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Regulation of Volume by Spermatozoa and Its Significance for Conservation Biology

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Regulation of Volume by Spermatozoa and Its Significance for
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A Dissertation

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By

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B.S. North Carolina State University, 2000

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This dissertation is dedicated to Molly,

my faithful companion.

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Table of Contents

List of Figures

List of Tables

Abstract

Reproductive science plays an important role in conservation biology. Quantitative studies of basic reproductive biology in wildlife are critical for the development of successful assisted reproductive technologies. Investigation of the volume regulatory mechanism of spermatozoa could produce options to improve the cryopreservation of spermatozoa and provide a non-hormonal contraceptive option for men, both of which could have significant impacts on global biodiversity preservation. Volume regulation of somatic cells involves the movement of osmolytes through various channels, including potassium channels. The potassium channels involved in volume regulation of human, monkey, and murine spermatozoa were investigated. Flow cytometry was used to gauge the sensitivity of the volume regulatory process of spermatozoa to various potassium channel inhibitors and a simultaneous hypotonic challenge. Channels potentially involved in regulatory volume decrease of spermatozoa varied with species but included voltage-gated (K_v) channels 1.4, 1.5, 1.7, 4.1, 4.2 and 4.3 as well as TWIK1, TWIK2, TASK1, TASK2, TASK3, TREK2 , and minK. The presence of some of these channels was confirmed by western blotting and immunocytochemistry. Changes in the motility patterns of human and monkey spermatozoa in the presence of potassium channel inhibitors during hypotonic stress were also observed, suggesting a relationship between volume regulation and motility. To evaluate potential organic osmolytes involved in, and compare effects of CPAs on, volume regulation, the isotonicity of murine epididymal spermatozoa was measured using a null point method. Spermatozoa were then exposed to high concentrations of various osmolytes and cryoprotective agents in isotonic medium to evaluate which compounds were able to penetrate the sperm plasma membrane. The osmotic responses of spermatozoa from strains of mice known to have spermatozoa of high (B6D2F1) and low (C57BL6) post-thaw fertility were compared during various osmotic challenges in various media. These experiments indicated that spermatozoa from B6D2F1 mice may have better volume regulation capabilities than spermatozoa from C57BL6 mice, suggesting that better post-thaw fertility of murine spermatozoa could be influenced by the volume regulatory process. The knowledge gained from these experiments could contribute to improved sperm handling and preservation techniques and be used to develop non-hormonal male contraceptives based on inhibiting volume regulation.

Chapter 1

General Overview

Conservation biology is a crisis discipline developed in response to the realization that we are in a period of unprecedented loss of biological diversity. While extinction can be a natural process, current extinction rates are 100 times those of historical background rates and 99% of modern extinctions, those occurring in the last 10,000 years since the Pleistocene epoch, can be attributed to human activity (Lawton and May 1995, Regan and others 2001, Pimm 2001). A mass extinction is the loss of a large number of taxa in a brief geological time frame (e.g. several million years). There have been five in the historical record (Meffe and Carroll 1997). The current crisis is unique in that the cause is a biological species rather than natural phenomena such as continental shifts or climate change. The connection between the current loss of biodiversity and human activities is well-documented and abundant (Ehrlich and Ehrlich 1981, Wilson 1989 and 1992, Myers 1990, Raven 1990, Soulé 1991). Conservation issues are gaining recognition and support from the general public and the response has been the expansion of conservation biology as a discipline. Scientists now recognize that the threats to biodiversity are intricate and require the attention of experts from a wide range of disciplines as well as public involvement. A concerted, immediate effort is required to prevent a mass extinction that rivals and surpasses the great extinction episodes of the prehistoric past (Meffe and Carroll 1997).

Causes of the Biodiversity Crisis

The root causes of the biodiversity crisis are threefold: i) the expanding human population, ii) the high per capita resource consumption in developed countries, and iii) low efficiency of resource use (Meffe and Carroll 1997). The world population has expanded from one billion in 1850 to just over 6.5 billion today, and is expected to reach 10 billion by 2050 (Primack 2004). The consequence of this rapid growth is a greater need for resources and space. The growth of the global population is not uncontrollable as countries such as Cuba, Mexico, Costa Rica, Venezuela, and Thailand have demonstrated by significantly lowering their population growth rates in a relatively short period (Meffe and Carroll 1997).

Equally important as the number of people on the planet is the rate at which resources are consumed. Resource consumption at some level is unavoidable as humans require natural resources to survive. Larger populations demand more resources that create a greater human impact on the environment, and ultimately higher rates of biodiversity loss (Primack 2004). For example, nitrogen pollution is greatest in rivers with the highest human population densities and rates of deforestation are greatest in countries where human population growth rates are highest (Primack 2004). Resource consumption rates are highest in the United States where 30% of the annual global resource consumption is achieved by 5% of the world's population (WRI 2003).

Linked to, yet distinct from, the consumption of natural resources is the efficiency of their use. In developed countries, convenience is the ultimate driving force behind product design and delivery. Single use household items are prevalent and create a "throw-away" mentality that is desirable for the average household. This attitude accelerates resource consumption that drives habitat loss, as the resources are extracted, and ultimately biodiversity loss. As the population of the world continues to grow, so does the number of people around the world aspiring to join the middle class and its associated consumption habits. With countries such as China, India, and the former Soviet Union, emerging into the world market, conservation of resources must become a platform issue for all governments and societies.

Direct causes of biodiversity decline

The three root causes listed above are the stimuli for the direct causes of biodiversity decline: loss of habitat (destruction, fragmentation, and alteration), introduced species, overexploitation/over-harvesting, pollution, increased spread of disease, and synergistic effects among these factors (Primack 2004, Meffe and Carroll 1997).

Habitat destruction is the primary direct cause of biodiversity loss (Primack 2004, Meffe and Carroll 1997). As the human population and human activities expand, suitable habitats that can sustain wildlife populations will shrink. In the United States, only 42% of natural vegetation remains and in regions of the East and Midwest, less than 25% remains (Stein and others 2000). Habitat loss for endangered species is due to several principal activities globally (in order of decreasing importance): agriculture (affecting 38% of endangered species), commercial developments (35%), water projects (e.g. dams and canals, 30%), outdoor recreation (27%),

livestock grazing (22%), pollution (20%), infrastructure and roads (17%), disruption of fire ecology (13%), and logging (12%) (Stein and others 2000). The World Conservation Union (IUCN) implicates physical destruction of habitat in 73% of all species listed under the categories of extinct, rare, endangered, and vulnerable (Campbell and others 1999).

Fragmentation is the separation of existing habitats into smaller isolated patches that cannot sustain the species within them indefinitely (Meffe and Carroll 1997). Aside from leading to smaller population size, fragmentation also prevents migration and thus gene flow (Frankham and others 2002). It is one of the leading causes of biodiversity loss due to habitat destruction and can increase the likelihood that a species will become endangered or extinct (Myers 1997). One such effect of fragmentation is the increased rate of exotic species invasion (discussed below) through the creation of disturbed habitats (Meffe and Carroll 1997). Species adapted to dispersed and fragmented habitats such as lakes, reefs, caves and rocky outcrops may be able to cope with increasing habitat fragmentation; however, others may not have the necessary phenotypic plasticity in life history traits that enable them to survive in small habitat patches (Orians and Soulé 2001).

The effects of invasive species on ecosystems are the second leading cause of biodiversity loss (Meffe and Carroll 1997). Invasive species are those that have been moved beyond their native ranges and flourish in a habitat to the detriment of other species or community structure and function. Some of the most well known examples include kudzu vines in the southeastern United States, zebra mussels in North America, exotic birds in Hawaii, rats and goats on many Pacific islands, and crop pests around the world (Myers 1997). Natural processes have always moved individuals across substantial environmental barriers and the biota of some environments (*e.g.* oceanic islands) have been created entirely by natural long-distance dispersal followed by *in situ* speciation (Orians and Soulé 2001). However, human activities are causing an unprecedented movement of thousands of species. Invasive species can cause the loss of native species by out-competing them for resources and upsetting the delicate balance of the ecosystem. Endangered native animals and plants can be especially vulnerable to newly introduced species (Cowan and Tyndale-Biscoe 1997). Not all species introduced into a new environment succeed. While there are many accidental introductions that fail and thus go undetected, a "normal" success rate for deliberate introductions is 10-40% (Lawton and Brown 1986). A species that successfully colonizes a new area is often one that can quickly and

aggressively out-compete the local species for habitat and resources, making their presence a serious threat. Major reductions in populations of native species, and in some cases extinction, due to invasive species are of considerable conservation concern.

