1 and CaV3.2 are fertile, and the remaining current in male germ cells from these mice displays characteristics that differ slightly from somatic cell T-type currents (Stamboulian et al., 2004). Pharmacological studies of T-type channels in mature sperm often require very high concentrations of inhibitors to elicit effects, and these high concentrations can affect sodium channels and other calcium channels as well (Bonaccorsi, Forti, & Baldi, 2001). Additionally, depolarization-induced calcium influx to simulate ZP-induced calcium rise in mature mouse sperm proved to be insensitive to blockers of L-, P/Q-, and T-type channels (Wennemuth, Westenbroek, Xu, Hille, & Babcock, 2000).

One common thread throughout reported studies on mature mouse sperm is the inhibition of AE by nickel and cadmium ions, which together would support the involvement of the $Ca_V2.3$ channel (Wennemuth et al., 2000). This channel is nearly indistinguishable from T-type channels with regard to its LVA gating properties, and is responsible for the majority of the residual, or R-type calcium current, (Ertel, 2004). R-type channels have been defined based on their resistance to inhibitors of other high-voltage activated (L-, N-, and P/Q-type) calcium channels and by their sensitivity to the spider venom peptide SNX-482 (Bourinet et al., 2001). Several studies have shown that R-type, $Ca_V2.3$ channels are modulated by phosphorylation and membrane binding partners and are also antagonized by peptides that antagonize $G\alpha_q$ and $G\alpha_i$ (R. A. Bannister, Melliti, & Adams, 2004; Cohen & Atlas, 2004; Kamatchi et al., 2003; Klockner et al., 2004; Toro-Castillo, Thapliyal, Gonzalez-Ochoa, Adams, & Meza, 2007). Such studies have defined physiologic roles for $Ca_V2.3$ in cerebellar neurotransmitter secretion, hormone secretion, neuronal plasticity and memory, and

morphine analgesia susceptibility (Cohen & Atlas, 2004; Fang et al., 2007; Muller et al., 2007).

In sperm, $Ca_V2.3$ has been localized to the membrane of the principal piece of the flagellum and the apical acrosome and equatorial region of the sperm head (Westenbroek & Babcock, 1999). $Ca_V2.3$ mRNA accounts for the greatest amount of voltage-operated calcium channel (VOCC) message in maturing sperm (Lievano et al., 1996). Despite the findings of both mRNA and protein, $Ca_V2.3$ channel activity was not detected in patch-clamp readings of immature spermatocytes (Stamboulian et al., 2004), suggesting that it might not function until later in maturation or in mature sperm. Imaging studies of depolarization-evoked calcium entry in mature sperm provided evidence that $Ca_V2.3$ currents are activated in sperm, but the lack of a specific inhibitors or genetic models prohibited further investigation of this finding at the time (Wennemuth et al., 2000).

Many maturational events of sperm capacitation, such as PKA and PKC phosphorylation, membrane fusion complex (SNARE) formation, and G-protein activity, have the potential to modify the $Ca_V2.3$ channel and regulate its activity (Bannister et al., 2004; Cohen & Atlas, 2004; Hell, Yokoyama, Breeze, Chavkin, & Catterall, 1995; Klockner et al., 2004). These activities are mediated in sperm in part by the reorganization of membrane rafts and increases in membrane fluidity that change the lipid microenvironment of the sperm (Bickel, 2002; Brazer, Singh, Liu, Swaim, & Ambudkar, 2003; Taverna et al., 2004; van Gestel et al., 2005). Here, we present evidence that focal clustering of the extracellular sugars of G_{M1} mediates AE through the activation of the $Ca_V2.3$ channel, and mice lacking this channel have severe defects in fertility. This is also the first report describing an essential role for

$Ca_V2.3$ as the previously-unidentified, initial voltage-gated calcium channel in zona pellucida-mediated AE.

Materials and Methods

Reagents and animals

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. CTB conjugated with Alexa Fluor 488 and 555 (Invitrogen, Carlsbad, CA) or conjugated with FITC was used as indicated. Male CD-1 mice were purchased from Charles River Laboratories (Kingston, NY). Male and female B6129SF/J mice were purchased from Jackson laboratories (Barr Harbor, ME). All experiments were repeated on both strains of mice. B6129SF/J mice carrying a null mutation for the α1E gene were a generous gift from the lab of Richard Miller, Northwestern University, IL. For indirect immunofluorescence, a monoclonal antibody against mouse α1E (Santa Cruz, Santa Cruz, CA) was used. Secondary antibody used was AlexaFlour 488- or AlexaFluor 555-conjugated goat anti-mouse IgG (Invitrogen). Changes in intracellular calcium concentrations were monitored using the membrane permeant fluorescent indicator dye Fluo-3 AM and Fluo-4 FF-AM (Molecular Probes, Invitrogen). All animal procedures were performed under the guidelines of the Institutional Animal Care and Use Committee at Cornell University.

Preparation of media

For murine sperm, a modified Whitten's medium (MW; 22 mM HEPES, 1.2 mM $MgCl_2$, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemi-

calcium salt, pH 7.35; (Travis et al., 2004)) was used for all incubations. Glucose (5.5 mM), $NaHCO_3$ (10 mM), and 2-hydroxypropyl-b-cyclodextrin (2-OHCD; 3 mM) were supplemented as needed. 2-OHCD supports sperm capacitation and *in vitro* fertilization by functioning as a sterol acceptor, and is preferred over the more potent methyl-β-cyclodextrin (Visconti et al., 1999).

Sperm collection and handling

Murine sperm were collected from the cauda epididymides of male CD-1 mice by a swim-out procedure as described previously (Travis et al., 2001). All steps of collection and washing were performed at 37˚C using MW medium, and large orifice transfer pipettes or large orifice pipette tips were used for handling sperm to minimize membrane damage. After the initial washes but prior to experimental incubations, motility assessment was carried out, and samples showing <60% motility were not used.

Sperm capacitation and induction of AE

$2x10^6$ sperm were incubated in 300 μl of MW with glucose as base medium alone as non-capacitating media or MW base media supplemented with both 10 mM $NaHCO_3$ and 1 mM 2-OHCD as capacitating conditions. The pH of media for all incubation conditions was adjusted to 7.35. The capacitating condition media has been shown to be sufficient to support IVF (Travis et al., 2004) and capacitation-induced tyrosine phosphorylation (Visconti et al., 1999a) in murine sperm. The dead spaces of tubes used for all incubations were filled with nitrogen to avoid the generation of bicarbonate anions in the aqueous media. Nitrogen had no effect on protein tyrosine phosphorylation events associated with capacitation (Travis et al., 2001; data not shown). Calcium channel inhibitors were added 10 minutes prior to the addition of AE

agonists. Progesterone (3 μg/ml final concentration, diluted in DMSO) was used as a positive control to induce AE in capacitated murine sperm after 50 minutes of incubation. CTB (1.5 μM final concentration, diluted in PBS) was added where indicated to assess its affect on AE. The exogenous lipids ceramide, asialo-G_{M1}, and G_{M1} (diluted in DMSO; 25 mM stock concentration) were also added to a final concentration of 25 μM where indicated. Sperm were then processed for Coomassie assessment of AE as described previously (Visconti, Stewart-Savage et al., 1999). Briefly, after a 10-minute incubation with the AE agonist, 300 μL 8% paraformaldehyde in PBS was added to each tube to a final concentration of 4 %. After a 10-minute fixation, sperm were washed with PBS and again with 500 mM ammonium acetate before being air dried on glass slides. The slides were stained with 0.22% Coomassie Blue G-250 in 50% methanol and 10% acetic acid for 10 minutes before being rinsed and mounted with GVA mount. To calculate the percentage of AE, all sperm in a given field were counted until at least 200 sperm were assessed per treatment condition for the presence or absence of a Coomassie stained acrosomal matrix. A Kruskal-Wallis test was run to look for differences among treatment groups and any that were found were subjected to a Wilcoxon Signed Rank test to differentiate between groups with significant differences.

Sperm capacitation and tyrosine phosphorylation

For murine sperm, incubation with different stimuli for capacitation was carried out with $2x10^6$ sperm in 300 μl of medium with 5.5 mM glucose under one of two conditions: (a) MW base medium ("non-capacitating conditions"), (b) MW with both 10 mM $NaHCO_3$ and 3 mM 2-OHCD ("capacitating conditions"), for 45 minutes. The pH of media for all incubation conditions was adjusted to 7.35 with HCl.

As a marker for capacitation-associated changes, phosphorylated tyrosine residues were detected using Western blotting techniques as described previously (Visconti et al., 1999). Briefly, the sperm were homogenized in 5X protease inhibitor cocktail containing 0.2 mM sodium orthovanadate and the proteins were solubilized by boiling in sample buffer and then separated by SDS-PAGE under reducing conditions. Immunoblotting was performed with an anti-phosphorylated tyrosine monoclonal antibody and signals were detected by chemiluminescence as described previously (Travis et al., 2001).

Collection and preparation of heat-solubilized zona pellucida

Zona pellucidae (ZP) were collected via ovarian homogenization as described previously (Buffone, Rodriguez-Miranda, Storey, & Gerton, 2009). All glass and plasticware were siliconized to reduce the loss of ZP by adherence to surfaces. Homogenization buffer (HB) contained 150 mM NaCl, 25 mM triethanolamine (TEA), 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 8.5, with 10 mg DNase, and 1 tablet Complete Protease Inhibitor added to 50 ml HB just prior to ovary collection. Approximately 70 ovaries from 6 to 12 week-old female mice were homogenized using 7-12 strokes in a Wheaten-Boroek tissue grinder on ice in 2 ml of HB. Ten percent Nonidet P40 (w/v) and 10 percent sodium deoxycholate (w/v) were added to the homogenate and briefly homogenized again. The homogenate was layered atop a discontinuous percoll gradient containing 3 ml of 22% percoll in HB, 2 ml of 10% percoll in HB, and 2 ml of 2% percoll in HB. The gradient was centrifuged for 2 hours at 400 g in a swinging bucket rotor at 4 °C. The 10 % percoll fraction was collected and diluted with 45 ml of HB. The diluted fraction was split into 24 2-ml conical centrifuge tubes and centrifuged for 10 minutes at 16,000 g at 4 °C. The top 1.7 ml of supernatant was removed and discarded, and the zonae were pooled into six tubes and washed again

via centrifugation at 16,000 g for 10 minutes as previously in HB, followed by two additional washes in MW. The final pellets were collected and combined. Three 1 μl aliquots were visually inspected under a dissecting microscope to determine the concentration (ZP per μl). The ZP solution was heated at 60 °C for 2 hours to solubilize the ZP and 12 μl aliquots were snap-frozen and stored at -80 °C until use.

Evaluation of changes in intracellular calcium concentrations

Intracellular calcium concentrations in a population of sperm were monitored using the membrane permeant fluorescent indicator dyes Fluo-3 AM and Fluo-4 FF-AM. 10 million sperm in both non-capacitating and capacitating conditions were incubated in 5 μM fluo-3 AM for 20 minutes at 37 °C in the dark. Excess dye was washed from sperm by centrifugation at 400 g for 2 minutes. Sperm were incubated for an additional 20 minutes to allow complete de-esterification of the intracellular dye with calcium channel inhibitors present where indicated. Fluorescence intensity was read at 10-minute intervals for 60 minutes using a Tecan SaFire fluorescent plate reader (MTX Lab Systems, Inc; Vienna, VA) and analyzed using Magellan software (MTX Lab Systems, Inc.).

In vitro fertilization

All steps of in vitro fertilization (IVF) were carried out in modified Kreb's bicarbonate (TYH) medium (Kito & Ohta, 2005). Sperm were allowed to swim out from the cauda epididymides for 15 minutes followed by a 1-hour incubation in TYH medium prior to IVF. Ovulation was induced in six-week-old B6129SF/J mice by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG) followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG). Cumulus oocyte complexes were collected from the oviducts 13 hours later using 20 gauge

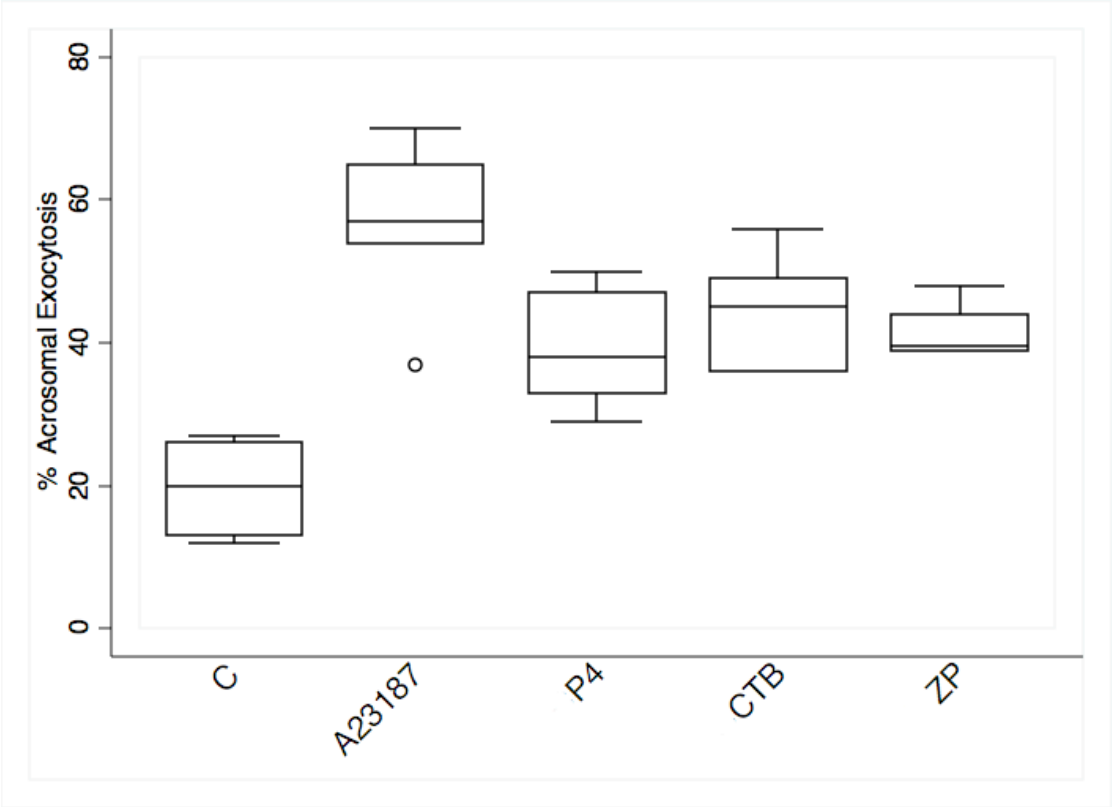
needles. Cumulus-oocyte complexes were either used directly for IVF or treated with 0.015% hyaluronidase for 1 minute and pipetted up and down to remove cumulus cells. Cumulus-free oocytes were washed in TYH and allowed to rest for 20 minutes prior to sperm addition at a concentration of 500,000 sperm/ml and incubation at 38 °C in 5% CO_2/5% O_2/90% nitrogen. Oocytes were transferred to sperm-free droplets after 6 hours and returned to the incubator. Percent of eggs fertilized was calculated by assessing 2-cell embryos after 24 hours. Embryos and unfertilized oocytes were fixed with 4 % paraformaldehyde and stained with 1:100 Hoechst to evaluate the occurrence of sperm binding and polyspermy.

Results

Exposure to CTB can induce AE in sperm incubated under capacitating conditions

Our prior characterization of G_{M1} segregation and enrichment within the plasma membrane overlying the acrosome and acrosomal membrane, as well as the temporary masking of G_{M1} to maintain quiescence, led us to hypothesize that this organization and dynamic was important for sperm function (Asano et al., 2009; Buttke et al., 2006; Selvaraj et al., 2006). Unexpectedly, in conducting those experiments, we noted that a portion of live, capacitated sperm exhibited a clearing of labeled CTB signal over the apical acrosomal area that appeared consistent with acrosomal exocytosis. Co-labeling with FITC-conjugated peanut agglutinin to assess acrosomal status confirmed that these sperm had in fact lost the plasma membrane overlying the acrosome in response to CTB (data not shown). To investigate whether this event involved changes in the membrane alone or was also associated with a loss of acrosomal matrix as occurs during physiologic acrosomal exocytosis, we assessed acrosomal status with

Figure 1. A. Box whisker plots of percentage of sperm undergoing acrosomal exocytosis in response to capacitating conditions alone (C), calcium ionophore A23187 (A23187), progesterone (P4), solubilized zona pellucida (ZP), or CTB as assessed by coomassie staining patterns (n=4 experiments, > 200 sperm per experiment). Sperm were incubated under capacitating conditions for 50 minutes and treated with P4 (3 μg/ml), ZP (2 ZP/μl), or CTB (1.5 μg/ml) to induce acrosomal exocytosis (AE) for 10 minutes prior to processing for Coomassie assessment as described above. As shown, CTB induced a significant increase in the percent of AE ($p < 0.0001$; $\alpha=0.05$) that was statistically similar to the rates of AE induced by both P4 and ZP, but lower than A23187.