In today's world, market demand for goods and resources is a driving force behind over-exploitation and over-harvesting of species. As the human population continues to grow, so will the demand for products that directly and/or indirectly threaten species worldwide. Currently one-third of endangered mammals and birds are impacted by over-exploitation (Hilton-Taylor 2000). Many restrictions on the harvesting of wild animals are not consistently enforced and, in countries rife with civil war and political unrest, restrictions do not exist (Primack 2004). In these countries, where poverty is a daily battle, harvesting the animals is a means of survival. As a result, countries such as Colombia, the Democratic Republic of the Congo and Haiti have seen declines of up to 80% in their populations of large primates, ungulates, and other mammals (Primack 2004). Meat from wild animals, or bushmeat, is a valued source of protein in poor and developing nations and has pushed many threatened species to the brink of extinction, especially in Africa (Primack 2004). Overharvesting of fish stocks has caused the collapse of numerous fisheries (e.g. cod in Newfoundland, sardines from Monterey Bay; Pimm 2001). The illegal trade of wildlife on the global market is a $10 billion/yr business not including the edible fish market (Hemley 1994). In each of these cases, as the animals become rarer they become more valuable, increasing the willingness of the local population to extract these species, and creating a strong incentive for their overexploitation. Legal harvesting also has its complications, the least of which is setting the maximum sustainable yield for a species; that is, the greatest amount of a resource that can be harvested each year and replaced through population growth without decreasing the fitness of the population (Essington 2001).

Pollution is perhaps the most subtle threat to wildlife but is no less potent. It can come in the form of pesticides, sewage, fertilizers from agricultural fields, industrial chemicals and wastes, emissions from factories and automobiles, and sediment deposits from eroded hillsides (Primack 2004). Each of these forms of pollution can affect an individual directly or indirectly by lowering the quality of the water, air, and land on which it depends. There is also growing evidence that pollution is altering the reproductive cycles, endocrine physiology and early-life

stage survival of many animals, especially teleost fishes (Rolland 2000). Global climate change may yet prove to be the most deadly challenge to wildlife as a result of pollution.

As available habitats dwindle, and population densities increase, there will be more intra- and inter-species (including humans and domestic animals) contact. This contact can expose species to new diseases and disease-carrying vectors to which they have no natural immunity (Hayden and others 2002). Having higher densities of animals in a confined space prevents them from migrating away from their feces, saliva, old skin, and other sources of infection (Primack 2004) and creates a favorable environment for a parasite as it can easily reach the next host within or between populations. Overcrowding can lead to a deterioration of environment and food availability that lowers nutritional status leading to weaker animals and a higher susceptibility to infection. Stress can also depress the immune system. Pollution may increase susceptibility to infection by introduction of pathogens, particularly in aquatic environments (Epstein 1998, Aguirre and others 2002). Each of these pathways to increased disease transmission has negative impacts on populations, and the synergistic effects can be devastating.

The threats facing biodiversity create a formidable task for conservation biologists. The increasing number of endangered species and the unlimited potential strategies for protecting them require prioritization and elaborate knowledge of the intricacies of the natural world. Thus, defining biodiversity and its various levels is a fundamental starting point for directing conservation efforts.

Levels of Biodiversity

Biodiversity, as defined by the World Wildlife Fund (1989), is "the millions of plants, animals, and microorganisms, the genes they contain, and the intricate ecosystems they help build into the living environment." All living organisms fall into a number of categories under the general umbrella of biodiversity. While there are many levels of biodiversity, three well-known categories are ecosystem, species, and genetic diversity. Beyond these three, one can discuss biodiversity in terms of higher taxa, evolutionary lineages, populations, etc. There is no "correct" level by which to measure biodiversity. Each level is critical for the continuation of life on earth (Purvis and Hector 2000).

Ecosystem

The most general classification of biodiversity is at the ecosystem level. This is composed of biomes, areas of land characterized first by their dominant vegetation followed by their precipitation and elevation gradients (Meffe and Carroll 1997). Preservation of this level of biodiversity is critical for its ability to accommodate the millions of different species with unique survival requirements. A current example is the Bicknell's thrush. This species breeds only in scrubby boreal forests above 2,800 feet on top of mountains in New Hampshire, Maine, New York, Vermont and eastern Canada, regions under high pressure from ski resort development, communications tower construction, wind energy projects, acid rain, mercury and global warming (Associated Press 2006). The many conservation efforts directed at the species level, such as captive breeding programs, are useless without the availability of suitable habitat into which the animals can be subsequently released. Discussion of conservation at the ecosystem level is valuable for presenting the importance of environmental goods and services performed by viable ecosystems on which human life depends (e.g. production of oxygen and removal of CO_2 by green plants) (Meffe and Carroll 1997).

Species

Species diversity includes the entire range of species found on earth (Primack 2004). There are approximately 1.5 million living and 300,000 fossil species described and named (Meffe and Carroll 1997). Estimates of the total number of the planet's species are between 5 and 30 million, although some estimates are much higher (Meffe and Carroll 1997). The species level of biodiversity is the most widely recognized classification of biodiversity by those outside the scientific community. Charismatic species, such as the giant panda, fascinate the public and encourage financial support of conservation efforts by public and private contributors for projects that benefit multiple species and habitats. It is also the level at which major pieces of conservation legislation are directed (e.g. the Endangered Species Act (ESA) and the Convention on International Trade of Endangered Species (CITES)). A large majority of conservation efforts are directed at the species level.

Genetic

The ultimate source of biodiversity at all levels is genetic variability (Meffe and Carroll 1997). This level of diversity includes all forms of all genes (i.e. alleles) found in all organisms

on Earth, and is the material upon which the agents of natural selection act (Meffe and Carroll 1997). Genetic studies have a wide range of applications to conservation biology as they are useful for identifying populations of conservation concern, detecting hybridization, understanding species biology, detecting inbreeding depression and loss of genetic diversity, especially for small populations, and resolving population structures (Frankham and others 2002). Inbreeding is the mating of individuals that are genetically related by descent; this often results in inbreeding depression (Frankham and others 2002). Data from bird and mammal populations indicate that inbreeding depression, a loss of fitness that results from the breeding of relatives that often occurs in small, isolated populations, significantly affects birth weight, survival, reproduction, and resistance to disease which puts them at risk of extinction (Keller and Waller 2002, Saccheri and others 1998). These issues make genetic management a key concern for conservation biologists, particularly for captive breeding programs where the mating population may be small and closely related.

Role of Reproductive Science in Conservation

Conservation biology is a dynamic, multidisciplinary field that combines a wide array of disciplines in an effort to manage and protect our finite natural resources from the genetic to the ecosystem level. Wildt and others (2003) appropriately portrayed the field of biodiversity conservation as a jigsaw puzzle (Fig. 1.1). Each discipline represented in Figure 1.1 can contribute strategic information for protecting biodiversity. As reproduction is the basis for the perpetuation of all species, reproductive science impacts conservation biology at all levels of biodiversity management.
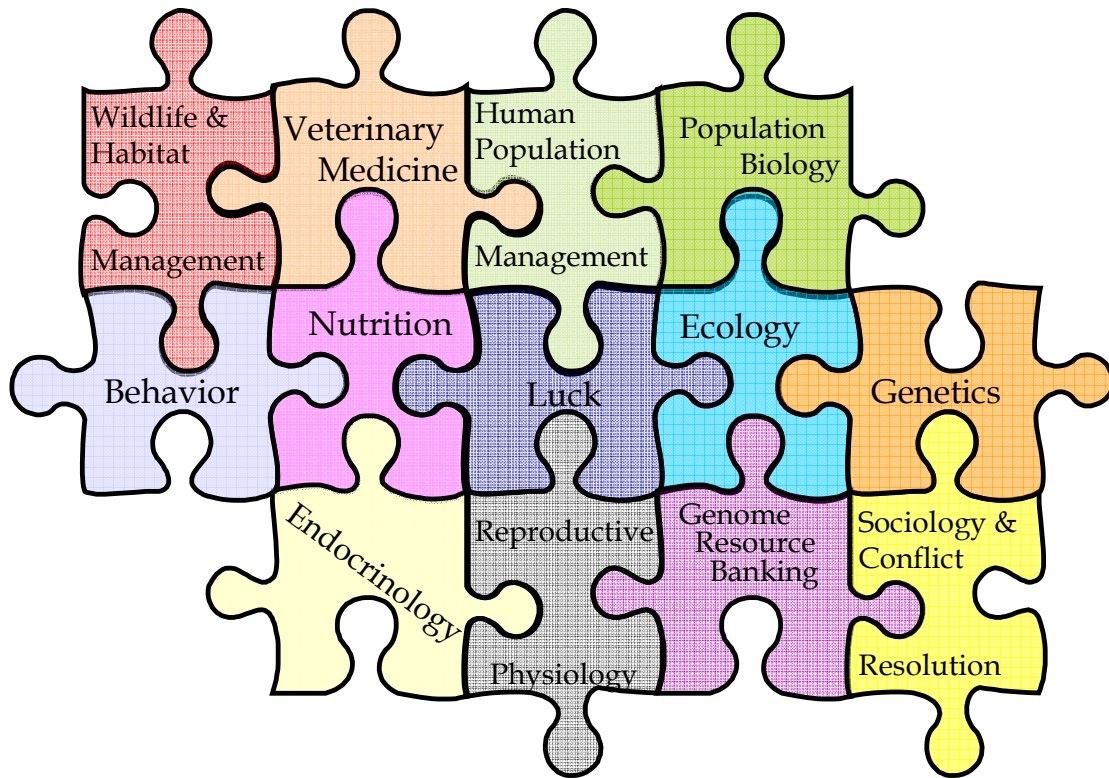
Figure 1.1. Representation of the multidisciplinary approach to conservation biology where professionals from various fields combine their expertise to preserve biodiversity (adapted from Wildt and others 2003).

Reproductive science and wildlife conservation

Reproductive strategies of wildlife vary extensively, from differences in courtship behaviors to gamete physiology. Within the framework of conservation biology, reproductive science embraces any and all methodologies and practices required to address priorities for understanding, monitoring, enhancing, or controlling reproduction (Wildt and others 2003). With regard to threatened and endangered wildlife, the ability to manipulate the reproductive process by promoting, or sometimes preventing, successful reproduction is particularly valuable. For some endangered species, intervention by scientists performing a variety of assisted reproductive technologies (ART) to promote reproduction coupled with *in situ* conservation efforts has played an integral role in preventing extinction. For example, the black footed-ferret, a native to the western great plains of the United States, was rescued from extinction when the last 18 individuals were removed from the wild and placed in a captive breeding program. Transabdominal-intrauterine artificial insemination via laproscopy and

cryopreservation methods for semen were two techniques that played a major role in a conservation project that involved the reintroduction of this species in its native range. As of 2001, 350 black-footed ferrets survive in wild populations (Howard and others 2003).

Contributions from the reproductive biology community to conservation efforts are principally through captive breeding programs. Understanding the reproductive biology of a species is critical for designing effective conservation strategies, especially when developing ART for captive populations. Herein lies one of the major problems when working with wildlife – a lack of knowledge of their basic reproductive biology. A study by Wildt and others (2003) revealed the shallowness of reproductive knowledge for most wildlife species. In a literature survey of wildlife reproductive biology studies, 75% of 256 species were represented by 3 or fewer articles (Wildt and others 2003). Because each species is unique and has specific reproductive strategies and physiology, ART cannot always be easily extrapolated to other species. There is a clear need for larger, comprehensive, multidisciplinary, and more coordinated research efforts to study basic reproductive biology of threatened wildlife species across many different taxa.

Despite the seeming paucity of research, ART have been developed and used on a variety of non-domestic species with varying degrees of success (Leibo and Songsasen 2002). Reproductive technologies such as artificial insemination, in vitro fertilization, embryo transfer, cryopreservation of gametes and embryos, gamete intra-fallopian transfer (GIFT), oocyte maturation, nuclear transfer, intracytoplasmic sperm injection, and sperm sexing can be used to support breeding programs. These technologies, however, are predominantly used for captive breeding programs with the intentions of contributing to the wild populations by introducing new individuals or new genes (e.g. via AI). The American Zoological Association has recognized the value of captive breeding programs for managing biodiversity by creating species survival plans (SSP). These plans coordinate efforts of accredited institutions to manage the breeding of a species in order to maintain a healthy and self-sustaining population that is both genetically diverse and demographically stable. There are currently active SSPs for 110 species (AZA 2006). Assisted reproductive technologies such as AI and cryopreservation can also be used to bolster the genetic diversity of isolated wild populations where these techniques can be applied in the field. This approach is more difficult as it requires close monitoring of the social dynamics and reproductive cycles within the population.

Stimulated by the realization that biodiversity loss is outpacing our ability to develop species-specific ART, the idea of the genome (genetic) resource bank (GRB) was developed. Genome resource banks for wildlife are organized collections of stored gametes, embryos, and tissues from individuals whose contribution to the gene pool is too valuable (genetically diverse) to lose (Holt and others 2003). These banks allow for controlled distribution of valuable genetic material aimed at maximizing a species' genetic variability in the wild and in captivity by protecting species against epidemics, natural disasters and social or political upheavals (Wildt and others 1997). Genome resource banks are also used to preserve lines of animals with desirable genetic characteristics such as transgenic mice. The growing number of transgenic mice used in research has been problematic for laboratories with limited space. Genome resource banks permit the creation of certain lines of mice in high demand, eliminating the space, money, and manpower required to sustain a live colony. Central to the functionality of these banks is the ability to create offspring using frozen-thawed gametes (Holt and others 2003).

Genome resource banks are also used to preserve genetic diversity within the plant kingdom. Although traditional cryopreservation is most common, vitrification and dehydration methods have been used on a variety of plant species, especially for tropical species that are unable to tolerate dehydration (Villalobos and others 1991). While large genetic banks have been developed for plant materials (e.g. seeds and shoots), especially food crops, a large majority of seeds lose germination vigor after 40 years of storage (Charles 2006, Graner and Börner 2006). To maintain reproductive potential, these stocks must be periodically germinated and re-stored.

With the concerted efforts of scientists, zookeepers, and veterinarians in identifying and manipulating factors that influence reproductive success, it seems that captive species should thrive. Indeed, this is sometimes the case, yet success can be fraught with the paradoxical occurrence of unbridled reproduction that can result in a saturated, overwhelmed captive habitat (Wildt and others 1993). In these circumstances, there is a need to manage the rate at which the individuals reproduce (i.e. by reversibly suppressing reproduction via contraception, until more suitable facilities are available). Contraception is also a valuable option when preventing reproduction can avoid a critical decline in a population or sub-population. This can mean protecting native species by preventing the reproduction of an invasive species or

preventing the unsustainable growth of an indigenous species that endangers the integrity of its own population and the populations of other species that share its habitat (e.g. African elephants).

Human reproductive science in conservation

Some scientists have strongly supported the notion that controlling the size of the global human population is the key to protecting biodiversity (Hardin 1993, Johns 2003). While this is not the panacea for preventing extinctions, slowing population growth, particularly in developing countries where biodiversity is highest, could ease the pressure on these valuable habitats. Methods of contraception have evolved over the last century and have resulted in several reliable forms, especially for women. Men, on the other hand, have very few available contraceptive options, most of which are unsatisfactory because of their cost, irreversibility, or general undesirability.

Humans are a slow breeding mammal in that they reach sexual maturity late, have a small chance of becoming pregnant during any given menstrual cycle, and have long natural intervals between births (Potts and Short 1999). Despite these biological disadvantages, the global human population continues to rise. There is no consensus about the predictions of the human population growth, but predictions of the earth's carrying capacity range from 1.5 to 100 billion with most figures falling between 15 and 30 billion (Cairns 2004). As the human population continues to increase, planetary resources will become strained and have a direct effect on the human quality of life. The use of contraceptives can make a significant contribution to slowing population growth, especially in countries where population growth rates are already high (Lutz and Qiang 2002).

For conservation biologists, these projections are of concern and make immediate action against loss of biodiversity even more imperative. Africa, a biodiversity hotspot and home to a large number of endangered species, is perhaps the most troubled continent. In 2000, Africa's human population was approximately 780 million. At current growth rates, the African population will increase 116% by 2050 (Rice 2006). If population growth does not drop to replacement level or lower before 2065, Africa's population is expected to reach 4.45 billion before stabilizing (Potts and Short 1999). This kind of unsustainable growth would be devastating for the already threatened populations of wildlife in Africa.