% Acrosomal Exocytosis
0
20
40
60
80
C
A23187
P4
CTB
ZP

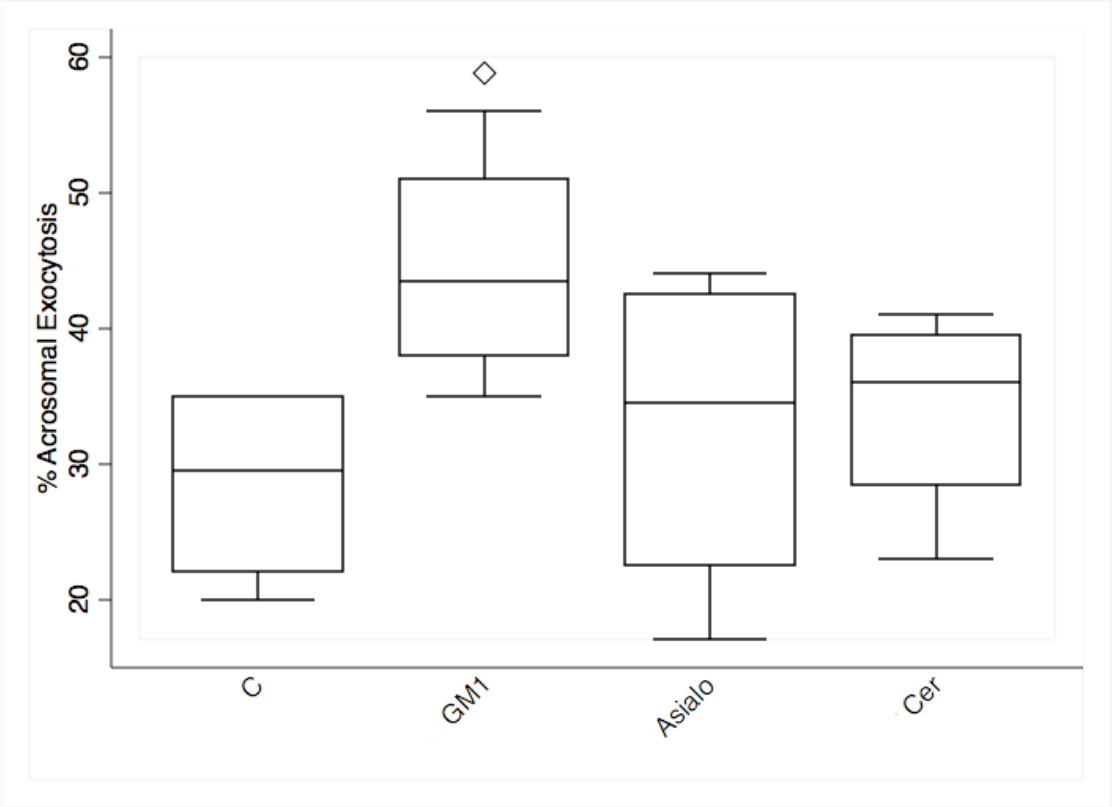
Coomassie staining after treatment with 1.5 μM CTB or commonly employed agonists for AE: progesterone (P4; 3 μg/ml), solubilized zona pellucida proteins (ZP; 2 ZP/μl), or calcium ionophore (A23187; 10 μM). As with the P4 and ZP controls, exposure to CTB induced AE in sperm incubated in capacitating conditions (Fig. 1), whereas no increase in AE was seen in those sperm incubated in base media alone (C).

The observed induction of AE by CTB in capacitated sperm might have been due to a non-physiologic membrane perturbation induced by the binding of the pentameric CTB (McCann, Mertz, Czworkowski, & Picking, 1997). Conversely, cross-linking of G_{M1} by CTB might have mimicked a physiologically relevant clustering of G_{M1} that could occur upon sterol efflux-induced increased membrane fluidity (Shoeb, Laloraya, & Kumar, 2004; van Gestel et al., 2005). If the effect were due specifically to G_{M1} clustering as opposed to non-specific membrane perturbations, then the addition of exogenous G_{M1} into the plasma membrane should mimic this clustering effect by increasing focal concentrations of G_{M1} in areas where it inserts (Gouy, Debre, & Bismuth, 1995). To address this question, we added 25 μM exogenous G_{M1} to sperm. This concentration of G_{M1} has been shown to stimulate G_{M1}–induced signaling events in somatic cells without affecting overall membrane integrity or cholesterol efflux (Yatomi, Igarashi, & Hakomori, 1996). As seen with the addition of CTB, exogenous G_{M1} significantly increased the rate of AE in capacitated sperm (Fig. 2), with no effect on viability or AE of non-capacitated sperm. Additionally, a bivalent antibody against G_{M1} with only two potential binding sites and, therefore, less clustering potential, was unable to induce AE in capacitated sperm (data not shown). This again suggests that it is the local concentration of G_{M1} in the membrane that is important in CTB induction of AE.

Studies from somatic cells have generated a model in which ligand-mediated clustering of G_{M1}'s extracellular sugars, specifically sialic acid, allow the open gating of calcium channels and/or sterically inhibit outward calcium transporters (Fang et al., 2002; Ledeen & Wu, 2007; Xie, Wu, Lu, & Ledeen, 2002; Zhang, Zhao, Duan, Yang, & Zhang, 2005). This is analogous to the regulation of the pore-forming subunit of VOCC through the heavily glycosylated α_2 portion of the $\alpha_2\delta$ subunits (Bannister et al., 2009). In such a model, proximity of the ceramide tail alone should not stimulate an increase in intracellular calcium, and a ganglioside lacking a sialic acid side chain (e.g. asialo-G_{M1}) might have decreased ability to stimulate calcium flux. To test this model in sperm, we evaluated the AE-inducing ability of the lipids asialo-G_{M1}, which lacks the extracellular sialic acid side-chain of G_{M1}, and ceramide, which lacks the entire extracellular portion of G_{M1}. This experiment addressed the specificity of G_{M1} clustering in the sperm membrane for induction of G_{M1}-mediated AE as opposed to non-specific membrane perturbations, which would be induced by other lipids. Neither asialo-G_{M1} (25 μM) nor ceramide (25 μM) was able to induce a significant elevation in AE as compared to exogenous G_{M1} (Fig. 2). These data suggest that the extracellular, carbohydrate portion of G_{M1} is important in the G_{M1}-mediated AE in sperm.

If the clustering of the extracellular sugars of G_{M1} could stimulate an increase in the activity of a plasma membrane-associated calcium channel, we reasoned that G_{M1}-mediated AE would require extracellular calcium. Indeed, the depletion of extracellular calcium prevented CTB- and P4-induced AE (Fig. 3B, C). This suggested that G_{M1} promoted AE by modulating intracellular calcium concentrations through the activation of a membrane-associated calcium channel or transporter. To further investigate this hypothesis, we employed several pharmacological inhibitors of

Figure 2. Box whisker plots of the percentage of sperm undergoing acrosomal exocytosis in response to base medium (C) or 25 μM of the exogenous lipids G_{M1}, asialo-G_{M1} (Asialo), ceramide (Cer), or solvent (DMSO, data not shown) (n=4 replicates, >200 sperm each replicate). Sperm were incubated under capacitating conditions for 50 minutes before the addition of exogenous lipids for 10 minutes. Coomassie staining was performed as described above, with no differences in motility seen among treatments after incubation. As shown, exposure to G_{M1} induced acrosomal exocytosis in in capacitated sperm at levels expected with exposure to progesterone, zona pellucida, or CTB (see Figure 1.), while asialo-G_{M1} and ceramide had no effect. This suggests that the extracellular portion of G_{M1}, particularly the sialic acid residue, is important in the G_{M1}-mediated effect on sperm acrosomal exocytosis. Diamond denotes statistical significance.

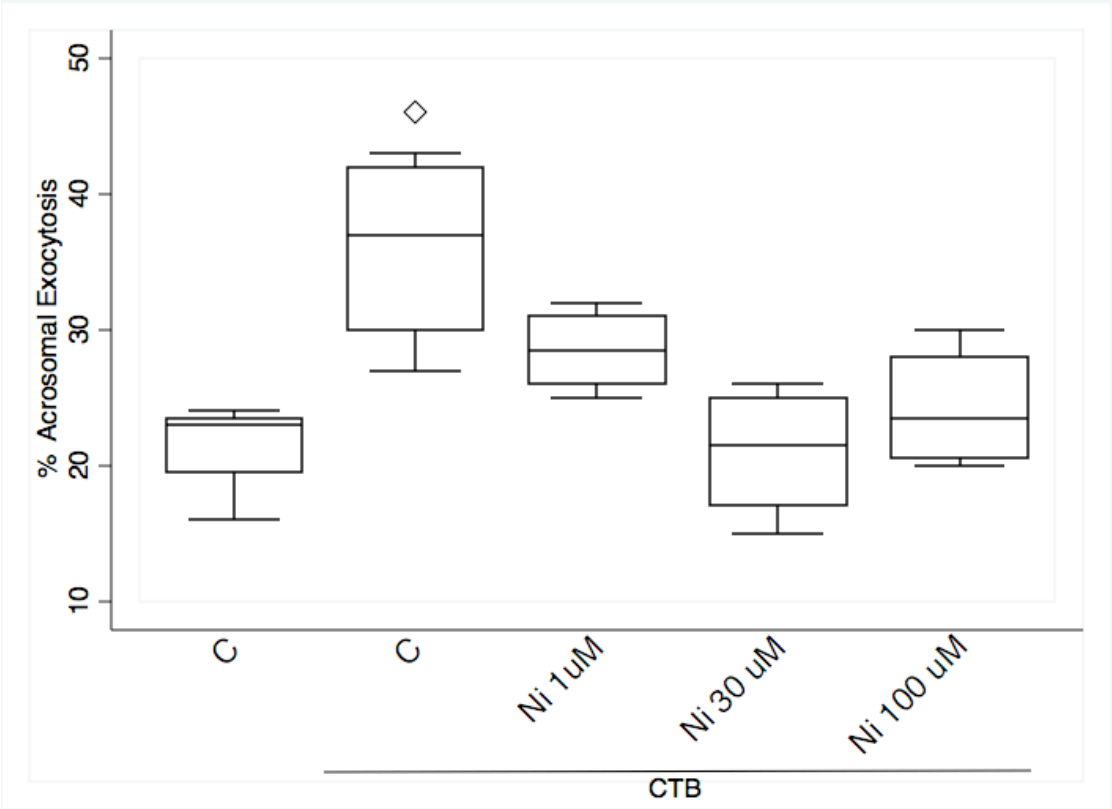

% Acrosomal Exocytosis
20
30
40
50
60
C
GM1
Asialo
Cer

known calcium channels and studied their effect on the CTB- and G_{M1}-induced AE observed through Coomassie assessment of acrosomal status. We attempted to inhibit the CTB-induced AE using nickel as a known inhibitor of low-voltage activated calcium channels, R-type calcium channels, and store-operated calcium channels (SOCC's), as well as calcium transporters (Ertel, 2004). The CTB-induced AE was inhibited by nickel in a concentration dependent manner (Fig. 3A). 100 μM nickel will inhibit T-type, R-type, and SOCC channels, while 30 μM is only fully effective for R-type and SOCC's. Inhibiting SOCC's with either cadmium (100μM; which also inhibits the $Ca_V2.3$, R-type calcium channels) or lanthanum (10 μM) prevented both the P4 and the CTB induced AE. The SOCC, TRP channel specific inhibitor, BPT2, had no effect on either CTB or progesterone-induced AE (data not shown).

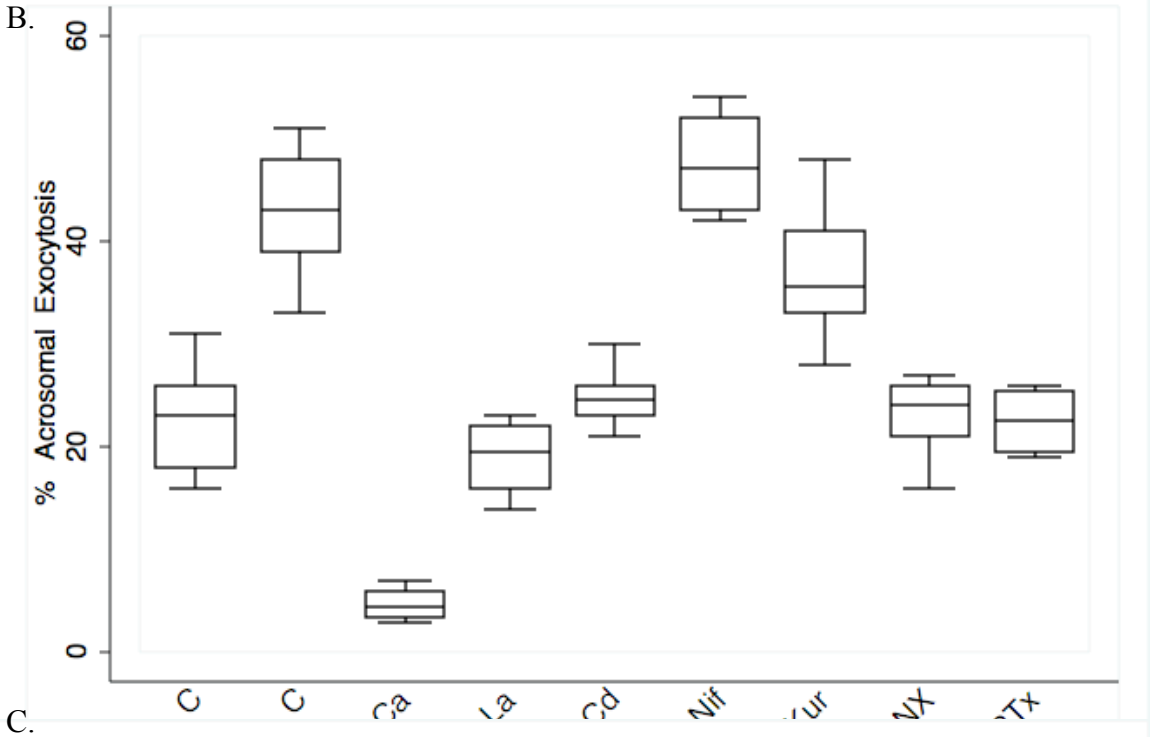
The effect of 30 μM nickel suggested that T- and L-type calcium channels are not important for G_{M1}-mediated AE. In support of this, the dihydropyridine nifedipine, which has reported selectivity for L-type channels, failed to inhibit either the CTB or the P4 induced AE even at concentrations of up to 100 μM (Fig. 3). Kurtoxin type KL1 (1 μM), which has been shown to inhibit T-type Ca_V 3.1 and 3.2 and HVA calcium channels in sperm, exhibited a non-significant reduction in P4-induced AE and had no effect on CTB-induced AE, suggesting that T-type channels are not important in G_{M1}-mediated AE. However, in agreement with our nickel and cadmium data, inhibition of the $Ca_V2.3$ or R-type calcium channel with SNX-482 (500 nM) prevented the CTB- and G_{M1}-induced AE, but had no significant effect on P4-induced AE. Together, these pharmacological data suggest that both the $Ca_V2.3$ R-type channel and SOCC activities are necessary for G_{M1}-mediated AE.

Figure 3. Box whisker plots of the percentage of sperm undergoing acrosomal exocytosis in response to CTB (panels A and B) or P4 (panel C) in the presence of inhibitors of various calcium channel inhibitors. A. Nickel (Ni) inhibited CTB-induced AE in a dose-dependent manner at concentrations that inhibit $Ca_V2.3$ channels (30 μM) as well as store-operated calcium channels (100 μM). B. The CTB-induced AE requires extracellular calcium, SOCC activity, and the $Ca_V2.3$ channel activity (n=4 replicates, $p< 0.005$). CTB-induced AE requires extracellular calcium as shown by the lack of response in calcium-depleted media (de-Ca). CTB-induced AE is inhibited by the SOCC inhibitor lanthanum (La; 10 μM), the L-, N-, P-type, and $Ca_V2.3$ channel inhibitor cadmium (Cd, 100 μM), but not LVA inhibitor nifedipine (Nif; 30 μM) or t-type kurtoxin KL1 (Kur; 1 μM). The $Ca_V2.3$ specific inhibitor SNX-482 (SNX; 500 nM) inhibits CTB-induced AE in addition to pertussis toxin (PTx). G_{M1}-induced AE showed the requirement for extracellular calcium and sensitivity to inhibitors as CTB-AE (data not shown). C. P4-induced AE also required extracellular calcium (de-Ca) and was inhibited by La, but was only partially inhibited by the same concentrations of Cd, nifedipine, and kurtoxin, and completely insensitive to SNX and pertussis toxin. The $GABA_A$ inhibitor picrotoxin (Picro, 50 μM) had no effect on CTB- or G_{M1}-induced AE (data not shown). Pertussis toxin (PTx, 0.1 μg/ml) inhibited CTB-, G_{M1}-, and ZP-induced AE but failed to inhibit P4-induced AE.