There are many issues that propel contraceptive research for humans that are not necessarily powerful driving forces of development for wildlife contraception, such as health issues and the desire for family planning. The desire to provide more readily available and diverse contraceptive options for humans provides a flow of information that can be used to form new ideas for contraceptive strategies in wildlife. Providing a variety of readily available and affordable contraceptive methods to more people will reduce unwanted pregnancies and decrease the incidence of unsafe abortions, which in underdeveloped countries contribute to the potentially preventable deaths of approximately half a million women worldwide every year (Hutchinson 1993).

Contraception affords couples the ability to delay or space the births of their children. The use of contraceptives for family planning has played a major role in curbing the birth rate of some populations over the past 40 years (*e.g.* China). The prospect of a contraceptive for men within the next 5 years (Kamischke and Nieschlag 2004, Heinemann and others 2005) will be a welcome addition to the limited possibilities currently available to men (condoms, vasectomy, withdrawal, periodic abstinence; Nieschlag and others 2000, Weber and Dohle 2003). Studies show that both partners would like the man to play a more active role in family planning and that, in many countries, willingness of men to adopt a contraceptive is high (Heinemann and others 2005, Bajaj 1999, Martin and others 2000, Glasier and others 2000, Weston 2002). The development of a male-directed contraceptive for humans is sure to spawn new contraceptive strategies directed at males in wild and captive animal populations.

Intertwined with the exponential growth of the human population and its consequences for the quality of human life are the effects that this growth will have on wildlife and the environment. While this may not be the leading cause for using contraception on an individual basis, it is certainly a cause for concern for humanity as a whole, and a legitimate rationale for governments, policy makers, and other institutions to promote contraceptive use. There are abundant examples of how human population growth is affecting wildlife. For example, the African wetlands inhabited by the Nile hippopotamus have been developed for human use and logging is displacing monkeys in Indonesian forests (Graham and others 2002, Wilson and Wilson 1975). Such encroachments greatly reduce the habitable ecosystems for impacted species, leaving habitats that are increasingly fragmented and isolated (Nave and others 2002). Curbing human population growth will slow habitat loss and the extinction of vulnerable

species while allowing for the development and implementation of more sustainable lifestyles that are beneficial for both wildlife and human populations.

Cryopreservation of Spermatozoa

Studies of gamete physiology have been, and will continue to be, fundamental to developing effective ART, especially in the use of frozen-thawed spermatozoa. Techniques to freeze spermatozoa were first developed to meet the demands of domestic animal breeders who have increasingly used artificial insemination to maintain their stocks (Gao and others 1997). With the discovery that glycerol could be used as an effective cryoprotectant (Polge and others 1949), development of successful cryopreservation schemes for spermatozoa from a variety of species became the focus of numerous empirical studies. Today the practical application of cryopreserved semen has expanded beyond agriculture. The flexibility that a successful cryopreservation scheme allows has several advantages that are also useful in endangered species conservation.

Advantages of cryopreservation

An important advantage of freezing spermatozoa is the ability to ship the semen of genetically valuable animals rather than the animals themselves, eliminating stress and the risk of injury to the animal, while simultaneously reducing costs and allowing for a wider distribution of selected semen. For endangered species this can be especially beneficial when the removal of individuals from the wild, in order to infuse new alleles into a captive population, may further weaken an already vulnerable wild population. When cryopreserved semen is used, ejaculation does not need to be timed with female reproductive cycles, eliminating the need to co-ordinate animal transport or availability with time-sensitive processes, an especially advantageous aspect when working with species of unpredictable or unknown breeding cycles and behaviors. In cases where a valuable animal dies prematurely, its genetic material can be stored and used to maintain the genetic variability of the population or reinforce that of others in the future.

Successes and areas of improvement

Despite decades of research, post-thaw sperm motility and conception rates from cryopreserved semen for most species are low. The notable exceptions are the dairy industry where AI is the method by which the majority of animals are produced (Iritani 1980) and in human fertility clinics where IVF and ICSI with cryopreserved spermatozoa are routine (Gunasena and Critser 1997). Surprisingly, some clinics have found that, when controlling for concentration and donor selection, results with frozen-thawed spermatozoa are similar to those with non-frozen spermatozoa (Bordson and others 1986, DiMarzo and others 1990). In addition to the differences between species, as with humans, there are distinct differences in sperm freezability among individuals within a species. Research on individual variability is in its early stages with several hypotheses such as a genetic determinant of freezability (Thurston and others 2002) and a critical provision of osmolytes needed to regulate cellular volume effectively during freeze-thawing (Cooper and Barfield 2006).

Low success rates (e.g. post-thaw motility, production of offspring) have been attributed to numerous factors with the foremost being the empirical development of these techniques despite a lack of knowledge about the fundamental cryobiology of mammalian spermatozoa (Gao and others 1997). In order to elucidate this problem, many scientists argue the need for more information about the complicated membrane structure of the spermatozoa and the varying species sensitivities to osmotic stress, cryoprotectants, and water influx/efflux (Watson and Fuller 2001). Others agree that biophysical properties such as sensitivity to cold shock, membrane composition, and permeability to water and cryoprotectants must be understood within and across species before advancements in sperm cryopreservation for wildlife are successful (Pukazhenthi and Wildt 2004). The research presented in this dissertation is one step of many required to elucidate the mechanisms by which spermatozoa cope with osmotic challenges. By understanding these mechanisms and the limits within which they can function, detailed protocols or methods can be designed that account for this volume regulation, thereby improving post-thaw motility and conception rates.

Sperm volume changes during cryopreservation

The viability of mammalian spermatozoa is highly sensitive to osmotic stress and the associated cell volume excursion (Gao and others 1997). The osmotic challenges and

accompanying volume changes that spermatozoa undergo during cryopreservation are dramatic relative to physiologically relevant osmotic fluctuations. To understand the extent of volume regulation needed by spermatozoa to survive the freeze-thaw process, the osmotic challenges during cryopreservation need to be considered.

The first osmotic challenge a spermatozoon encounters during the freezing process is the addition of a cryoprotective agent (CPA). This is a compound that minimizes the formation of intracellular ice crystals by decreasing the chemical potential of the solvent water, physically expressed as the osmolality and freezing point of the solution (Karow 1997). There are two types of CPAs: penetrating (e.g. glycerol and dimethylsulfoxide (DMSO)) and non-penetrating (e.g. sucrose). The most commonly used CPA is glycerol. When high concentrations of glycerol are used, the hyperosmotic solution drives water egress from the cell. The initial shrinkage is fast because water leaves the cell quicker than the CPA enters. Hyperosmotic stress reduces sperm motility and although the mechanism is unknown, it is generally believed that 'cryoinjury' is related to hyperosmotic stress (Gao and others 1993). Indeed, studies have shown that a change in the osmotic environment by exposure to CPAs for as little as 15 min, without cryopreservation, causes a loss of motility (Critser and others 1988). As glycerol enters the cell, the water lost during the initial shrinkage is regained.

The second osmotic challenge occurs as extracellular water freezes during the cooling process. As cells cool to -5°C, the cells and the surrounding medium remain unfrozen and supercooled (Gao and others 1997). Supercooled water has, by definition, a higher chemical potential than the partially frozen extracellular solution, causing water to flow out of the cells osmotically and freeze externally leaving high concentrations of salts (Gao and others 1997). Between -5° and -10°C ice forms extracellularly but the cell contents remain unfrozen and supercooled. As cooling proceeds to approximately -40°C, the solution becomes increasingly concentrated, reaching osmolality values greater than 20 times isotonic (Mazur and others 1981). If cooling is sufficiently slow, the intracellular water content will reach equilibrium with the extracellular solution so that cell volume is diminished and ice crystals do not form. However, if cooling is too slow, the cells will be exposed to extremely hypertonic conditions for a prolonged period resulting in cell death.

Upon thawing the cells go through reverse volume changes. As water is converted from the solid to liquid state it lowers the extracellular solute concentrations and the osmotic gradient

pulls water into the cells. Several studies have indicated that any lethal osmotic injury likely occurs during warming and rehydration rather than cooling (Watson and Fuller 2001), yet the mechanism is currently unknown. This may be the result of the inability of the cell membrane to cope with rapid changes in volume which disrupt cytoskeletal elements (Watson and Fuller 2001). Regardless of the mechanism, understanding cellular damage sustained in the thawing process requires a focus on how osmotic stress can be modified or how the membrane can be made more resistant. However, without first understanding sperm volume regulation and its mechanism across the membrane in general, overcoming complications of the cryopreservation process will be unachievable.

Finally, during cryoprotectant removal, a large change in volume occurs as water flows into the CPA-containing cell because the water influx is faster than the glycerol efflux. Rapid cryoprotectant removal is especially harmful causing loss of membrane integrity and motility (Gao and others 1993, 1995). Theoretically, if cryoprotectant is not removed before insemination the spermatozoa will undergo a 4.3-fold expansion of osmotically active internal water as the cells are transferred from a solution of approximately 1300 mmol/kg to the female tract which is approximately 300 mmol/kg (Hammerstedt and others 1990), although the extent of cellular expansion due to the movement of cryoprotectant and water is temperature-dependent. Extensive swelling can be overcome with a multi-step dilution removal of the CPA. Diluting the CPA-loaded suspension once with CPA-free medium causes a 1.6-fold increase in cell size. By diluting the suspension eight times with fixed volumes of CPA-free medium, there is only a 1.2-fold increase in volume. If the eight dilutions are of fixed molarity rather than fixed volume, then the increase is less than 1.1 times isosmotic, an increase that is inconsequential (Gao and others 1993).

Each step of the cryopreservation process forces spermatozoa to cope with drastic osmotic challenges and potentially large changes in cell volume. Volume regulation must be functional during the challenges to maintain membrane integrity. It is clear that for spermatozoa undergoing cryopreservation, the ability to maintain a critical volume is extremely important for cell viability in ART techniques, with the exception of ICSI.

Conclusion

Reproductive science has an important role in conservation biology. Most of the contemporary threats to biodiversity are caused by human action and any effort to reduce the current global population rate will be beneficial. While reproductive biology cannot provide a solution to the biodiversity crisis itself, it will undoubtedly contribute integrally to the multi-disciplinary effort required to preserve threatened and endangered species. Assisted reproductive technologies represent a valuable avenue of research, especially for species in captive breeding programs and wild populations with low numbers. Descriptive studies of basic reproductive biology in wildlife are critical for the development of successful ART, especially cryobiological research. Investigation of the volume regulatory mechanism in spermatozoa is an avenue of basic research that could produce options for improving the preservation of spermatozoa, and also a non-hormonal contraceptive option for men, both of which could have significant impacts on global biodiversity preservation.

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<u>Chapter 2</u>

The Study of Cell Volume Regulation

The regulation of cellular volume is an extremely narrow aspect of sperm physiology but a vital one, although very little is known about the process. The contribution of knowledge on sperm volume regulation to conservation biology is not immediately obvious but within the framework of reproductive sciences, and assisted reproductive techniques or technology (ART) in particular, this area of study could be of great importance. This dissertation contributes to the field of conservation biology by examining an aspect of sperm physiology that, if understood, could play an important role in improving sperm preservation techniques as well as serving as a potential target for male-directed contraception. The volume regulatory process of spermatozoa is an under-studied phenomenon and the experiments described in this dissertation serve as a starting point for future research that could make the potential applications of these studies a reality.

Volume Regulation by Somatic Cells

Regulation of cellular volume is vital for a wide range of physiological functions. Exposure to anisotonic conditions challenges a variety of cells during normal cellular processes. Some cells are challenged as a result of a pathological condition, maturation (erythrocytes), cell growth, differentiation, hypertrophy, and apoptosis (Wehner and others 2003). Cell migration and shape changes require volume adaptations and cell volume has been identified as a regulatory mechanism of cell metabolism (Lang and others 1998).

Regulatory adjustments

Because a homeostatic cell volume is critical to many facets of cellular function, any change in cell volume must be dealt with rapidly and efficiently. As a result, somatic cells have many different mechanisms to cope with osmotic challenges, and the activated mechanisms vary according to the magnitude of osmotic challenge, the time since the challenge occurred, and the nature of the challenge. Changes in osmolality can be hyper- or hypotonic, and they require the appropriate cell-mediated responses of regulatory volume increase (RVI), regulatory

volume decrease (RVD), respectively, and in some cases, isovolumetric (isovolumic) regulation (IVR).

Regulatory volume increase is the augmentation of intracellular osmolyte content and associated water that increases cell volume after a hyper-osmotic challenge (see Fig. 2.1). Sodium channels are typically the first to be activated. During RVI, the main inorganic osmolyte taken up by cells is sodium (Wehner and others 2003). There are four mechanisms by which this happens: the simultaneous activation of the Na^+/H^+ antiporter and the Cl^-/HCO_3^- antiporter, operation of the Na^+-K^+-$2Cl^-$ symporter (NKCC), activation of the Na^+-Cl^- symporter (NCC), and operation of Na^+ channels (Fig. 2.1, Okada 2004). The predominant pathway that operates varies with cell type and environment. For example, in the absence of HCO_3^- most cells utilize the Na^+-K^+-$2Cl^-$ symporter, while availability of HCO_3^- activates the Na^+/H^+ antiporter and the Cl^-/HCO_3^- antiporter (Okada 2004). Mechanisms of RVI will not be fully discussed here as mechanisms of RVD are more relevant to the topic of this dissertation. However, the reader is referred to several excellent reviews for more complete information on RVI (Lang and others 1998, Okada 2004, Wehner and others 2003)

RVD is the reduction of intracellular osmolyte content and associated water to reverse swelling caused by a decrease in extracellular osmolality. Ion channels, specifically K^+ and Cl^-, have received the most attention in volume regulatory investigations. Rapid and efficient adjustments to cellular osmolality are most easily achieved by transporting ions across the membrane and as a result, ion channels are the most frequently activated transport system during cell swelling events (Lang and others 1998). A wide variety of channel proteins from different families is used in RVD responses, both selective and non-selective. The non-selective channels are indirectly involved in volume regulation by allowing the passage of cations into the cell, as dictated by the negative membrane potential, which can activate more selective channels involved in RVD, such as Ca^{2+}-activated K^+ channels (Lang and others 1998). These channels and other mechanisms of RVD are discussed below.
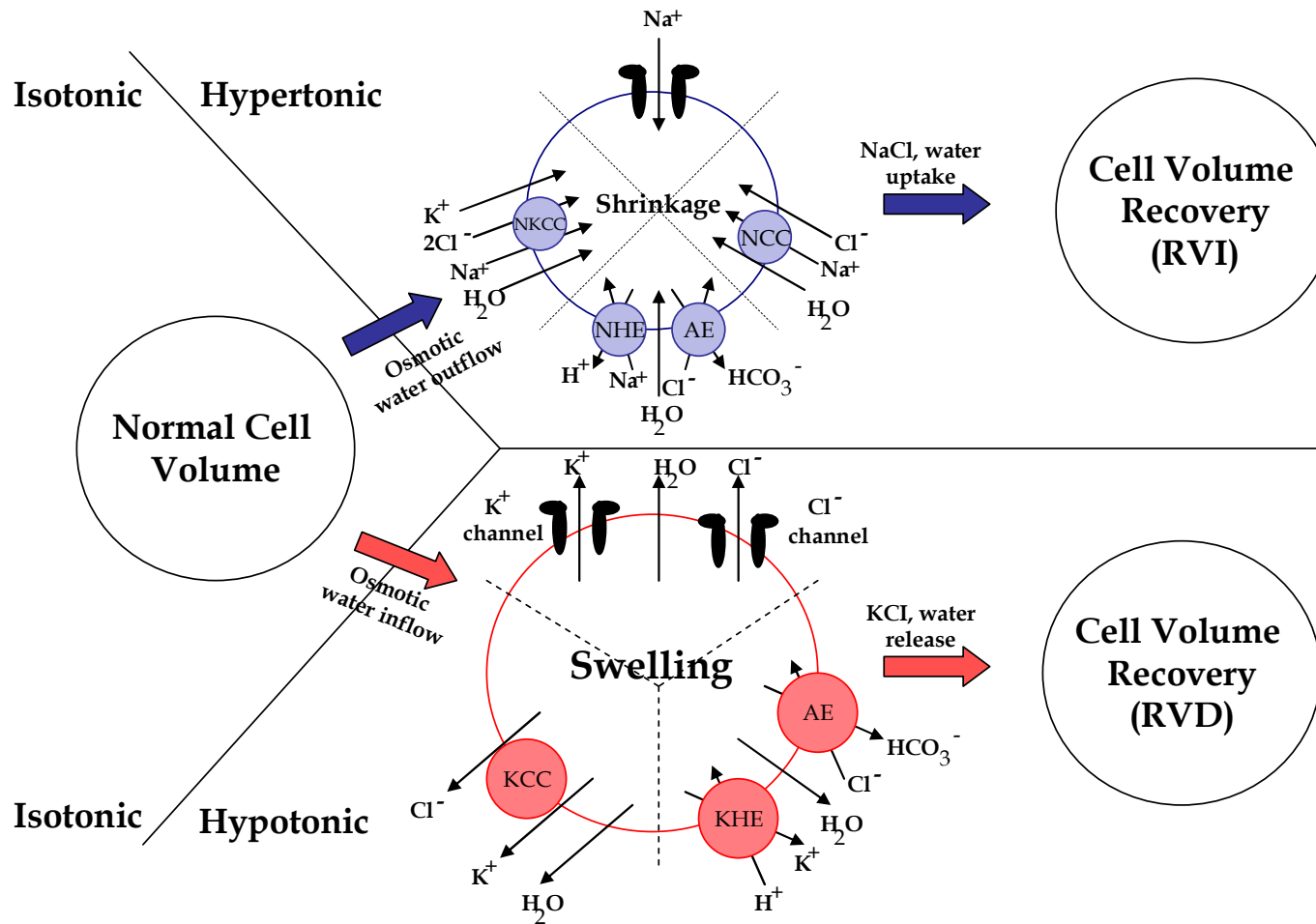
Figure 2.1. The mechanisms involved in RVI and RVD of somatic cells during hyper- and hypo-osmotic challenges, respectively. See text for details. Adapted from Okada (2004).