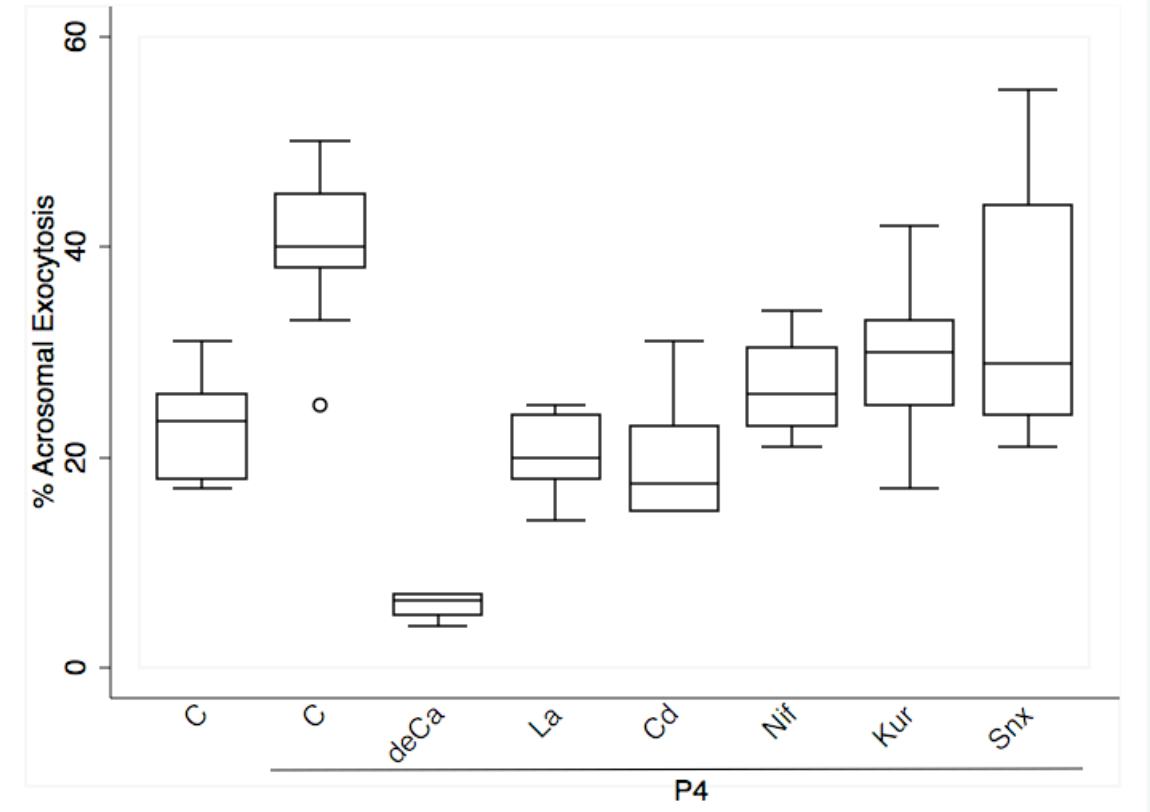
A.


% Acrosomal Exocytosis
10
20
30
40
50
C
C
Ni 1uM
Ni 30 uM
Ni 100 uM
CTB

B.


% Acrosomal Exocytosis
0
20
40
60
C
C
Ca
La
Cd
Nif
Kur
NX
Tx

C.


% Acrosomal Exocytosis
0
20
40
60
C
C
deCa
La
Cd
Nif
Kur
Snx
P4

The difference in SNX-482 susceptibility between CTB- and P4-induced AE suggested a difference in signaling pathways for these two agonists. No further increase in the percent of sperm undergoing AE was seen with the combination of progesterone and CTB and/or G_{M1}, suggesting that both agents were acting on the same physiologically relevant population of cells (data not shown). Like CTB, solubilized zona-pellucida proteins (ZP) also acts on the same physiologically relevant population of cells as P4, but utilizes a different initial signaling cascade for AE. Therefore, we wanted to characterize potential G_{M1}-mediated AE signaling components common to P4 and/or ZP.

SOCC are necessary for later steps of both of the physiological P4 and ZP induced AE (Bhandari, Bansal, Talwar, & Gupta, 2010; Jungnickel, Marrero, Birnbaumer, Lemos, & Florman, 2001; Sutton et al., 2004). However, like the G_{M1}-mediated AE, the initial ZP-induced calcium rise appears to differ from that in P4. Previous studies have shown that the P4-induced AE is inhibited by blocking $GABA_A$ channel activity with picrotoxin, while $GABA_A$ blockade has no effect on ZP induced AE (Bhandari et al., 2010; Murase & Roldan, 1996). Conversely, P4-induced AE is pertussis toxin insensitive, whereas ZP-induced AE is inhibited by pertussis toxin (0.1 μg/ml), providing a means to differentiate the initial steps of these two distinct pathways to AE (Endo, Lee, & Kopf, 1987; H. M. Florman, Tombes, First, & Babcock, 1989; Tesarik, Carreras, & Mendoza, 1992). Like the ZP-induced AE, both the CTB- and G_{M1}-induced AE are unaffected by the presence of the $GABA_A$ antagonist picrotoxin (100 μM), while conversely, pertussis toxin inhibits CTB- and G_{M1}- induced AE (Fig. 3B). This suggests that G_{M1}- mediated AE shares more common pathways with the ZP-induced AE than with P4. In support of this, SNX-482 inhibited ZP-induced AE,

suggesting that the $Ca_V2.3$ channel is essential for ZP induced AE as well as the G_{M1} mediated AE (Fig. 5).

Characterization of sperm lacking $Ca_V2.3$

In order to confirm the importance of the $Ca_V2.3$ channel in AE, we took a genetic approach. Sperm from mice lacking the α1E subunit of the $Ca_V2.3$ channel have normal intracellular calcium concentrations, but altered calcium dynamics (Sakata et al., 2002). This translates into a straighter flagellar wave-form and delayed and lower calcium rises in response to mannosylated-BSA, an agent that can induce AE in human sperm (Amin et al., 1996). No reports investigating ZP or P4 responses in $Ca_V2.3$ null sperm have been published. We therefore wanted to characterize the response of α1E, $Ca_V2.3$ null mice to address the potential role of this channel in AE and sperm function, especially given our pharmacologic data supportive of an important role of this channel in AE and G_{M1}-regulated sperm function.

The $Ca_V2.3$ channel is found in the apical acrosome and flagellum of mature sperm (Fig. 4). Tyrosine phosphorylation cascades in $Ca_V2.3$ null sperm were indistinguishable from wild-type sperm (data not shown), and motility and morphology were grossly normal. As reported previously (Sakata et al., 2002), the increase in intracellular calcium during capacitation was identical in normal and homozygous null mice as measured by fluo-3 and -4 calcium indicator fluorescence, suggesting that other calcium homeostasis and flux machinery are functional in the knockout mice (data not shown). However—and in agreement with our pharmacological data—sperm from $Ca_V2.3$ null mice failed to undergo AE in response to P4, CTB, G_{M1}, or ZP (Fig. 5). AE in response to calcium ionophore was

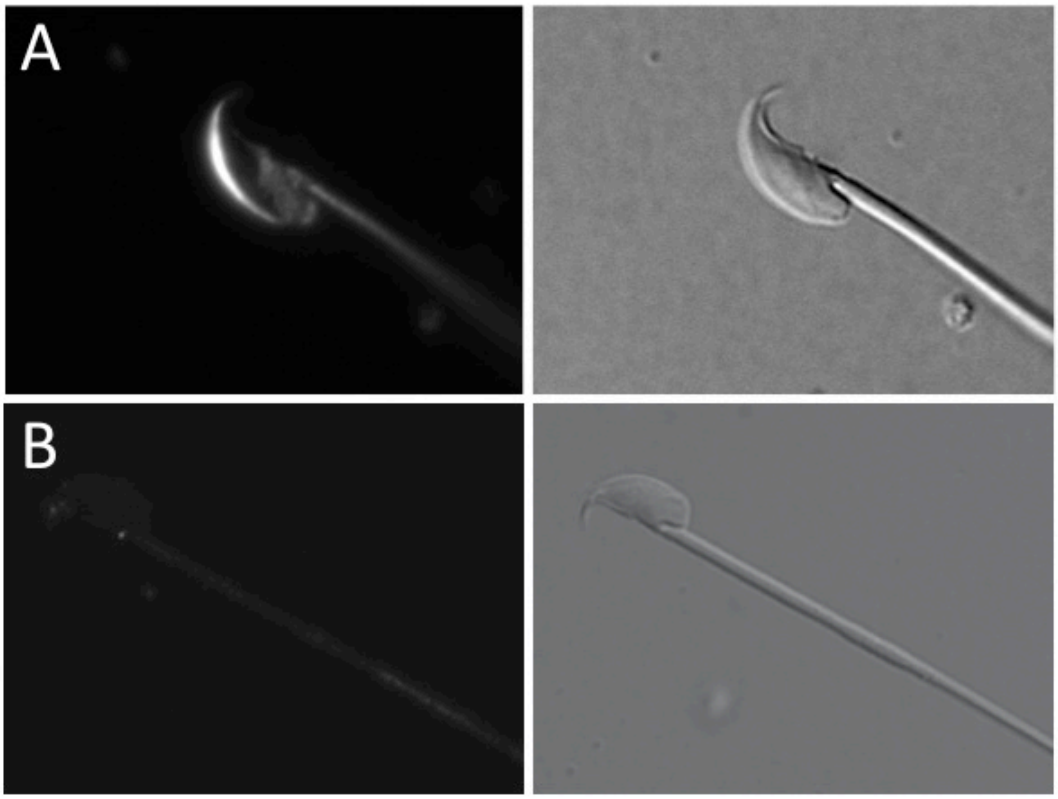


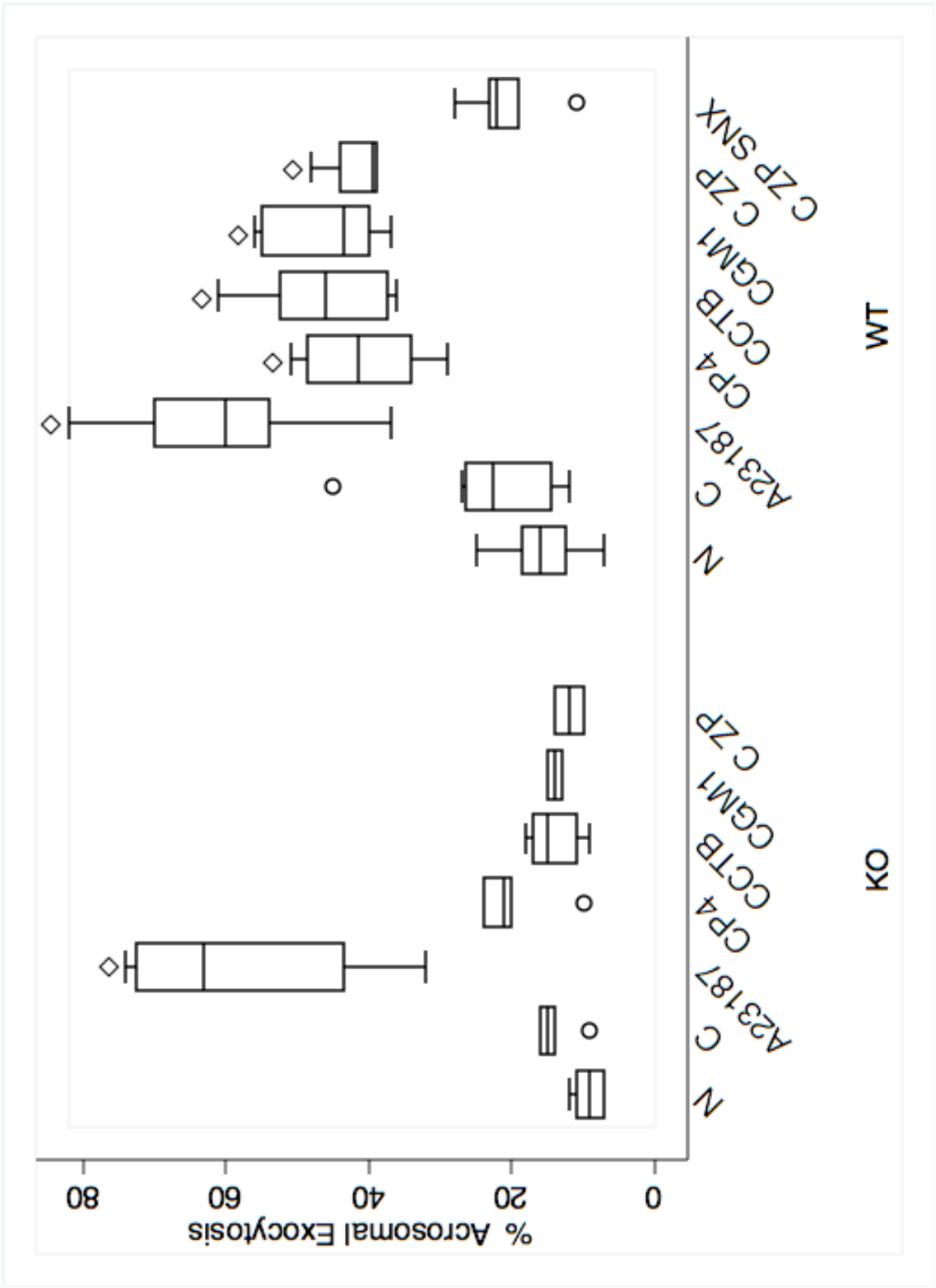
Figure 4. Indirect immunofluorescence images of strain-matched A) wild-type sperm stained with antibodies against the α1E subunit of the $Ca_V2.3$ calcium channel showing apical acrosomal and midpiece localization and a phase-contrast image of the same spermatozoon. B) A spermatozoon from an α1E homozygous null mouse stained with the same antibodies against the α1E subunit showing a complete lack of signal and a phase contrast image of the same spermatozoon.

normal in these sperm, suggesting that the AE membrane fusion machinery is intact and functional in these sperm (Fig. 5). Defects in $Ca_V2.3$ knockout fertility were evident in significantly smaller litters than strain-, age- and season-matched control mice (4 pups versus 7.1 pups, n= 12, p=0.001; Table 1) and impaired IVF rates with cumulus-intact oocytes (61% 2-cell embryos for wildtype sperm versus 4% 2-cell embryos for $Ca_V2.3$ null sperm; n=2 days, 4 female mice). These findings are supportive of our hypothesis that it is the failure of the initial calcium response in these sperm that prevents AE, and the $Ca_V2.3$ channel is responsible for the initial, voltage-operated calcium influx during regulated exocytosis in sperm.

Table 1. Average litter sizes for homozygous and heterozygous matings of wildtype and $Ca_V2.3$ null mice

Genotype Pairing	**Mean litter size**	**St. Deviation**	**N**
WT male/WT female	**7.1**	**1.6**	**11**
Null male/Null female	**4**	**2.1**	**13**
WT male/Null female	**7.25**	**1.25**	**8**
Null male/WT female	**4**	**6**	**2**

Figure 5. Box whisker plots of the percentages of AE in α1E null sperm (KO) and wildtype sperm (WT) in response to calcium ionophore A23187 (A23187), P4, CTB, G_{M1}, and ZP (n=4). KO sperm fail to respond to physiologic agonists for AE, but can undergo AE when intracellular calcium is artificially increased by using calcium ionophore A23187. ZP-induced AE in WT sperm is also inhibited by SNX-482 (CZP SNX) ($p > 0.0001$; $\alpha=0.05$). Diamond denotes statistical significance.


% Acrosomal Exocytosis
0
20
40
60
80
KO
WT
N
C
A23187
CP4
CCTB
CGM1
C ZP
N
C
A23187
CP4
CCTB
CGM1
C ZP
C ZP SNX

Discussion

The identification of the $Ca_V2.3$ channel as the voltage-operated channel responsible for the depolarization-induced rise in calcium at AE resolves several important conflicts and questions in the field of sperm biology, most notably, the reported differences between patch-clamp recordings on spermatogenic or uncapacitated sperm (Arnoult et al., 1998; Stamboulian et al., 2004) and reports from calcium imaging and pharmacologic studies (Sakata et al., 2001; Wennemuth et al., 2000). The necessity for PKA- (Hell et al., 1995) and for PKC-dependent phosphorylation and potential interaction with the raft-associated chaperone protein Hsp70 (Krieger et al., 2006) or syntaxin1A (Cohen & Atlas, 2004) for $Ca_V2.3$ current might explain the lack of $Ca_V2.3$ current found in uncapacitated or spermatogenic sperm cells. The physiologic maturation events of PKA and PKC activity and SNARE assembly have not occurred in these immature cells, and the sterol content maintains a high degree of membrane rigidity to limit diffusion of membrane signaling molecules. The lack of $Ca_V2.3$ activity without these membrane-remodeling events, such as an increase in fluidity and G_{M1} clustering, highlights the importance of capacitation in priming the sperm for the signaling processes of AE (Visconti, Stewart-Savage et al., 1999).