The response evoked by gradual changes in osmolality is called isovolumetric (or isovolumic) regulation (IVR). Under IVR, cells compensate for changing osmolality in such a way that cell volume adjustments are undetectable. There is evidence that the mechanisms used during IVR are different from those used in response to abrupt changes in extracellular osmolality (i.e. RVI and RVD; Souza and others 2000, Pasantes-Morales and others 2000, Franco and others 2000). While the stimuli for these changes can occur over the same range of osmolalities, the difference is the rate of change in osmolality. Organic osmolyte channels are activated as a method of augmenting intracellular inorganic electrolytes only if an osmotic challenge lasts for an extended period of time (i.e. hours or days, Wehner and others 2003) and thus are an important component of the IVR process. During epididymal transit, spermatozoa are exposed to changes in osmolality that occur over days or weeks, depending on the species (Cooper 1998). It is hypothesized that IVR is the mechanism by which spermatozoa accumulate osmolytes that are used for volume regulation upon ejaculation into the female reproductive tract (Cooper and Yeung 2003).

Associated with the mechanisms involved in RVI, RVD and IVR is the movement of water. In principle, any changes in volume of animal cells under anisotonic conditions reflect the permeability of the plasma membrane to water (Wehner and others 2003). This permeability is increased by water channels or aquaporins, specialized membrane proteins which mediate the facilitated diffusion of water (Maunsbach and others 1997, Marinelli and LaRusso 1997, Ma and Verkman 1999). The presence of these proteins enhances osmotic behavior in response to deviations from isotonicity; a movement of water will occur changing cell volume until the difference in osmolality is abolished and a new chemical equilibrium across the membrane is achieved (Wehner and others 2003). This would suggest that the cell responds according to the Boyle van't Hoff plot, which describes a linear relationship between cell volume and extracellular osmolality. However, the cell is not a perfect osmometer because of the presence of osmotically-inactive, intracellular water (Lucké and McCutcheon 1932). By plotting the reciprocal of extracellular osmolality against relative volume, the percentage of osmotically inactive water can be determined (Wehner and others 2003).

Mechanisms of RVD

During RVD, somatic cells utilize the K^+-Cl^- symporter, coupled activation of the K^+/H^+ antiporter and the Cl^-/HCO_3^- antiporter, and separate conductive ion channels (Fig. 2.1). In addition to ionic mechanisms, efflux of organic osmolytes has been discovered to be an important component of the RVD response.

The K^+-Cl^- symporter (KCC), while also activated by oxidizing agents and low Mg^{2+} concentrations, can mediate KCl efflux from swollen cells (Lauf and Adragna 2000). Swelling-induced activation has been demonstrated in the red blood cells of many species and several other cell types (Okada 2004). Some evidence suggests that swelling-induced activation is produced by a phosphorylation-dephosphorylation event, although in human erythrocyte ghosts, the absence of ATP does not prevent swelling-stimulated K^+-Cl^- symporter activity (Sachs and Martin 1993). Nevertheless, as long as there is a difference between the chemical potentials for K^+ and Cl^- across a membrane favoring efflux of electroneutral K^+ and Cl^-, the K^+-Cl^- symporter may have a role in volume regulation, and has been implicated in volume regulation of murine spermatozoa (Klein and others 2006). There is considerably less evidence for the involvement of the K^+/H^+ antiporter and the Cl^-/HCO_3^- antiporter. *Necturus* erythocytes require operation of these transporters to accomplish RVD (Okada 2004).

To maintain electroneutrality with the efflux of K^+ through channels engaged in the RVD process, there is significant involvement of Cl^- channels. In cases of extreme osmotic challenge, cells will release Cl^- by all available anion channels (Okada 2004). However, there are some specific channels that are unequivocally associated with RVD and cell-volume expansion. For example, CLC-2, a member of the chloride channel (CLC) gene family of Cl^-channels, is activated by hyperpolarization and cell swelling (Grunder and others 1992). The most common Cl^- channel identified in volume regulatory Cl^- efflux is a volume-sensitive outwardly-rectifying Cl^- channel (VSOR). While extensive research has been conducted on this channel, the activation mechanisms are largely unknown or controversial (Okada 2004). In addition, VSOR channels are activated during RVI and under conditions of isotonicity, as in swelling induced by active solute transport (Okada 2004). Other Cl^- channels implicated in RVD are the cloned BRI-VDAC (volume-dependent anion channel), I_{Cln}, and the P-glycoprotein (multi-drug resistance (MDR) protein; Lang and others 1998).

Volume regulation does not depend solely on the conductance of inorganic ions. Large changes in ion concentration within the cell can disrupt cell structure and interfere with the function of macromolecules (Lang and others 1998). Imbalance in the ion gradient can also affect the transporters mentioned above, further altering intracellular osmolality and disrupting cell function (Trump and Berezesky 1995). In recent years, the role of organic osmolytes in RVD has become more evident. Organic osmolytes are molecules that provide osmolality without compromising cellular processes (Lang and others 1998). There are three types of organic osmolytes present in mammalian cells: polyalcohols (or polyols), methylamines, and amino acids and their derivatives (Lang and others 1998). In addition to their being used in the volume regulatory response, organic osmolytes also counteract the adverse effects of inorganic ions and can protect the cell from harmful effects of high temperatures and dehydration. From a review of the literature, it is obvious that there is a ubiquitous presence in vertebrates of an organic osmolyte channel that responds during the volume regulatory process (Perlman and Goldstein 1999).

A wide variety of osmolytes has been identified in RVD of somatic cells including sorbitol, *myo*-inositol, glycerophosphocholine (GPC), taurine and betaine. There is strong evidence that all of these osmolytes may move through the same channels (Wehner and others 2003). The most well studied osmolyte is taurine of which it is estimated some 30-50% of the intracellular content leaves the cell during RVD (Wehner and others 2003). Channels involved in taurine transport have been termed VSOR-Cl$^-$, a voltage-sensitive organic anion channel (VSOAC) and a voltage-responsive anion channel VRAC. Although osmolytes may utilize the same channel, their efflux from the cell is dictated by osmotic thresholds and the length of exposure to hypo-osmotic conditions. For example, studies on rat and rabbit collecting duct cells indicate that taurine release is at 50% activation when the difference from isotonic (Δ) reaches 250 mmol/kg (Kinne 1998). Taurine is the second osmolyte to be released in a sequence of activation (betaine Δ 200 mmol/kg, *myo*-inositol and taurine Δ 250 mmol/kg, sorbitol Δ 350 mmol/kg, GPC Δ 400 mmol/kg; Kinne 1998). In addition, each osmolyte is associated with a particular lag period, the time between exposure to hypo-osmolality and activation of release. For sorbitol, the lag time is 20 seconds while it is 7.5 min for GPC. Other osmolytes fall within this range (*myo*-inositol and taurine 2 min, betaine 5 min; Kinne 1998).