Precisely how changes in membrane G_{M1} heterogeneity result in opening of the $Ca_V2.3$ channel is unknown. G_{M1} is known to regulate calcium fluxes in somatic cells through voltage-operated channels (Buckley, Su, Milstien, & Spiegel, 1995; Muthing, Maurer, & Weber-Schurholz, 1998; Nakatani et al., 2009), as well as nuclear sodium/calcium exchangers and activation of second messenger signals such as IP_3 generation (see Mocchetti, 2005, for review). Clustering of G_{M1} might promote

voltage-operated calcium current in two main ways: through modification of secondary signaling molecules (Chen et al., 2003; Maehashi et al., 2003), or through direct interaction with the calcium channel itself (Xie, Wu, Lu, Rohowsky-Kochan, & Ledeen, 2004; Zhang et al., 2005; Zhao et al., 2004).

In support of the direct interaction hypothesis, many voltage-operated calcium channels are regulated by the highly glycosylated, extracellular α_2 component of the α_2-δ auxiliary subunit (see Davies et al., 2007, for review; Bannister et al., 2009). The δ portion of this subunit is a GPI-linked protein, so the sugars on the α_2 portion are held in a conformation that can interact with the pore-forming, α_1 subunit and is segregated to membrane rafts. Indeed, the conformation and interaction of G_{M1} with membrane calcium channels alone has been shown to promote the open gating conformation (Fang et al., 2002; Xie et al., 2004; Zhang et al., 2005), providing a mechanism by which G_{M1} might stimulate $Ca_V2.3$ calcium flux in sperm. Clustering of G_{M1} around a membrane calcium channel, either $Ca_V2.3$ or an additional channel, might initiate a small influx of calcium into the cell, similar to the effect of the extracellular portion of the $\alpha_2\delta$ auxiliary subunit association, that could cause sufficient depolarization to open additional voltage-operative $Ca_V2.3$ channels.

As another possibility, clustering of G_{M1} might influence membrane depolarization by changing the activity of the alkalinizing Hv1 proton pump (Lishko, Botchkina, Fedorenko, & Kirichok, 2010), the hyperpolarizing Slo3 channel (Santi et al., 2010), or BSA-activated calcium current through CatSper (Xia & Ren, 2009a; Xia & Ren, 2009b). The upstream activity of each of these ion transporters is necessary for AE and likely contributes to the membrane depolarization that facilitates $Ca_V2.3$ activity. Other upstream modifiers, such as PKC activity, G-protein activation, and/or SNARE

assembly are potential avenues for G_{M1}-mediated changes. For example, in Neuro2a cells, G_{M1} has been shown to mediate calcium influx through an R- or T-type channel dependent upon pertussis toxin-sensitive G-proteins (Ravichandra & Joshi, 1999). A similar G_{M1}-mediated mechanism might also account for the G_{M1}- and ZP3-induced AE sensitivity to pertussis toxin.

Our characterization of sperm membrane organization and dynamics have led to a model through which either 1) removal of inhibitory factor SVS2 (Kawano & Yoshida, 2007), 2) an increase in membrane fluidity with sterol efflux (Jones, 2010), or 3) point fusion events between the plasma membrane and acrosomal membrane (Kim & Gerton, 2003) and lipid transfer allows the clustering of G_{M1} focally in the plasma membrane around a signaling target to activate calcium influx. Some combination of these three possibilities is also quite likely.

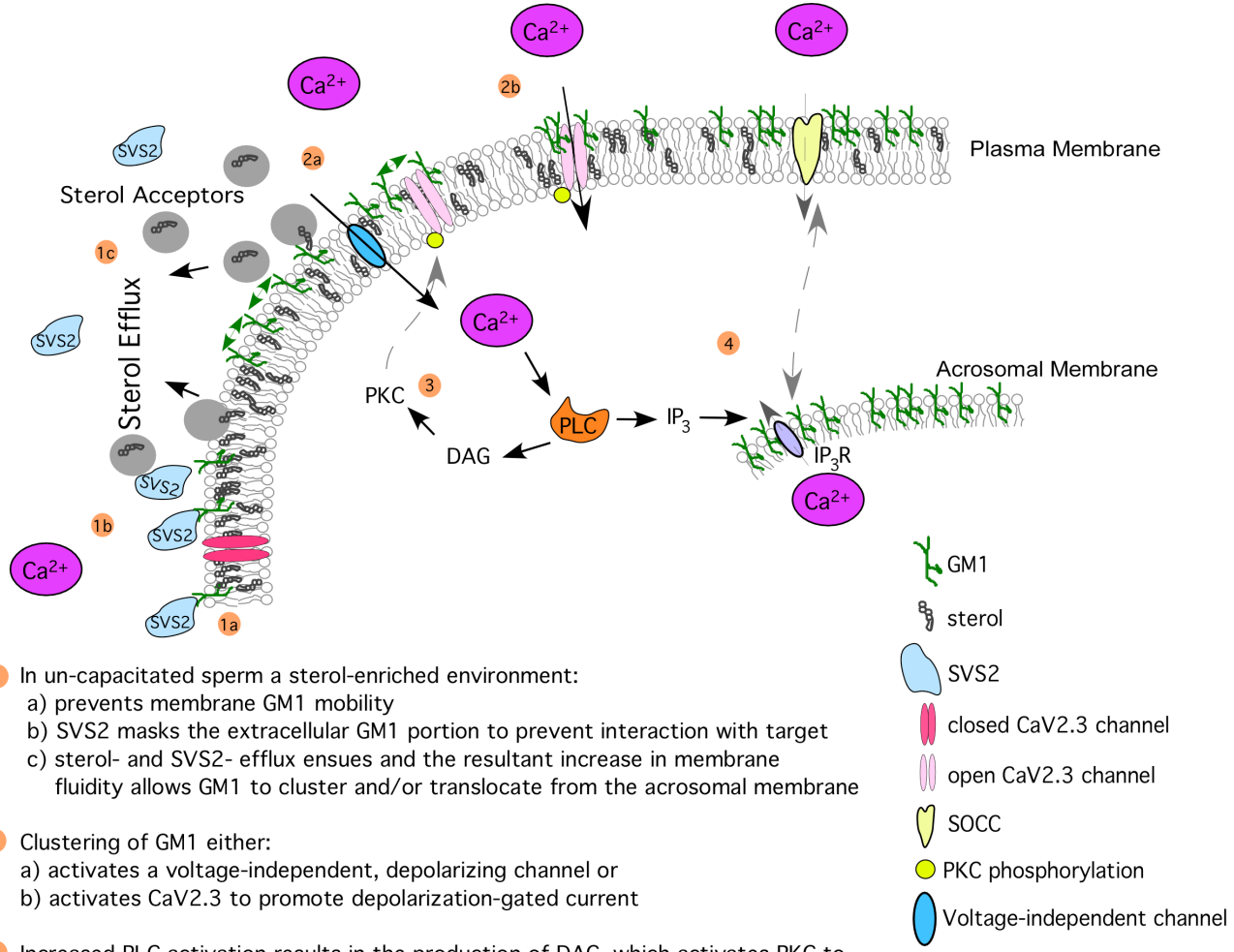
The decapacitation factor SVS2 specifically binds G_{M1} during the process of ejaculation and is gradually lost from the sperm surface during transit through the female reproductive tract (Kawano & Yoshida, 2007; Kawano, Yoshida, Iwamoto, & Yoshida, 2008). The evolution of a conserved protein that specifically and transiently masks the extracellular portion of G_{M1} to prevent capacitation is highly suggestive of an important role for G_{M1} in physiologic signaling processes important for fertilization. The decapacitation effects of SVS2 might be accomplished by preventing G_{M1} interaction with membrane targets, such as $Ca_V2.3$. Such a mechanism of "masking" G_{M1} might act as an additional means for sperm to regulate a gradual clustering of G_{M1}. This unmasking is likely accompanied by other membrane changes that occur with sterol efflux.

Although the requirement for sterol efflux in fertilization has been known for decades, the exact mechanisms through which sterol efflux influences sperm signaling have not been fully elucidated. Efflux of sterols from the plasma membrane might facilitate G_{M1} stimulation of $Ca_V2.3$ activity in sperm. Capacitation-induced changes in lipid diffusibility within the acrosomal sub-domain have suggested that sterol efflux mediates an increase in membrane fluidity, allowing the formation and/or diffusion of smaller raft units within the larger sub-domain (Smith, McKinnon-Thompson, & Wolf, 1998; van Gestel et al., 2005; Wolf, Maynard, McKinnon, & Melchior, 1990; Wolf, 1995; Wolfe, James, Mackie, Ladha, & Jones, 1998). Association of raft-associated syntaxin 1A and/or synaptotagmin with $Ca_V2.3$ dramatically increases current through this channel (Cohen & Atlas, 2004). Likewise, raft-dependent interaction of the α_2-δ subunit with the pore-forming calcium subunits has been hypothesized to prevent channel activation until raft-association of the pore-forming subunit occurs with membrane reorganization (Davies et al., 2010; Dickman, Kurshan, & Schwarz, 2008). A similar raft-orchestrated regulation of G_{M1}-$Ca_V2.3$ dynamics would provide a mechanism both for the regulation of $Ca_V2.3$ activity, as well as the requirement for sterol efflux in AE. Such an increase in fluidity, coupled with the loss of SVS2, might allow the movement and clustering of the extracellular sugars of G_{M1} within the plasma membrane, similar to the experimental clustering induced with CTB or the addition of exogenous G_{M1} (Fig. 6). Clustering of other potential oocyte or ZP interaction molecules, such as β-1,4-galactosyltransferase-1 (Gong, Dubois, Miller, Shur, & 5231, 1995), on the sperm surface might also facilitate the clustering of G_{M1} around the $Ca_V2.3$ channel.

Alternatively or concomitantly, the efflux of sterols from the plasma membrane and the resultant increase in membrane fluidity might allow point fusion events to occur

between the plasma membrane and the outer acrosomal membrane. These point fusion events might allow G_{M1} enriched in the acrosomal membrane to move to the plasma membrane, as seen with the acrosomal protein sp56 (Kim & Gerton, 2003). Although it has been known for decades that the process of AE itself has several steps (Saling, Sowinski, & Storey, 1979), only recently has it been proposed that "kiss-and-run" fusion events, similar to those observed in neurons, occur between the APM and OAM during the process of sperm capacitation (Buffone, Foster, & Gerton, 2008; Gerton, 2001; Kim & Gerton, 2003). In either the membrane fluidity or membrane point fusion scenario, the data presented here suggest that the resultant local enrichment of G_{M1} upon sterol efflux allows the activation and opening of the $Ca_V2.3$ channel, resulting in AE.

Figure 6. Schematic drawing of a sperm plasma membrane and acrosome depicting the potential roles of G_{M1} in sperm signaling pathways leading to AE. In ejaculated sperm, the plasma membrane overlying the acrosome is highly enriched in sterols. Seminal plasma components, such as SVS2, have adsorbed to the plasma membrane by binding G_{M1}. During transit through the female reproductive tract, sterols and seminal plasma components, including SVS2, are removed from the plasma membrane, resulting in an increase in membrane fluidity. The increased membrane fluidity, coupled with the loss of SVS2 binding to G_{M1}, might allow increased movement and clustering of G_{M1} in the plasma membrane. Alternatively or concomitantly, the changes in membrane fluidity facilitate point fusion events between the plasma membrane and the outer acrosomal membrane, allowing further enrichment and focal clustering of G_{M1} in the plasm membrane. Focal clustering of G_{M1} around a membrane target such as the $Ca_V2.3$ calcium channel allows open gating of the channel and a resultant increase in intracellular calcium within the spermatozoon. This increase in calcium stimulates PLCδ4 production of IP_3, which stimulates IP3 receptors to release calcium stores and generate store-operated calcium influx. The sustained calcium influx results in membrane fusion complex formation and exocytotic release of the acrosomal vesicle.



1 In un-capacitated sperm a sterol-enriched environment:
a) prevents membrane GM1 mobility
b) SVS2 masks the extracellular GM1 portion to prevent interaction with target
c) sterol- and SVS2- efflux ensues and the resultant increase in membrane fluidity allows GM1 to cluster and/or translocate from the acrosomal membrane

2 Clustering of GM1 either:
a) activates a voltage-independent, depolarizing channel or
b) activates CaV2.3 to promote depolarization-gated current

3 Increased PLC activation results in the production of DAG, which activates PKC to phosphroylate CaV2.3 and further increase current amplitude

4 PLC generation of IP_3 activates acrosomal IP_3R, resulting in acrosomal calcium release and SOC activation in the plasma membrane to stimulate acrosomal exocytosis

These potential pathways are not mutually exclusive and likely work in concert to facilitate optimal sperm responsiveness. Further work characterizing the chronology and nature of the signaling events leading to $Ca_V2.3$ mediated calcium influx will greatly contribute to the knowledge of this enigmatic but crucially important step in sperm-egg communication.

ACKNOWLEDGEMENTS

We would like to thank the lab of Dr. Richard Miller for the generous gift of the α1E null mice used in these studies as well as Dr. Claire Fewtrell and Dr. Roy Cohen for helpful discussions on calcium channel pharmacology.

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CHAPTER 6

THE TRKA KINASE PATHWAY IS ACTIVE DURING CAPACITATION OF MURINE SPERM AND REGULATED BY THE GANGLIOSIDE GM1

Abstract

Previous studies have shown that the ganglioside G_{M1} is highly enriched in and stably segregated to the acrosomal membrane and plasma membrane overlying the acrosome in human, mouse, and bull. G_{M1} is also specifically bound by the decapacitation factor SVS2 from seminal vesicle fluid upon ejaculation and is progressively lost from the membrane as the sperm transits the female reproductive tract. The mechanism of the decapacitating effect of SVS2 is currently unknown. In order to elucidate this mechanism and the role of G_{M1} in sperm membrane dynamics, we investigated the effects of modulating G_{M1} concentrations in murine sperm. Exogenous G_{M1} accelerated tyrosine phorphorylation of sperm proteins even without the presence of a sterol acceptor in the medium and bypassed the decapacitating effects of SVS2. This hastening of phosphorylation was blocked specifically by the TrkA inhibitor tyrphostin AG 879. Supporting the specificity of the inhibitor, TrkA mRNA and protein were identified in sperm. Activation of PLC by TrkA appears essential for these effects. This is the first report documenting the presence of TrkA kinase activity in sperm and its potential role in capacitation. These findings have important clinical implications regarding the presence of G_{M1} in current semen extenders and implications in cryocapacitation of mammalian sperm.

Introduction

The ability of the lipid microenvironment to influence the composition and accessibility of signaling molecules effectively regulates the responsiveness, efficacy, duration, and reliability of signaling cascades (Lingwood & Simons, 2010). Although described relatively recently, membrane domains termed membrane rafts are essential components of cellular signaling processes. Membrane rafts are defined as "small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" (Pike, 2006). Cells utilize transmembrane proteins, lipid binding proteins, cytoskeletal elements and active vesicular trafficking mechanisms to maintain the heterogeneity of membrane rafts (Fan, Sammalkorpi, & Haataja, 2009). This interplay of components results in an organization more sophisticated than lipid phase separation would allow on its own.

Membrane rafts have been described in sperm and appear to play an integral role in signaling and maturation based on their dynamic reorganization during capacitation (Bou Khalil et al., 2006; Jones et al., 2010; Nixon & Aitken, 2009; Asano et al., 2009; Travis et al., 2001; van Gestel et al., 2005). The process of capacitation has not been fully described but includes the removal of sterols and seminal plasma constituents from the plasma membrane with a concomitant influx of calcium and bicarbonate into the cell (Darszon et al., 2005; Demarco et al., 2003). The rise in intracellular bicarbonate activates a sperm-specific soluble adenylate cyclase (sAC) to produce cAMP, resulting in PKA activation (Breitbart, 2003; Wuttke, Buck, & Levin, 2001). Through an unknown mechanism, these changes are transduced into a series of

tyrosine phosphorylation events on a specific subset of protein residues (Visconti, Stewart-Savage et al., 1999).

Several kinases have been identified in sperm, and these enzymes play redundant roles. The activation of calmodulin dependent kinase (CAMKII) is essential for hyperactivated motility patterns (Ignotz & Suarez, 2005). Protein kinase C appears necessary for the pathways of capacitation resulting in exocytotic release from the single sperm vesicle, the acrosome, upon contact with the egg coat (Breitbart, Lax, Rotem, & Naor, 1992). Still different tyrosine phophorylation pathways appear active during the process of cryocapacitation, during which the freeze-thaw process results in a slightly different subset of phosphorylated proteins (Cormier & Bailey, 2003; Hammerstedt, Graham, & Nolan, 1990; Huo, Yue, & Yang, 2002; Pommer, Rutllant, & Meyers, 2003). Freeze-thawed sperm display altered membrane properties, tyrosine phosphorylation, and are able to undergo acrosomal exocytosis without prior incubation with capacitating stimuli, an effect which limits the longevity and fertility of these sperm.

Important roles for membrane rafts in tyrosine phosphorylation cascades are known to exist in many excitable cells, and rafts have been hypothesized to play a role in organizing signaling pathways involved in capacitation (Guan, 2004; Harder, 2004; Janes, Ley, & Magee, 1999; Parpal, Karlsson, Thorn, & Stralfors, 2001; Sato et al., 2003; Ushio-Fukai et al., 2001). Membrane rafts organize lipid-protein complexes that facilitate epidermal and nerve growth-factor receptor phosphorylation cascades in many cells, as well as calcium-dependent kinase activity and other calcium dependent processes (Salzer, Hinterdorfer, Hunger, Borken, & Prohaska, 2002; Sato et al., 2003).

Lipid constituents of membrane rafts can also act as signaling molecules themselves. The importance of phosphoinositides in regulating kinase pathways and intracellular calcium release is well documented in sperm (Berruti & Franchi, 1986; Domino & Garbers, 1989; Roldan & Harrison, 1989), and glycosphingolipids can act both as cell surface receptors as well as transducers of intracellular signals in somatic cells (McNamara et al., 2001; Rusnati et al., 2002; Tsai et al., 2003; Yu et al., 2002). Most notably, lipid constituents of membrane rafts have been hypothesized to play an important role in the organization of membrane fusion machinery that is essential for sperm-egg recognition (see Nixon & Aitken, 2009, for review).

The glycosphingolipid G_{M1} is commonly associated with membrane rafts and is involved in many signaling processes in somatic cells. The pentameric B subunit of cholera toxin binds up to five G_{M1} molecules in a membrane with high specificity and affinity (Cuatrecasas, 1973; Lauer, Goldstein, Nolan, & Nolan, 2002). This experimental tool has contributed greatly to studies of G_{M1} in live cells and cell signaling pathways. G_{M1} has a role as a receptor for cholera toxin, SV40 and polyoma virus, and as a co-receptor for FGF2 (Cuatrecasas, 1973; Fukumoto et al., 2006; Tsai et al., 2003). Through these and other pathways, G_{M1} is involved in cell differentiation and growth, tyrosine phosphorylation cascades, and modulation of calcium homeostasis and flux (Brodsky et al., 2003; Chen et al., 2003; Fang, Xie, Ledeen, & Wu, 2002; Rusnati et al., 2002). G_{M1} can interact either directly or through binding partners to increase calcium flux through several calcium transporters and channels (Buchwald et al., 2007; Buckley, Su, Milstien, & Spiegel, 1995; Ledeen & Wu, 2007; Yatomi, Igarashi, & Hakomori, 1996). Likewise, exogenous G_{M1} will also induce calcium influx in sperm (see chapter 5). This has dramatic and important effects in calcium dependent processes in sperm, including acrosomal exocytosis.

In mammalian sperm, G_{M1} is stably segregated to and highly enriched in the plasma membrane overlying the acrosome of several species (Buttke, Nelson, Schlegel, Hunnicutt, & Travis, 2006; Selvaraj et al., 2006). G_{M1} is specifically bound by the decapacitation factor SVS2, which is gradually removed from the plasma membrane as sperm transit the female reproductive tract (Kawano, Yoshida, Iwamoto, & Yoshida, 2008). In this report we investigate the role of G_{M1} as a signaling molecule in murine sperm and present a model in which G_{M1} enhances signaling pathways important in capacitation and acrosomal exocytosis. These findings have important implications not only to our understanding of mammalian sperm capacitation, but also to the formulation and use of cryopreservation and semen extension media.

Materials and Methods

Reagents and animals

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. Male CD-1 mice were purchased from Charles River Laboratories (Kingston, NY). Purified G_{M1}, ceramide, and asialo-G_{M1} were purchased from Matreya, LLC (Pleasant Gap, PA). A monoclonal antibody against phosphorylated tyrosine, clone 4G10 was purchased from Invitrogen (Carlsbad, CA). Antibodies against TrkA and phosphorylated TrkA were obtained from Cell Signaling Technology (Danvers, MA). All animal procedures were performed under the guidelines of the Institutional Animal Care and Use Committee at Cornell University.

Preparation of media

For murine sperm, a modified Whitten's medium (MW: 22 mM HEPES, 1.2 mM $MgCl_2$, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, 4.8 mM lactic acid hemi-calcium salt, pH 7.35) (Travis et al., 2001) was used for all incubations. Glucose (5.5 mM), $NaHCO_3$ (10 mM), and 2-hydroxypropyl-β-cyclodextrin (2-OHCD; 3 mM) were supplemented as needed. 2-OHCD supports sperm capacitation and in vitro fertilization by functioning as a sterol acceptor, and is preferred over the more potent methyl-β-cyclodextrin (Visconti et al., 1999).

Sperm collection and handling

Murine sperm were collected from the cauda epididymides of male CD-1 mice by a swim-out procedure as described previously (Travis et al., 2001). All steps of collection and washing were performed at 37˚C using MW medium, and large orifice transfer pipettes or large orifice pipette tips were used for handling sperm to minimize membrane damage. After the initial washes but prior to experimental incubations, motility assessment was carried out, and samples showing <60% motility were not used.

Sperm capacitation and tyrosine phosphorylation

For murine sperm, incubation with different stimuli for capacitation was carried out with $2x10^6$ sperm in 300 µl of medium with 5.5 mM glucose under one of four conditions: (a) MW base medium, (b) MW supplemented with 10 mM $NaHCO_3$, (c) MW supplemented with 3 mM 2-OHCD and (d) MW with both 10 mM $NaHCO_3$ and 3 mM 2-OHCD, for 45 minutes. The pH of medium for all incubation conditions was adjusted to 7.35. The medium in incubation condition (d), has been shown to be

sufficient to support IVF (Travis et al., 2004) and capacitation-induced tyrosine phosphorylation in murine sperm.

Western blotting to detect phosphorylated tyrosine residues as a marker for capacitation associated changes was conducted as described previously (Travis et al., 2001). Briefly, the sperm were homogenized in 5X protease inhibitor cocktail containing 0.2 mM sodium orthovanadate and the proteins were solubilized by boiling in sample buffer and then separated by SDS-PAGE under reducing conditions. Immunoblotting performed with an anti-phosphorylated tyrosine monoclonal antibody and detection by chemiluminescence were performed as described previously (Travis et al., 2001).

Isolation of SVS2

Native SVS2 was purified according to (Kawano & Yoshida, 2007). Seminal vesicle fluid was collected from the seminal vesicles of retired male CD-1 mice by manual expression of dissected glands into 1 ml PBS pH 7.4. The fluid was centrifuged at 10,000 x g for 10 minutes at 4˚ C and the supernatant was collected and placed over a P-30 gel filtration column (1 x 50 cm, Bio-Rad Japan, Tokyo) and centrifuged for 2 minutes at 200 x g in a swinging bucket rotor. The eluate was desalted and run on a polyacrylamide gel and stained with Coomassie Blue to confirm the presence of a single 40-kDa protein before the purified SVS2 was lyophilized and stored at -80 ˚ C until use.

Indirect immunofluroescence

Sperm were fixed and treated as described previously for detection of proteins on whole cells (Selvaraj et al., 2006). Cells were fixed with 4% paraformaldehyde with

0.2 % gluteraldehyde for 10 minutes. After fixation, cells were washed with PBS and blocked overnight in 3% bovine serum albumin at 4 ˚ C. Cells were incubated in primary antibody at a 1:50 dilution for 2 hours, washed three times in PBS, and incubated in anti-rabbit Alexa fluor 488 at a 1:200 dilution. As a negative control, cells were incubated in secondary antibody alone after the blocking step. Imaging was done using a Nikon Eclipse TE 2000-UY microscope (Nikon, Melville, NY) equipped with a Photometrics Coolsnap HQ CCD camera (Roper Scientific, Ottobrunn, Germany) and Openlab 3.1 automation and imaging software.

Molecular characterization

Germ cells were isolated from the testes of male CD-1 retired breeders by a 20-minute incubation in collagenase followed by a 10-minute incubation in 0.5 mg/ml trypsin with 1 µg/ml DNAse I. The cell suspension was filtered through a mesh filter, and cells were collected and lysed for RNA extraction using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription PCR was performed in the germ cell RNA using the superscript III kit (Invitrogen) and primer (5' – TCG CCT CAG TGT TGG AGA G) corresponding to the C-terminal intracellular kinase domain of TrkA. Resulting cDNA was amplified using GoTaq enzyme with amplification reactions performed in volumes of 25 µl using TrkA specific primer (3' AGG TGG CTG CTG GTA TGG T 5') containing 50 ng template DNA, reaction buffer (10 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$), 200 µM dNTP (Fisher Scientific, Pittsburgh, PA, USA), and 0.5 U Taq DNA polymerase (Invitrogen) in a Mastercycler gradient PCR machine (Eppendorf, Westbury, NY, USA). The protocol was as follows: initial denaturation for 2 min at 94 °C; then 35 cycels of 94 °C for 20 s, 58.5 °C for 15 s, and 72 °C for 1.5 minutes, followed by a final extension cycle of 5 min at 72

°C. PCR products were purified using agarose gel electrophoresis. A single band was excised from the germ cells and submitted to the Cornell Biological Resource Center (Ithaca, NY) for sequence analysis.

Results

We have previously found that the plasma membrane overlying the acrosome and outer acrosomal membrane of mammalian sperm is highly enriched in G_{M1} (Buttke et al., 2006; Selvaraj et al., 2006; Selvaraj et al., 2009), and that G_{M1} influences calcium dynamics crucial for AE (see chapter 5). Because calcium flux is a key component of other signaling pathways important for sperm function, we next investigated the impact of exogenous G_{M1} on calcium-dependent tyrosine phosphorylation events in sperm. We evaluated the effect of exogenous G_{M1} on tyrosine phosphorylation cascades over 0, 15, 30, 45, and 60 minutes of incubation in capacitating and non-capacitating conditions. Exogenous G_{M1} accelerated tyrosine phosphorylation cascades beyond levels seen under capacitating conditions alone (Fig. 1). As shown in Figure 1, G_{M1} induced phosphorylation in the same subset of proteins as seen under capacitating conditions, suggesting that the same targets and pathways are active. After 15 minutes of incubation, levels of phosphorylation in sperm treated with exogenous G_{M1} equaled levels of phosphorylation seen only after 60 minutes of incubation under capacitating conditions without exogenous G_{M1} in the media.

Interestingly, G_{M1} appeared to bypass the need for a sterol acceptor in the media, because bicarbonate and G_{M1} alone were able to induce tyrosine phosphorylation consistent with capacitation (Fig. 2, lane B5). The extracellular sugars of G_{M1} appear

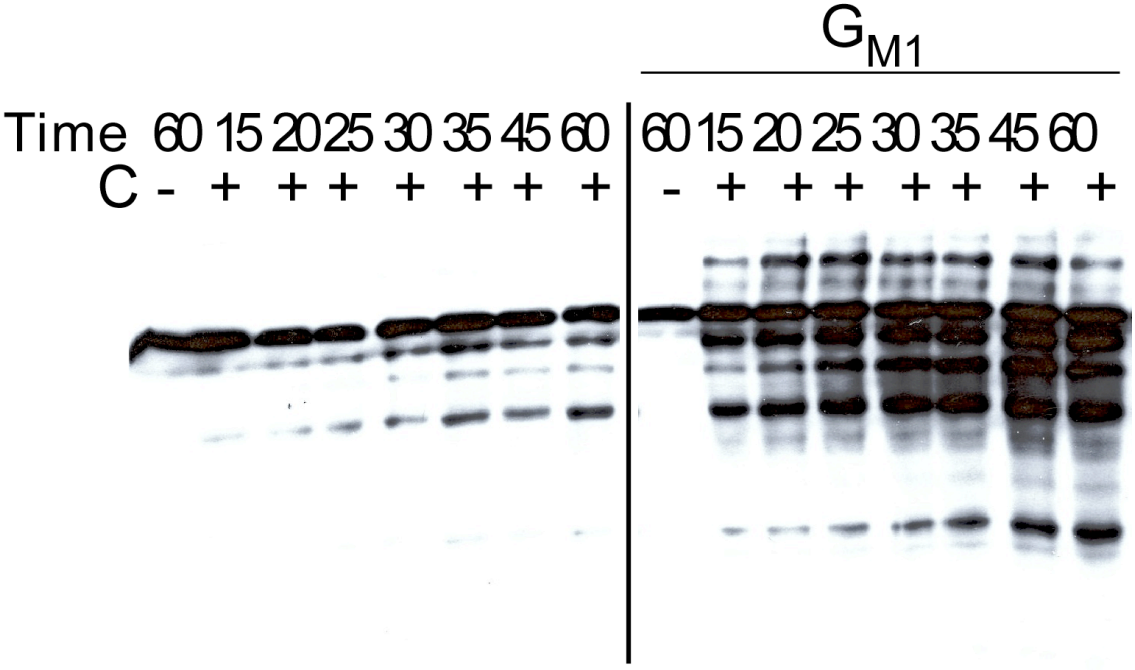


Figure 1. A Western blot of phosphorylated tyrosine residues in sperm incubated under capacitating conditions without and with 25 μM G_{M1} in the media, showing G_{M1}-mediated acceleration of tyrosine residues beyond levels seen under capacitating conditions alone. C=capacitating conditions.

important in mediating this effect, as ceramide (which lacks these sugars) failed to induce this effect (lanes B3, D3) and asialo- G_{M1} increased phosphorylation to a lesser extent and only in the presence of a sterol acceptor in the media (lanes B4, D4). Additionally, the increased efficacy of G_{M1} in inducing phosphorylation was apparent despite the fact that the increased hydrophobicity of ceramide and asialo- G_{M1} compared to G_{M1} result in a higher percent of these lipids inserting into a membrane as compared to exogenous G_{M1} (O'Keefe & Cuatrecasas, 1977; Scheel, Schwarzmann, Hoffmann-Bleihauer, & Sandhoff, 1985; Spiegel, Schlessinger, & Fishman, 1984). The requirement for sialic acid in ganglioside-mediated effects in sperm is in agreement with reports from somatic cells where the extracellular sialic acid residue of G_{M1} is necessary to induce conformational changes in membrane-associated targets such as tyrosine kinases (Duchemin, Ren, Mo, Neff, & Hadjiconstantinou, 2002a; Woronowicz et al., 2007).

Cells can regulate membrane lipid composition in a variety of ways. Transfer of G_{M1} between membranes can occur through both spontaneous and facilitated transport mechanisms and result in focal enrichments of G_{M1} (Brown & Thompson, 1987; Brown & Hyland, 1992; Depauw et al., 1990). Conversely, regulation through the acquisition or loss of binding partners can change the movement and accessibility of membrane lipids. One way in which sperm might regulate G_{M1}'s interactions in vivo is through the gradual loss of the seminal vesicle protein SVS2 that binds specifically to G_{M1} during ejaculation and is lost during transit through the female reproductive tract (Kawano et al., 2008). This would provide an additional level of physiologic regulation of G_{M1} dynamics. We tested whether the addition of G_{M1} could bypass the SVS2 mediated suppression of tyrosine phosphorylation cascades that occurs during

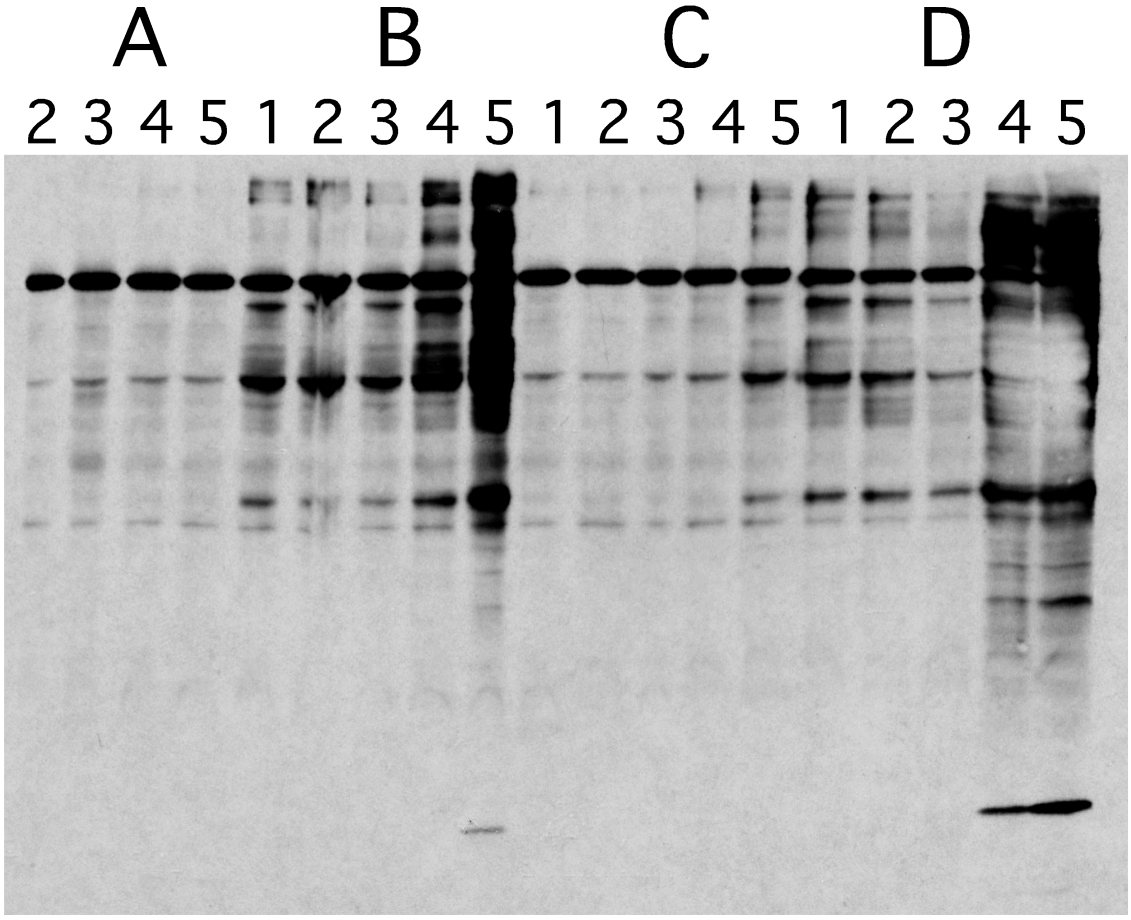


Figure 2. Exogenous G_{M1} accelerates the phosphorylation of tyrosine residues in sperm. In panel A) sperm were incubated in MW base media alone, B) MW supplemented with 10 mM bicarbonate, C) MW supplemented with 3 mM 2-hydroxypropyl cyclodextrin (2-OH CD), and D) MW supplemented with both 10 mM bicarbonate and 3 mM 2-OH CD. Lane 1) base media, 2) DMSO solvent control, 3) 25 uM ceramide, 4) 25 uM asialo G_{M1}, 5) 25 uM G_{M1}.