The contribution of organic osmolytes versus that of ions in a given volume regulatory response varies from species to species. Skate erythrocytes exposed to hypo-osmotic conditions efflux far more taurine than K^+, the latter being some 10% of the former (Dickman and Goldstein 1990). Equine erythrocytes, on the other hand, have very small taurine effluxes compared to the large amounts of K^+ lost during a hypo-osmotic challenge (Gibson and others 1993). This difference could be due to the initial levels of taurine or K^+ in the cell or, since many of the experiments included a pre-incubation period for the uptake of taurine (Perlman and Goldstein 1999), a difference in the rate of taurine uptake over the same incubation time.

Potassium Channels and RVD

Potassium channels are the most widely investigated component of the volume regulatory process. Activation of potassium channels brings about volume regulatory potassium efflux resulting from the outwardly-directed electrochemical gradient.

Structure and conductance

The architecture of the potassium channel was established from *Streptomyces lividans*, with a nucleotide sequence showing similarity with all described potassium channels (Doyle and others 1998). All K^+ channels are tetramers (i.e. comprised of four α-subunits each of which is composed of two, four, or six membrane-spanning segments) with four-fold symmetry about a central pore. Two of the membrane-spanning α-helices of each subunit form the pore region, which consists of roughly 30 amino acids. This pore region contains the turret (loops that connect putative membrane-spanning segments), the pore helix, and the selectivity filter. An α-subunit is situated such that two α-helices face the inner wall of the pore while any others face the lipid membrane. The helices that face the pore are slightly tilted so as to form a teepee shape, yet slightly kinked so that they open as a flower would, facing the extracellular surface. The area in the opening of the flower, or the inner helices, encircles the pore region, which is composed of the K^+ channel signature sequence, otherwise known as the selectivity filter. The outer helices are also tilted in the same manner as the inner helices and are positioned in a staggered configuration between, yet just beyond, the inner helices, with their carboxyl ends in close proximity near the center of the channel (Doyle and others 1998).

Just below the ends of the outer helices, in the center of the channel, is a large cavity filled with polarizable water (Fig. 2.2). This arrangement serves two purposes. First, when an ion passing through the straight narrow pore reaches the center of the plasma membrane, it must be able to pass through the maximum energy barrier. Normally, a cation will bring the negative ends of dipoles closer with its electrostatic field in an attempt to stabilize itself. However, at the center of the membrane, the polarizability of the surrounding environment is minimal, and the energy of the cation is very high. This cavity in the center of the potassium channel overcomes this electrostatic instability by simply surrounding the ion with polarizable water. Second, the arrangement of the outer helices centers their carboxyl ends at a point that coincides with this large cavity. The ends of these helices create a negative electrostatic potential that attracts cations to this point and thus into the selectivity filter just beyond the water filled cavity. This arrangement effectively solves the problem of an electrostatic barrier facing a cation crossing a lipid bilayer and ensures that the ions continue to move along the channel properly (Doyle and others 1998).
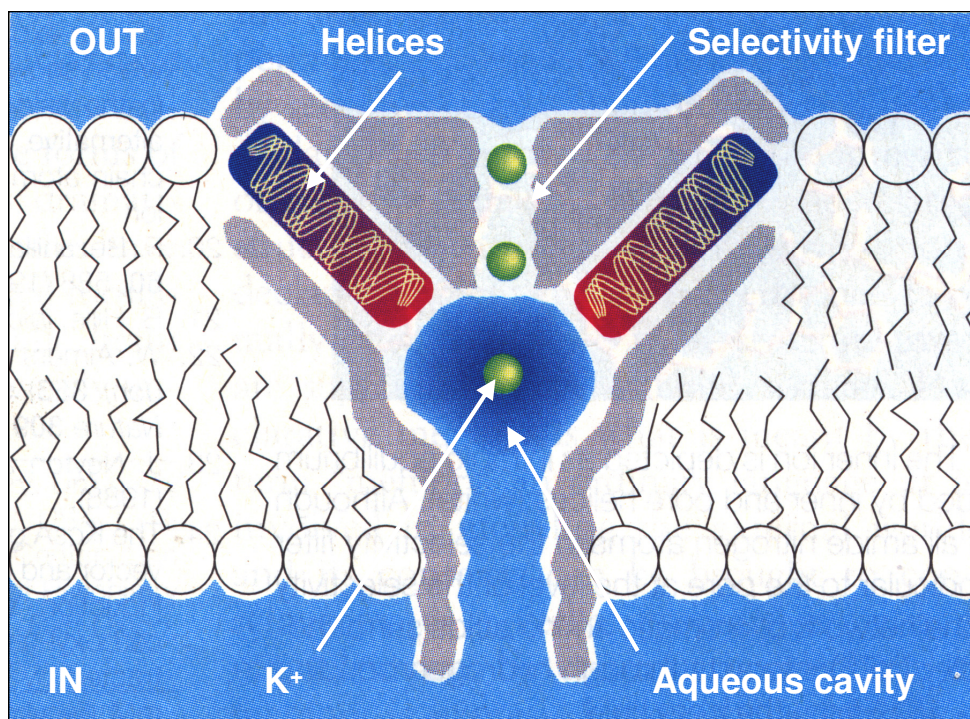
Figure 2.2. Diagram of the polarizable water center and orientation of the outer helices of a potassium channel. This structure negates the electrostatic barrier that a cation would face when crossing a lipid bilayer making conductance rapid and efficient. Modified from Doyle and others (1998).

A K$^+$ ion in the cavity in the middle of the channel is fully hydrated, bound to four water molecules. In order to enter the selectivity filter, the ion must shed these molecules in order to fit. The selectivity filter is constructed so that the oxygen rings from the helices stack up in a configuration that just fits the dehydrated potassium ion (Fig. 2.3). Therefore, when an ion enters the filter it must become dehydrated. The carbonyl oxygen atoms lining the filter take the place of the water oxygen atoms (Hille 1973). This arrangement is highly selective for potassium. Even dehydrated ions that are smaller than potassium (1.33 Å) cannot pass through this filter (e.g. Na$^+$). The carbonyl oxygens may be able to replace the oxygens of one or two water molecules, but because the dehydrated Na$^+$ molecule is so small (0.95 Å), the remaining two water oxygens cannot be replaced because there is not the required proximity to the carbonyl oxygens. The filter is only 12 Å (Angstroms) long or 0.0012 μm, minimizing the distance over which K$^+$ must interact strongly with the channel.
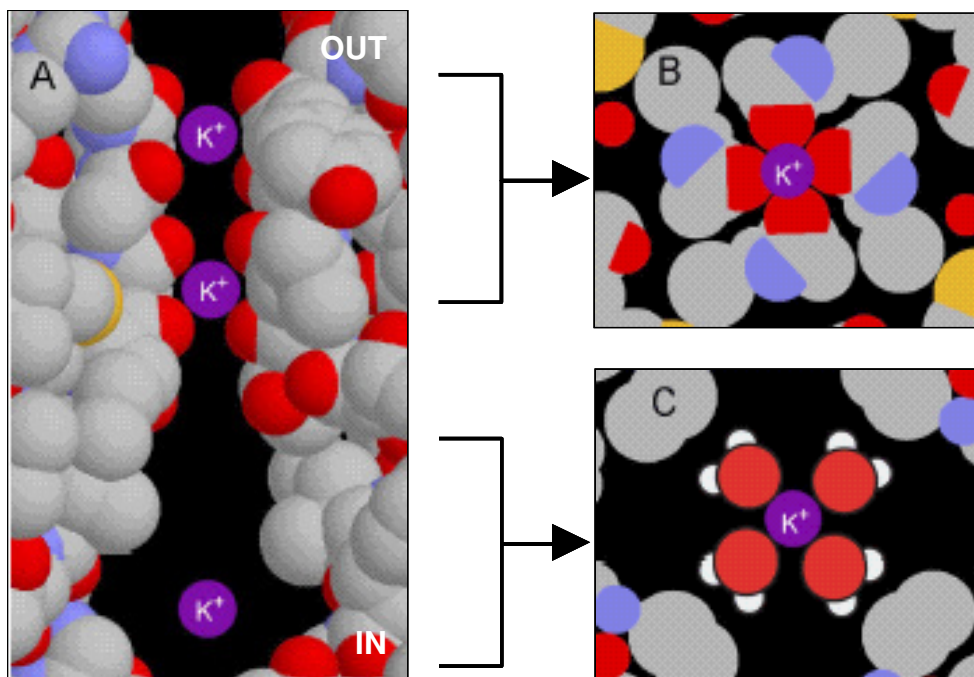
Figure 2.3. Longitudinal view of ions passing through the pore of a potassium channel (A) with cross-sectional views at the corresponding levels where ions are in close contact with carbonyl oxygens within the filter (B) and in the aqueous cavity where the ion is surrounding by its water of hydration (C). Adapted from Armstrong 2003.