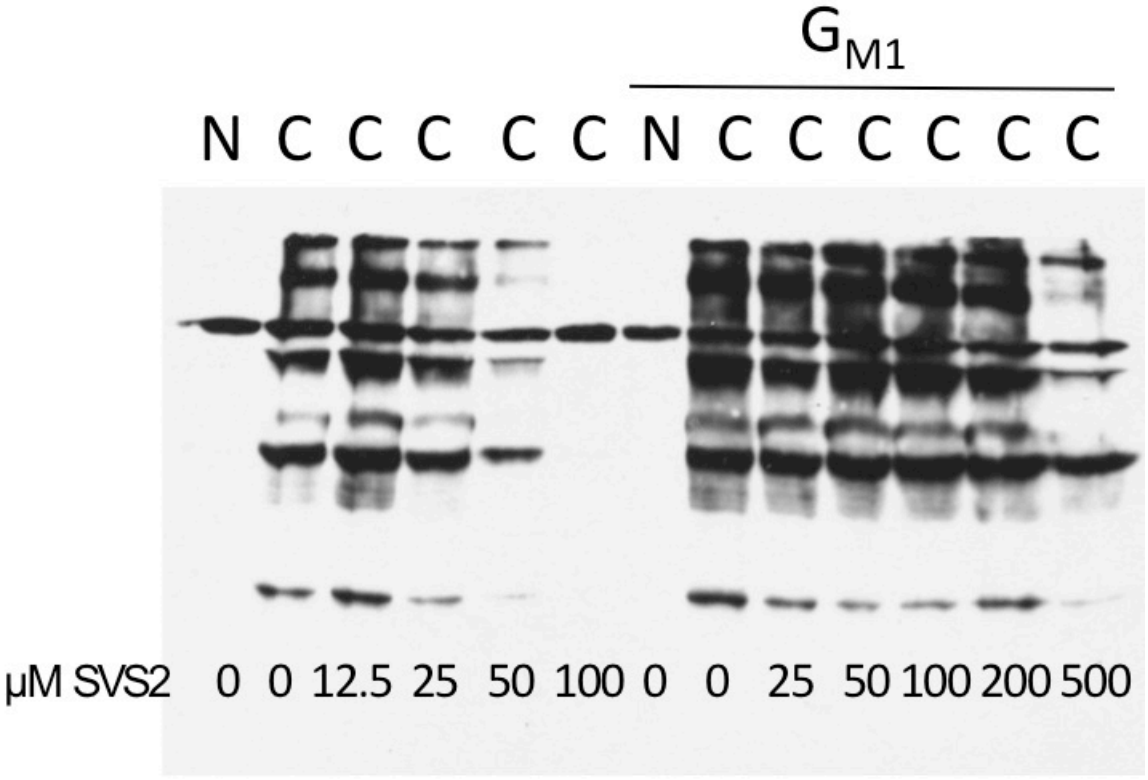


Figure 3. Exogenous G_{M1} overcomes SVS2 mediated inhibition of tyrosine phosphorylation. A 20-fold increase in SVS2 concentration was needed to overcome the effects of exogenous G_{M1} in the media. Physiologic concentrations of SVS2 are reported to be approximately 25 μM according to Kawano and Yoshida, 2008. N= MW base media alone. C= MW supplemented with 10 mM bicarbonate and 3 mM 2-OHCD.

normal fertilization. As shown in Figure 3, SVS2 inhibits tyrosine phosphorylation in a dose-dependent manner. This inhibition was overcome with the addition of G_{M1} to the media. A twenty-fold increase in SVS2 concentration was necessary to inhibit the G_{M1} effects on tyrosine phosphorylation.

In vitro and in vivo studies of murine neurons have shown that clustering of G_{M1} in the plasma membrane results in the transactivation of the tyrosine kinase and NGF receptor TrkA (Duchemin, Neff, & Hadjiconstantinou, 1998a; Duchemin, Ren, Mo, Neff, & Hadjiconstantinou, 2002a; Farooqui, Franklin, Pearl, & Yates, 1997). Importantly, this activation occurs irrespective of the presence of NGF through association with G_{M1} with or without G-protein coupled receptors. TrkA has been localized by immunohistochemistry to the head and flagellum of mature sperm in rat, human, and macaque (Jin et al., 2006; Li et al., 2005; Muller et al., 2006), but has not been investigated in murine sperm. Therefore, we wanted to confirm and characterize the presence of TrkA in murine sperm. We conducted PCR on cDNA from mixed male germs cells of mice and sequenced the corresponding 218 base pair fragment of TrkA. Immunoblotting revealed the presence of an approximately 130 kDa protein recognized by a polyclonal antibody directed against the N terminus of somatic cell TrkA near alanine 225 (Fig. 4). An additional band of approximately 10 kDa was identified that was not present in brain. The antibody used for Western blotting was not able to immunolocalize TrkA in fixed sperm (data not shown). Conversely, a different commercial antibody against TrkA labeled the flagellum and apical acrosome of sperm but failed to recognize TrkA in Immunoblotting techniques. Although these data agree with previous reports localizing TrkA in the acrosomal area of rat and macaque sperm, they should be interpreted with caution given the application-limited

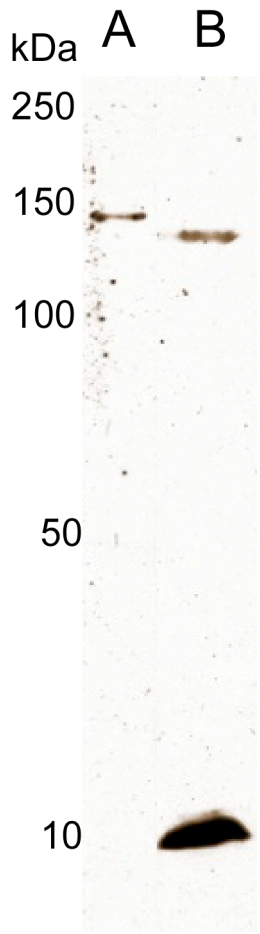
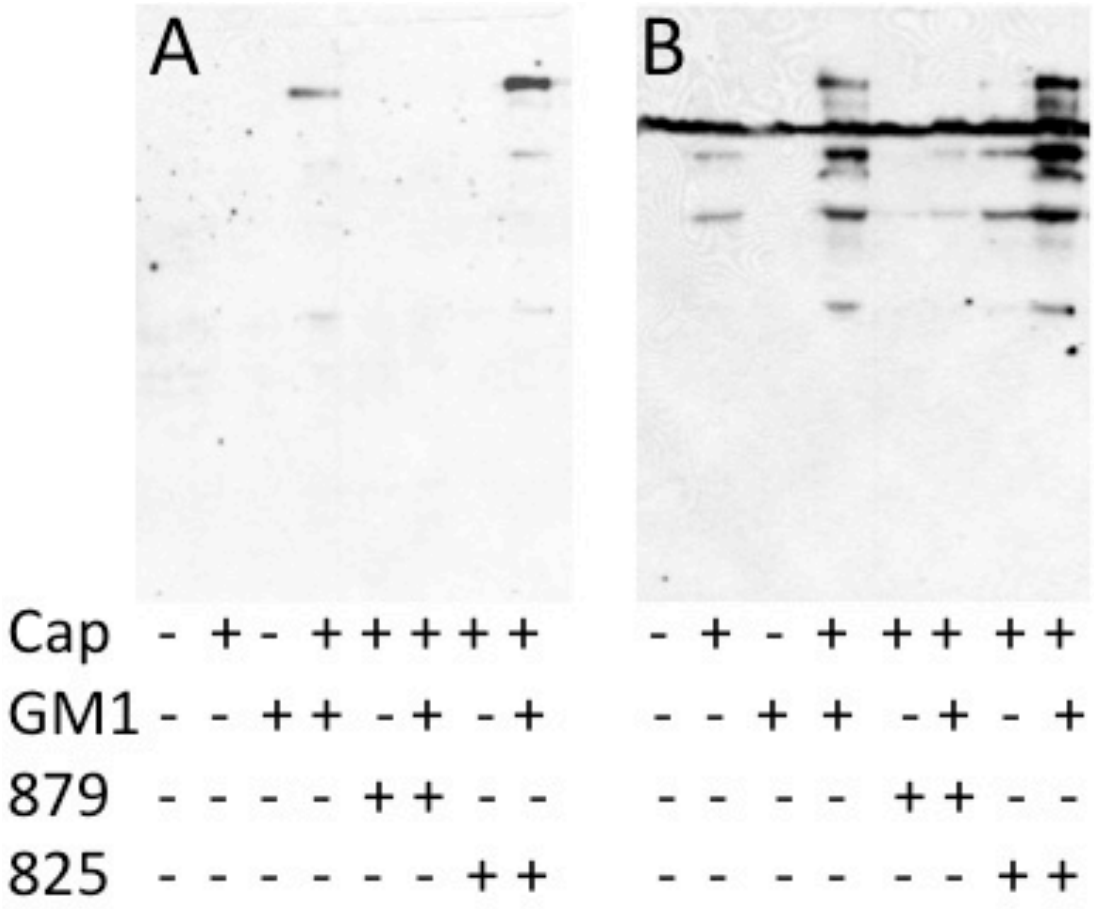


Figure 4. Immunoblot of total brain and sperm protein extracts using a polyclonal antibody against the extracellular portion of TrkA. Lane 1) brain, lane 2) sperm. The smaller band seen in the sperm extracts is at the level of the dye front.

nature of the antibodies and noted potential differences in the antigenic domain of TrkA.

Having confirmed the presence of the kinase domain of TrkA in sperm, we next attempted to characterize TrkA activity during sperm signaling pathways stimulated by exogenous G_{M1}. The binding of TrkA to its ligand or interaction with G_{M1} induces TrkA dimerization and trans-phosphorylation of the kinase domain in neurons, resulting in its activation. Using an antibody directed against the active phosphorylated TrkA, we found TrkA to be strongly activated by exogenous G_{M1} (Fig. 5). This phosphorylation occurred in the absence of G_{M1} only after prolonged incubation under capacitating conditions and did not yield as robust a response. Importantly, we were able to inhibit G_{M1}-induced tyrosine phosphorylation in sperm through the use of the specific TrkA inhibitor tyrphostin AG 879 (Fig. 5B; 25 μM). Although less dramatic, TrkA inhibition reduced tyrosine phosphorylation under normal capacitating conditions as well. This appeared to be specific to TrkA inhibition, because use of tyrphostin AG 825, to inhibit Her2 (a protein whose transcription is reported to be down-regulated by tyrphostin AG 879) had no effect on tyrosine phosphorylation or sperm viability at any timepoint (25 μM; data not shown). The nitric oxide synthesis pathway has also been implicated in tyrosine phosphorylation cascades in sperm, but appears not to play a role here based on insensitivity to the inhibitor N (G)-nitro-L- arginine methyl ester, L-NAME (data not shown). These data further support the hypothesis that TrkA activation is responsible for the G_{M1}-induced tyrosine phosphorylation effects.

Figure 5. Inhibition of TrkA activity prevented the G_{M1}-induced acceleration of tyrosine phosphorylation and reduced phosphorylation levels in sperm under normal capacitating conditions. Panel A) immunoblot using antibody against phosphorylated TrkA . Panel B) The same membrane shown in panel A was stripped and re-probed using antibody against phosphorylated tyrosine residues. Cap: MW supplemented with 10 mM bicarbonate and 3 mM 2-OHCD. G_{M1}: supplemented with 25 μM G_{M1}. 879: MW supplemented with 50 μM tyrphostin AG 879, a specific inhibitor of TrkA activation. 825: MW supplemented with 50 μM tyrphostin AG 825, a specific inhibitor of Her2 that has been shown to have no effect on TrkA activation.


A
B
Cap - + - + + + + + - + - + + + + +
GM1 - - + + - + - + - - + + - + - +
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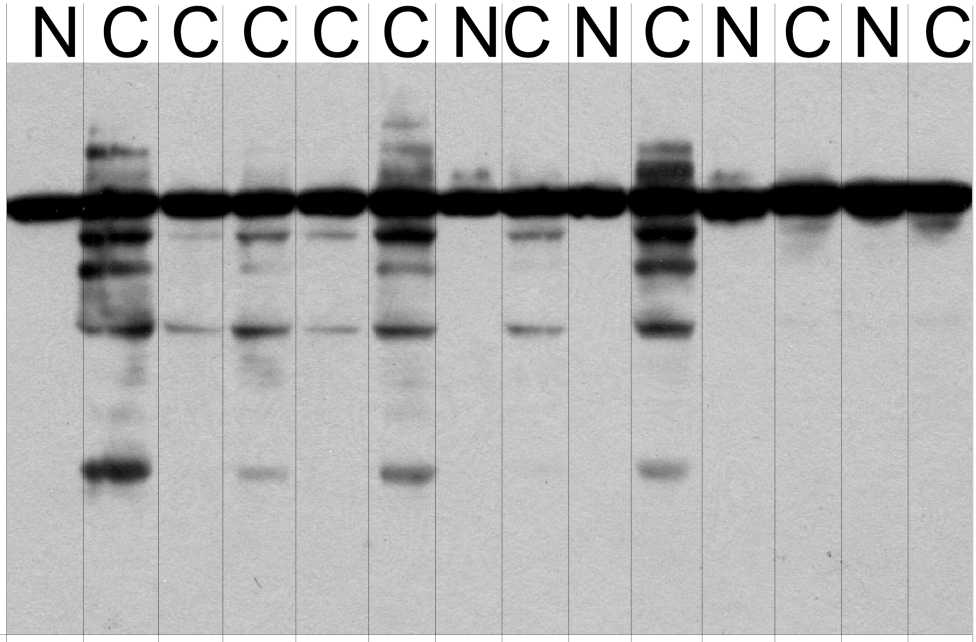
825 - - - - - - + + - - - - - - + +

Activation of TrkA results in the activation of PLCγ, PI_3K, and the Ras-ERK-MAPK pathway (Duchemin, Ren, Mo, Neff, & Hadjiconstantinou, 2002a; Willard et al., 2007). Therefore, we next investigated if signaling pathway(s) initiated with TrkA activation were important in sperm. Inhibiting PI_3K with wortmannin (20 nM) failed to ablate G_{M1}-induced tyrosine phosphorylation. Blockade of IP_3 receptors using 2-aminoethoxydiphenyl borate (2- ABP; 20 μM) had no effect on tyrosine phosphorylation, nor did inhibition of Ras via the inhibitor GW-5074 (10 μM). However, prevention of PLC activity with the inhibitor U73122 (10 μM) prevents G_{M1}-induced tyrosine phosphorylation. Surprisingly, the use of U73122 also significantly reduced the amount of phosphorylated TrkA, suggesting a positive feedback mechanism between TrkA and PLC activity, or a role of PLC in maintaining TrkA activation. These data suggest that the tyrosine phosphorylation induced by exogenous G_{M1} occurs downstream of TrkA activation and likely involves PLC activity.

Discussion

The ganglioside G_{M1} is an amphipathic glycosphingolipid with diverse cellular functions orchestrated from its distinct association with membrane rafts. G_{M1} is essential in several somatic cell signaling pathways (see (Mocchetti, 2005), for review), including tyrosine phosphorylation and regulatory pathways that are also critical for sperm function. The discovery and characterization of TrkA activity in sperm during physiologic and G_{M1} accelerated capacitation provides a mechanism for activation of essential signaling pathways, such as PLC, PI_3K, and other tyrosine kinases.

Figure 6. Downstream activation of the TrkA effector PLC, but not PI_3K, is necessary for observed effects of exogenous G_{M1}. Sperm were incubated with or without the given inhibitor for 60 minutes prior to processing for immunoblotting. Only experiments where sperm had >50% motility after 60 minutes of incubation were used. U7: inhibitor of PLC activity U73122 50 μM; WM: inhibitor of PI_3K wortmannin 10 nM; 825: Her2 inhibitor tyrphostin AG 825 25 μM 879: TrkA inhibitor tyrphostin AG 879 25 μM.



G_{M1}	+	+		+		+			+	+			+	+
U7											+	+	+	+
WM							+	+	+	+				
825					+	+								
879			+	+										

G_{M1} is stably segregated to membrane rafts in mammalian sperm (V. Selvaraj et al., 2009; V. Selvaraj et al., 2006), where its signaling functions are suppressed by G_{M1}'s specific interaction with seminal vesicle protein SVS2 (Kawano et al., 2008). While present on the sperm surface, SVS2 prevents tyrosine phosphorylation cascades and acrosomal exocytosis. The decapacitation effects of this molecule are relieved gradually; as the sperm transit the female reproductive tract, SVS2 is lost and capacitation ensues. We hypothesize that the loss of SVS2 allows focal clustering of the extracellular sugars of G_{M1} to facilitate TrkA activity during capacitation, as evidenced by the ability of TrkA inhibition to decrease tyrosine phosphorylation both with and without exogenous G_{M1}. This likely occurs in concert with G_{M1}-mediated calcium influx (see chapter 5), because the loss of sterols and seminal plasma proteins from the sperm membrane increases membrane fluidity and clustering of G_{M1} in the membrane (James, Hennessy, Berge, & Jones, 2004; Jones et al., 2010; Shadan, James, Howes, & Jones, 2004). G_{M1}-mediated calcium influx provides an additional potential mechanism for TrkA potentiation of tyrosine phosphorylation.

TrkA activity in sperm provides a plausible mechanism for both the decapacitating effects of SVS2 and the observed cryocapacitation effect noted in mammalian sperm. The activity of this kinase is seen under normal capacitating conditions and accelerated with the addition of exogenous G_{M1}, as might be seen with exposure to some cryopreservation media. Many media utilized for semen extenders or cryopreservation purposes in assisted reproduction technologies rely upon undefined components, such as egg yolk or milk, that are known to contain the ganglioside G_{M1} (Ford et al., 1992; Phelps et al., 1999). The presence of G_{M1} in cryopreservation media may in part account for the cryocapacitation phenomena observed as precocious tyrosine phosphorylation and membrane changes upon freeze thaw in the absence of in

vitro capacitating conditions (Thomas et al., 2006). The findings presented here could help to refine cryopreservation media to improve freeze-thaw outcomes and assisted reproduction techniques in humans and other species alike.

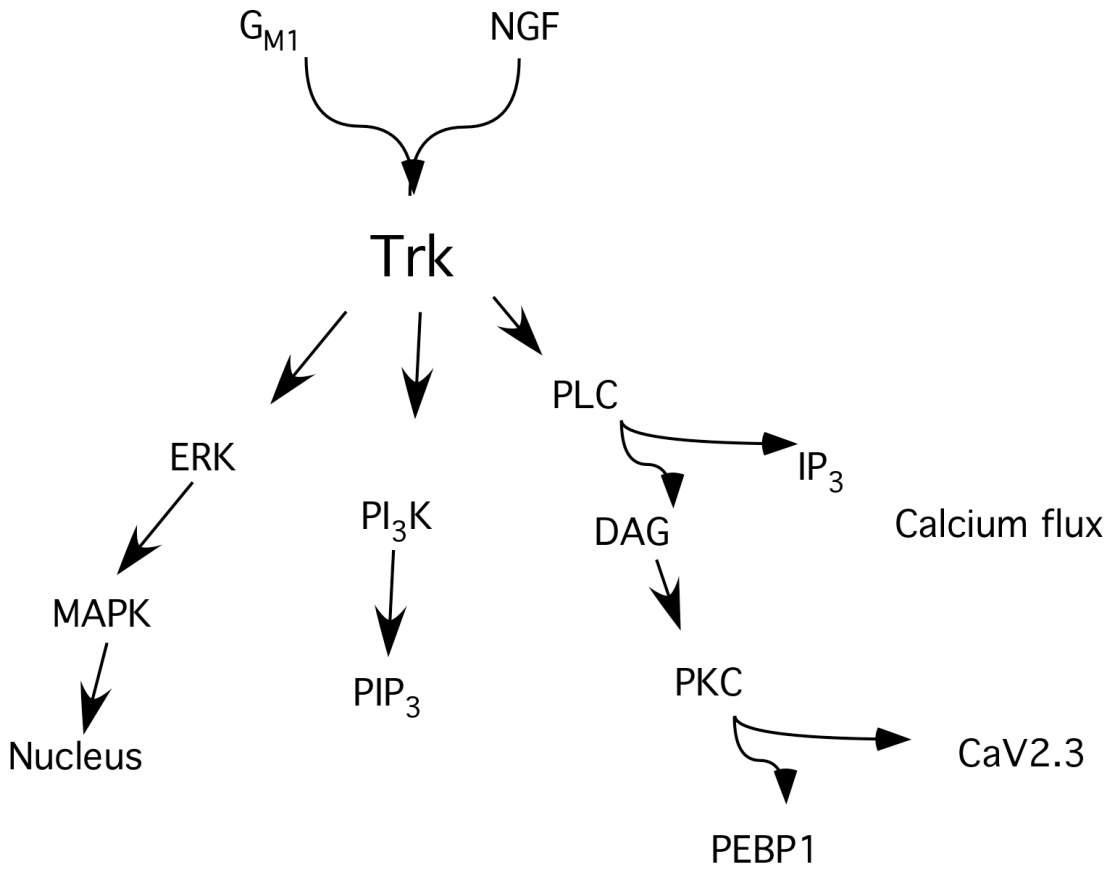
Previous reports have identified two isoforms of TrkA expressed in the testis, the long form found in neuronal tissues and responsive to NGF, as well as a shorter form which lacks exons 2-3 corresponding to the leucine-rich repeats of the protein (Dubus et al., 2000). This shorter isoform is hypothesized to have decreased to abolished affinity for NGF based on structure models and known binding properties of the short isoform of TrkB, which also lacks these leucine-rich repeats. Based on our immunoblotting results for TrkA protein and the lack of sequence for exons 2 and 3 despite numerous attempts with various primers and 5' RACE techniques, we hypothesize that the shorter form is present in sperm, where activation by G_{M1} might bypass the need for NGF binding and thus the need for the extra leucine rich repeats.

Investigations into potential G_{M1}-TrkA interactions in somatic cells stemmed from the observations that both exogenous G_{M1} and clustering of endogenous G_{M1} mimics and potentiates the actions of neurotrophic factors in vivo and in vitro (Hakomori & Igarashi, 1993; R. W. Ledeen & Wu, 2002; Zeller & Marchase, 1992). These G_{M1}-induced effects include the prevention of ischemia-induced neuronal death (Karpiak, Wakade, Tagliavia, & Mahadik, 1991), neurite outgrowth, treatment of neurodegenerative diseases (Fadda, Negro, Facci, & Skaper, 1993; Hadjiconstantinou & Neff, 1998a; Hadjiconstantinou & Neff, 1998b; Vyas et al., 2002), and cell migration assays (Fukumoto et al., 2006; Hakomori & Igarashi, 1993). Additionally, TrkA and G_{M1} have been co-immunoprecipitated, showing a tight and specific interaction of G_{M1} with the glycosylated, membrane-associated TrkA (Duchemin,

Neff, & Hadjiconstantinou, 1998a; Mutoh, Tokuda, Miyadai, Hamaguchi, & Fujiki, 1995). The clustering of G_{M1} in the membrane results in TrkA phosphorylation to activate signaling pathways (Avrova et al., 2010; Duchemin, Neff, & Hadjiconstantinou, 1998b; Duchemin, Ren, Mo, Neff, & Hadjiconstantinou, 2002b; Woronowicz et al., 2007). Although we report here that similar TrkA signaling pathways are also active in sperm, the interaction of G_{M1} and TrkA has not been documented in sperm, as we were unable to co-immunoprecipitate the two molecules. Additionally, clustering of G_{M1} through CTB addition was not sufficient to stimulate tyrosine phosphorylation cascades or TrkA phosphorylation and activation, suggesting potential differences in G_{M1} dynamics of the highly-tethered and sharply-curved flagellar plasma membrane. Further characterization of the relationship between sperm TrkA and G_{M1} is needed.

While evidence exists for an association between G_{M1} and TrkA activation in sperm, it appears as though TrkA is not the only effector in G_{M1}-induced cell signaling. The ability of G_{M1} to induce a smaller but still significant pro-survival pathway in PC12 cells lacking TrkA expression suggests that although TrkA activity is important for G_{M1} effects, it is not the sole transducer. One significant effect of G_{M1} that can account for a number of signaling effects, including calcium-dependent cytoskeletal remodeling, neurotrophin exocytosis (and thus further Trk receptor activation), and tyrosine phosphorylation cascades is the ability of G_{M1} to increase calcium influx (Buchwald et al., 2007; Buckley et al., 1995; Carlson, Masco, Brooker, & Spiegel, 1994; Gouy, Deterre, Debre, & Bismuth, 1994; R. Ledeen & Wu, 2007; Yatomi et al., 1996). G_{M1} induces a rise in intracellular calcium in all neuronal cell types investigated, including those lacking TrkA transcripts and protein (Mocchetti, 2005).

Figure 7. The TrkA kinase pathway can be activated by nerve growth factor (NGF; Dubus et al., 2000) or G_{M1} to stimulate the activity of the MAPK, PI_3K, and PLC pathways. Each of these signaling pathways has been implicated in sperm biology. In the present report, we find evidence for involvment of the PLC pathway in sperm capacitation and acrosomal exocytosis. Inhibition of TrkA activity or PLC activity prevented tyrosine phosphorylation cascades both with and without exogenous G_{M1} in the media. The activation of PLC results in the generation of diacylglycerol (DAG), which can stimulate protein kinase C (PKC) activation. Phosphorylation of the $Ca_V2.3$ calcium channel increases current through this channel. Phosphorylation of the decapacitation factor, PEBP1, might facilitate its release from the sperm plasma membrane to further facilitate capacitation, and has been found to be enhanced by exogenous G_{M1} (data not shown).


G_{M1}
NGF
Trk
PLC
ERK
IP_3
PI_3K
DAG
Calcium flux
MAPK
PIP_3
PKC
Nucleus
CaV2.3
PEBP1

Although it is known that G_{M1} induces tyrosine phosphorylation cascades through TrkA activation in somatic cells and likely in sperm, how activation of TrkA targets affects other signaling pathways active during capacitation is not yet understood. Roles for Ras, Raf, ERK, and MAPK have been suggested in sperm function (Fig. 7; Luconi et al., 1997; Nixon et al., 2010), and PKC and PLC activity is known to be essential for fertilization in mammalian sperm (Breitbart, 2003). Roles for PLC are especially well established during acrosomal exocytosis (Fig. 7; Jungnickel, Marrero, Birnbaumer, Lemos, & Florman, 2001). Redundant pathways for the activation of PKC and PLC appear present in sperm (Breitbart, 2003). Each of these pathways is directly activated by TrkA, which provides an attractive means for their activation in sperm. Further understanding of how membrane microdomain organization and lipid signaling in sperm influences these pathways and functions during capacitation and fertilization is needed. This would greatly improve our understanding of how a sperm can respond to its extracellular environment in a timely and precise manner and holds promise for improving assisted reproduction outcomes.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

The organization of G_{M1} in sperm membranes

The localization of G_{M1} has been an active topic in sperm biology due to its association with membrane rafts and the putative role of membrane rafts in sperm signaling and fertilization. However, the localization of G_{M1} in mammalian sperm has also been a contentious issue, with conflicting studies reported in the literature (Roberts, Wamstad, Ensrud, & Hamilton, 2003; Shadan, James, Howes, & Jones, 2004; Trevino, Serrano, Beltran, Felix, & Darszon, 2001). Studies from our lab have largely reconciled or explained the reported inconsistencies, with differences in localization attributed to fixatives (Selvaraj et al., 2006) and/or exposure to seminal plasma (Buttke, Nelson, Schlegel, Hunnicutt, & Travis, 2006). In addition to these studies of mammalian sperm membrane organization, further characterization by our lab has identified a second pool of G_{M1} in the acrosomal membrane (Selvaraj et al., 2009). Biochemical characterization concurs with this finding, identifying two distinct membrane fractions enriched in G_{M1}: one possessing density and composition properties consistent with membrane rafts of the plasma membrane, and the other, less buoyant fraction consistent with the acrosomal membrane (Asano et al., 2009). This was a surprising finding, as the acrosomal membrane is not enriched in sterols or other raft markers (Clark & Koehler, 1990; Seki, Toyama, & Nagano, 1992; Toshimori et al., 2001), and the golgi vesicle of somatic cells lacks significant amounts G_{M1} (Lencer, Hirst, & Holmes, 1999). Together, these experiments further piqued our interest in the functions of this versatile ganglioside in sperm biology.

These descriptive studies beg the question, why would a sperm need such stable, dramatic segregation and enrichment of G_{M1}? What role does this subdomain play in sperm-egg communication? Why is the acrosomal membrane, a membrane of very

different composition and properties compared to the plasma membrane, also enriched in G_{M1}? The studies described in this dissertation provide a strong foundation towards understanding these questions.

The discovery of a de-capacitating protein in seminal plasma, named SVS2, that specifically binds G_{M1} accounts for the ability of seminal vesicle fluid to mask G_{M1} (Kawano & Yoshida, 2007; Kawano, Yoshida, Iwamoto, & Yoshida, 2008). Importantly, the interaction of SVS2 with G_{M1} occurs during ejaculation as a sperm is deposited in the female reproductive tract, when cellular quiescence is desirable (Fraser, Adeoya-Osiguwa, Baxendale, & Gibbons, 2006). This binding maintains low intracellular calcium and cAMP levels, while preventing the tyrosine phosphorylation cascades acquired during capacitation (Kawano & Yoshida, 2007). As sperm transit the female reproductive tract, SVS2 is gradually lost from the membrane in the distal oviduct and capacitation ensues. One can hypothesize that this loss of SVS2 allows the interaction of G_{M1} with membrane targets important for signaling processes of sperm-egg interaction.

Many seminal plasma components are known to adsorb to the sperm plasma membrane (Acott & Carr, 1984; Adeoya-Osiguwa & Fraser, 1996; Carr & Acott, 1984; Fraser et al., 2006; White, Rodger, Murdoch, Williams, & Abney, 1975). The removal of these molecules, as well as membrane sterols and phospholipids, dramatically changes the membrane microenvironment of the spermatozoon. An increase in membrane fluidity has been described (James, Hennessy, Berge, & Jones, 2004; Jones et al., 2010; Shadan, James, Howes, & Jones, 2004; van Gestel et al., 2005), but it is likely that many other processes and attributes are altered during this dramatic change in membrane composition. For example, the activity of many

membrane ion-transporters is affected by membrane composition in somatic cells and is likely to be affected in sperm as well (Brady et al., 2004; Lingwood & Simons, 2010; Romanenko et al., 2004; Romanenko, Rothblat, & Levitan, 2004).

One potential consequence of this increase in membrane fluidity and reorganization is the formation and/or restructuring of membrane rafts (Asano et al., 2009; Cross, 2004; Jones et al., 2010; Shadan, James, Howes, & Jones, 2004; Tanphaichitr et al., 2007). This is a hypothesis that has been difficult to observe in vitro due to the small size and variable nature of membrane rafts (van Meer, 2004). However, biochemical characterization of raft molecules has been a promising tool in defining these changes. An increase in zona pellucida-binding affinity of isolated membrane rafts has been reported to occur following sterol efflux and capacitation (Bou Khalil et al., 2006). A coalescence of raft-associated G_{M1} at the apical ridge of the acrosome occurs during capacitation in bull (Selvaraj et al., 2007) and boar (Jones et al., 2010). The relationship between sterol efflux-mediated raft formation and changes in signaling processes and ion channels warrants further investigation.

In addition to raft reorganization, sterol efflux and seminal plasma removal have been hypothesized to induce point-fusion events between the plasma membrane overlying the acrosome and outer acrosomal membrane (Kim, Cha, & Gerton, 2001; Kim, Foster, & Gerton, 2001; Kim & Gerton, 2003). Acrosomal exocytosis is thought to be a graduated process (Gerton, 2001). Priming of the membranes might occur through these point fusion events analogous to kiss-and-run fusion events observed in neurons. Such membrane communication accounts for the appearance of molecules of acrosomal origin on the plasma membrane of sperm (Kim et al., 2001). This is observed late in capacitation and prior to acrosomal exocytosis. Additionally, these

point fusion events might explain the observation that plasma membrane G_{M1} is internalized during capacitation. This prolonged membrane communication and reorganization could then allow a sperm to regulate its responsiveness to the extracellular environment, preventing premature exocytosis and thereby maximizing fertilizing potential.

Differences in membrane composition between the plasma membrane overlying the acrosome versus the acrosomal membrane might facilitate these point fusion events, or the differences between membranes might rely on these communication events for functional relevance, ie, a concentration gradient might facilitate lipid or protein transfer. It is tempting to speculate that the sterol-poor acrosomal membrane is poised to transfer G_{M1} to the sterol-rich, raft environment of the plasma membrane overlying the acrosome, which represents a more favored environment for G_{M1}. Such transfer could occur during point fusion events preceding acrosomal exocytosis, allowing plasma membrane enrichment of G_{M1} and initiation of G_{M1}-mediated signaling processes.

Effects of G_{M1} dynamics

The formation of focal enrichments of G_{M1} might occur through three potential mechanisms, with synergism of these three mechanisms likely. First, unmasking of G_{M1} through the removal of SVS2 might allow interaction and clustering of G_{M1} through the extracellular carbohydrate groups. The evolution of this specific masking protein makes this an attractive hypothesis. Second, the removal of SVS2 occurs in concert with an increase in plasma membrane fluidity. Increased diffusion of G_{M1} within the acrosomal domain of the plasma membrane upon sterol efflux might be sufficient to facilitate G_{M1} clustering. This could occur through increased diffusion of

G_{M1} alone, or via a sialic acid binding molecule, such as egg glycans. Again, the unmasking of G_{M1} would occur simultaneously with changes in membrane fluidity and allow G_{M1}-target binding to occur synergistically. Alternatively, the point fusion events between the plasma and acrosomal membranes might allow the translocation of acrosomal G_{M1} to the plasma membrane, increasing focal concentrations of plasma membrane G_{M1}. It is likely that a combination of these three mechanisms occurs.

An increase in the focal concentration of G_{M1} would translate to an increased potential for raft-molecule interaction with G_{M1}, as occurs with α_1 and $\alpha_2\delta$ subunit of calcium channels (Davies et al., 2007; Davies et al., 2010). α_1 subunits are found in both raft and non-raft membrane fractions of somatic cells. However, the $\alpha_2\delta$ subunit is a GPI-anchored protein found primarily in membrane rafts, where the highly glycosylated, extracellular α_2 portion of the subunit interacts with the α_1 subunit to dramatically change current properties (Bannister et al., 2009; Dickman, Kurshan, & Schwarz, 2008; Hahm et al., 2009). We hypothesize that the extracellular sugars of G_{M1} mimic the effects of the glycosylated $\alpha_2\delta$ subunit. Clustering of G_{M1} around the α1E subunit could increase current and responsiveness of this channel to changes in the membrane environment due to its similarity to other modifying subunits.

Changes in membrane concentrations of G_{M1} have significance for many signaling processes in somatic cells as well as key events of a spermatozoon's life. We have determined that exogenous G_{M1} results in phosphorylation and activation of the tyrosine kinase TrkA. This may occur through direct binding of G_{M1} and TrkA, as seen in neuronal cells (Duchemin, Ren, Mo, Neff, & Hadjiconstantinou, 2002; Farooqui, Franklin, Pearl, & Yates, 1997), or through intermediate binding partners (Ledeen & Wu, 2007; Mocchetti, 2005). The activity of this kinase is seen under

normal capacitating conditions and accelerated with the addition of exogenous G_{M1}, as might be seen with exposure to some cryopreservation media. Downstream effectors of TrkA kinase activity, such as PLC, PI_3K, and PKC, are required for capacitation (Evans & Florman, 2002; Visconti et al., 2002) and acrosomal exocytosis (Jungnickel, Marrero, Birnbaumer, Lemos, & Florman, 2001), so it is plausible that TrkA activation of these pathways is important for sperm function. Precisely when activation of these pathways is necessary and how their activity is regulated is not known and is an important area for future research.

One possible role for G_{M1}-induced TrkA activity is the induction of phosphorylation of $Ca_V2.3$ by PKC. As presented in this dissertation, the clustering of G_{M1} in the sperm plasma membrane results in acrosomal exocytosis through calcium influx via the $Ca_V2.3$ channel. Although we have demonstrated this dependence of G_{M1} effects on $Ca_V2.3$ activity using pharmacologic and genetic mechanisms, the potential intermediate steps and interactions in this pathway are currently unknown. $Ca_V2.3$ is a voltage-gated calcium channel, likely dependent upon an initiating membrane depolarization event (Ertel, 2004). Whether G_{M1} initiates this depolarization event through another mechanism or through direct activation of $Ca_V2.3$ current is unknown.

Additionally, membrane hyperpolarization and depolarization is dependent upon the intracellular alkalinization of sperm (Florman, Tombes, First, & Babcock, 1989). Changes in the activity of the sperm proton pump Hv1 occurs during capaciation and results in intracellular alkalinization of the sperm (Lishko, Botchkina, Fedorenko, & Kirichok, 2010). This rise in pH occurs together with potassium flux through sperm Slo3 channels, which ultimately leads to the hyperpolarization of the sperm membrane (Martinez-Lopez et al., 2009; Santi et al., 2010; Schreiber et al., 1998). This

hyperpolarization allows transitioning of the $Ca_V2.3$ channel from an inactive state to an active, closed state in which the channel is primed for opening in the event that depolarization occurs (Fang et al., 2007). Interaction of G_{M1} with this upstream proton pump or Slo3 channels is another potential target for the observed G_{M1} effects. Additionally, Slo3 null sperm fail to undergo calcium ionophore A23187-induced acrosomal exocytosis or fertilize zona-intact or zona-free oocytes, suggesting that this channel is important in other, later-stage pH and/or voltage dependent processes (Santi et al., 2010).

Membrane depolarization, such as occurs through the Slo3 channel, and alkalinization, such as occurs with Hv1 activity, is a requirement for CatSper and $Ca_V2.3$ channel activity (Carlson et al., 2003; Wennemuth, Westenbroek, Xu, Hille, & Babcock, 2000). The relationship between the two identified voltage-dependent calcium channels and the chronology of calcium influx during acrosomal exocytosis is unknown. Sperm from CatSper null mice are infertile, a phenotypic defect attributed to the inability of these sperm to remain motile or undergo hyperactivation and penetrate the viscous cumulus cell and zona pellucida matrix (Carlson et al., 2003; Quill et al., 2003), but CatSper also appears necessary for initial ZP-induced calcium influx (Xia & Ren, 2009). In addition to its role in hyperactivated motility, CatSper activation has been reported (Carlson et al., 2003) and predicted (Olson, Suarez, & Fauci, 2010) to induce calcium influx in the head of the sperm, and is pH dependent. This CatSper-mediated calcium influx might be the pH-dependent depolarization event necessary for $Ca_V2.3$ activity. Potential CatSper-evoked activation of $Ca_V2.3$ is also a possibility that needs further investigation. Such a model would provide for Slo3 and Hv1 activity priming of the sperm for ZP-induced CatSper activity, resulting in $Ca_V2.3$ activity and final store-operated calcium entry and exocytosis.

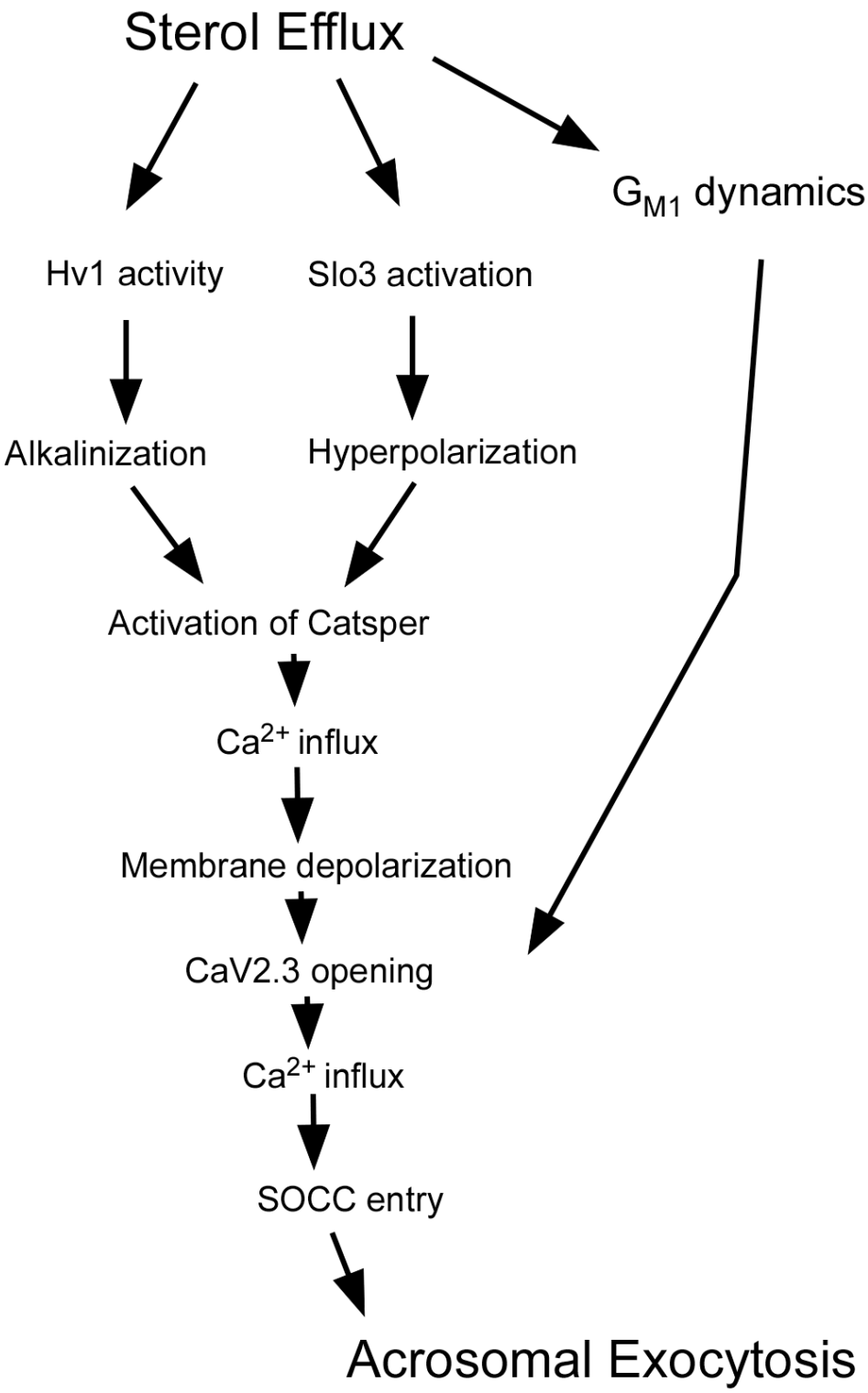
The initial calcium rise of acrosomal exocytosis is short-lived, discrete, and apparently voltage-independent (Florman, Arnoult, Kazam, Li, & O'Toole, 1998). Several alternative voltage-independent candidates for calcium rise have been identified in sperm, such as the $GABA_A$ channel in progesterone-medicated AE (Murase & Roldan, 1996) or the glycine receptor/chloride channel in ZP-induced AE (Bray, Son, Kumar, Harris, & Meizel, 2002; Melendrez & Meizel, 1995; Sato, Son, & Meizel, 2000), but no single candidate appears necessary for all pathways of AE. Roles for the nicotinic- or muscarinic-acetylcholine receptor channels have been hypothesized, but neither has been fully characterized (Bray, Son, & Meizel, 2005; Ward, Kopf, & Storey, 1994). Activation of any of these channels might have the potential to induce membrane depolarization through chloride efflux or calcium influx, respectively. Indeed, coupling between glycine receptor/chloride channel activation and VOCC influx has been described in somatic cells (Bannister, Melliti, & Adams, 2004; Tai, Kuzmiski, & MacVicar, 2006).

Potential indirect targets for G_{M1} in acrosomal exocytosis also merit further investigation. Regulatory molecules are important for several aspects of signaling in excitable cells. Acrosomal exocytosis induced by either zona pellucida or G_{M1} clustering requires inhibitory G-protein activity through an unknown mechanism. $Ca_V2.3$ activity is stimulated and enhanced by G-protein interaction as well as phosphorylation by PKC (Bannister et al., 2004; Krieger et al., 2006; Toro-Castillo, Thapliyal, Gonzalez-Ochoa, Adams, & Meza, 2007). Each of these mechanisms is a potential avenue for G_{M1} regulation of $Ca_V2.3$, as TrkA phosphorylation stimulates PKC activity (Duchemin, Ren, Mo, Neff, & Hadjiconstantinou, 2002) and G-proteins

can be activated either directly through G_{M1} (Chen et al., 2003) or through alternative downstream signaling processes (Rajagopal, Chen, Lee, & Chao, 2004).

The potential signaling intermediates through which G_{M1} might stimulate $Ca_V2.3$ activity and the chronology of this signaling is a promising area of research that has potential for clinical interventions as well as elucidating a fundamental mechanism of mammalian fertilization. The interplay of intracellular alkalinization, membrane hyperpolarization and subsequent depolarization leading to VOCC activity has been a long present and puzzling question in sperm biology. Moreover, while $Ca_V2.3$ null sperm undergo normal tyrosine phosphorylation cascades in response to capacitating stimuli and G_{M1}, other potential defects and differences in these knockout sperm merit further evaluation. Initial

Figure 1. Sterol efflux mediates membrane remodeling events that are known to stimulate changes in several ion transporters. The proton pump Hv1 is stimulated by sterol efflux to increase the intracellular pH of the sperm. This pump is sensitive to depolarization as well. Activation of Slo3 hyperpolarizes the sperm plasma membrane and may cause sufficient hyperpolarization to transition the voltage-operated $Ca_V2.3$ channel from an inactive state to an active, closed state. The sperm-specific CatSper channel is both weakly depolarization and more strongly pH sensitive, and has been shown to become active with BSA-mediated sterol efflux. G_{M1} may influence the activity of any of these or other membrane channels to ultimately result in activation of the $Ca_V2.3$ calcium channel for initiation of acrosomal exocytosis. Calcium influx into sperm can occur by changes in activity of a number of channels and transporters, and it is likely that different cohorts of channel activity can result in successful fertilization. This concept is highlighted by the ability of $Ca_V2.3$ null mice to produce viable litters while still unable to respond to physiologic agonists of acrosomal exocytosis or successfully fertilize oocytes in vitro.


Sterol Efflux
G_{M1} dynamics
Hv1 activity
Slo3 activation
Alkalinization
Hyperpolarization
Activation of Catsper
Ca^{2+} influx
Membrane depolarization
CaV2.3 opening
Ca^{2+} influx
SOCC entry
Acrosomal Exocytosis

reports on the fertilization competence of $Ca_V2.3$ null sperm described a more straight flagellar wave form and path velocity (Sakata et al., 2002), and empirical observations of sperm exposed to exogenous G_{M1} suggest that G_{M1} can influence sperm motility patterns (personal observation). Roles for $Ca_V2.3$ and G_{M1} in motility are another avenue of study requiring more thorough characterization.

Furthermore, proteomic studies from our lab have identified raft-associated phospholipase B that is activated upon sterol efflux (Asano et al., in preparation). This enzyme cleaves phospholipids in the plasma membrane and has the potential to increase focal membrane curvature. We hypothesize that this change in membrane curvature could act to mediate point fusion events that facilitate the transfer of acrosomal G_{M1} to the plasma membrane overlying the acrosome. This provides a physiologic regulatory mechanism by which a sperm might control G_{M1} enrichment for activation of exocytotic machinery.

The highly compartmentalized mammalian spermatozoon undergoes a significant amount of maturational events and signaling changes without the aid of many somatic cell tools, such as protein transcription and translation. As presented in this dissertation, the organization of membrane rafts and tethering of signaling complexes within the membrane serve as an attractive mechanism for sperm to accomplish these changes in a timely and precise manner. The ganglioside G_{M1} is stably segregated to distinct membrane domains in mammalian sperm where it appears to effect dynamic signaling processes for capacitation and acrosomal exocytosis important for successful fertilization. The work presented here suggests that focal enrichments of G_{M1} ocurring during membrane remodeling of capacitation can activate TrkA signaling pathways during physiologic capacitation processes. This effect is likely accelerated during

exposure to ill-defined cryopreservation media and may adversely affect assisted reproduction outcomes. This work therefore may have direct applications for improving assisted reproduction technologies. Also activated during focal clustering of G_{M1} is calcium influx and $Ca_V2.3$ actvity. This ascribes significant function to the previously under-described $Ca_V2.3$ channel and may have corrollaries in somatic cell $Ca_V2.3$ function. Further definition of precisely where and how G_{M1} activates signaling processes for acrosomal exocytosis will have important consequence for our understanding of sperm function and dysfunction for potential intervention strategies. This report on G_{M1} in mammalian sperm function is likely just the beginning for the story of how this biologically active sphingolipid regulates one of the first and most fundamental events in cell-cell recognition and communication.

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