In a 150 mM K^+ solution, two ions can be found in the selectivity at any one time. One ion is at the end nearest the central cavity and the other closest to the external environment. This structure suggests that when one ion is present, it is bound very tightly; however when a second ion is present it creates a repulsion within the filter. Thus, when a second ion enters the filter, the attractive force between a K^+ ion and the selectivity filter becomes perfectly balanced by the repulsive force between the two ions, releasing the K^+ from its tight association with the filter and thus allowing for conduction (Doyle and others 1998). In this way, ions pass through the filter in single file.

Classification

Potassium channels are classified according to their structure. All K^+ channels are tetramers, that is, they have four α-subunits (Fig. 2.4A, Coetzee and others 1999). The structural differences of these subunits and the number of pores formed by them define the classification of a particular channel. There are three groups of K^+ channels: those which have subunits with six transmembrane domains (TMD) and form one pore (Fig. 2.4B), those that have 2 TMDs per subunit and form one pore (Fig. 2.4C), and those that have four TMDs per subunit and form two pores (Fig. 2.4D, Wickenden 2002).

Within these groups are families of K^+ channels categorized by specific properties, determined by their α-subunit. Within the subclass of channels having 6 transmembrane domains and one pore, channels are grouped into one of two families, voltage-gated (Kv) and Ca^{2+}-activated. The voltage-gated family has eight subfamilies including Kv1-Kv6 and Kv8-Kv9 (Coetzee and others 1999). Members of these families include *shaker* (Kv1), *shab* (Kv2), *shaw* (Kv3), *shal* (Kv4), silent (Kv5-9), EAG, and KvLQT-related channels. The second family in this group is that of Ca^{2+}-activated K^+ channels, which includes subfamilies reflecting the extent of charge conductance: large (BK_{Ca}), intermediate (IK_{Ca}), and small conductance (SK_{Ca}) channels.

Channels having 2 transmembrane domains and one pore are all inwardly rectifying, that is, they pass currents over a hyperpolarized voltage range (e.g. the cell becomes more negative internally so these channels allow the influx of potassium to balance the hyperpolarization event). This family has seven subfamilies with varying degrees of inward rectification, including channels which are G-protein regulated, ATP-sensitive, or those belonging to the ROMK1 group (Wickenden 2002, Coetzee and others 1999). K^+ channels

having four transmembrane domains and two pores do not yet have any further classifications, as there are only 14 members. These channels are often referred to as "leak channels," some of which can be extensively modulated by factors such as pH (Coetzee and others 1999).
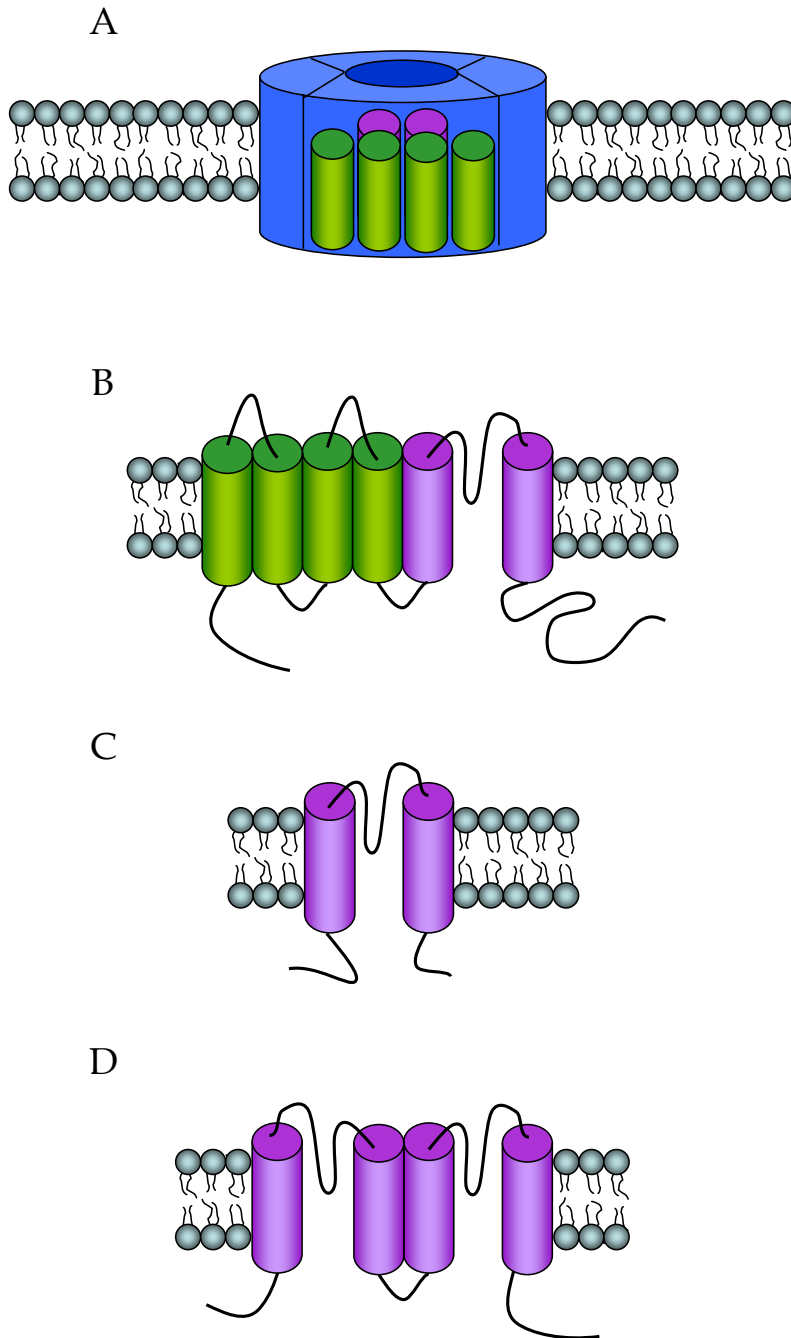
Figure 2.4. A diagram of the orientation of the TMDs around the single pore of a 6TMD potassium channel (A). Each section of the blue cylinder surrounding the pore (the darker circular center) represents one of the four subunits. All subunits are oriented identically to that of the displayed section with two TMDs in contact with the pore. Sketches of the transmembrane domains of one α-subunit of 6TMD-1 pore (B), 2TMD-1 pore (C), and 4TMD-2 pore potassium channels (D). Purple TMDs indicate those that are in contact with the channel pore.

A further classification of each group can be made according to their modulatory β-subunits, which include cytoplasmic proteins, single transmembrane spanning proteins (minK- and minK-related proteins) and large ATP-binding cassette transport-related proteins (Wickenden 2002). β-subunits play crucial roles in the regulation of channel activation (time and voltage dependency of channel opening) and inactivation (channel closure in response to a change in membrane potential or other factor). For voltage-gated potassium channels, one β-subunit is bound to the intracellular surface of each of the tetrameric α-subunits. β-subunit configurations differ for Ca^+ and Na^+ channels (Hanlon and Wallace 2002).

Channels involved in somatic cell RVD

Several K^+ channels have been implicated in regulatory volume decrease of somatic cells. Among these are large-conductance, calcium-activated K^+ channels (BK_{Ca} or maxi-K^+), intermediate conductance, calcium-activated K^+ channels (IK_{Ca}), small conductance, calcium-activated K^+ channels (SK_{Ca}), two voltage-gated delayed rectifier channels (Kv1.3 and Kv1.5), minK channels, and TASK2, a two-pore domain K^+ channel (Wehner and others 2003).

Large-conductance calcium-activated potassium channels (BK_{Ca}), also known as maxi-K^+ channels, are so named for their high range of conductance of 100-250 pS (at 0 mV) (Wehner and others 2003). Various blockers can inhibit conductance via these channels such as Ba^{2+}, quinine, tetraethylammonium (TEA), charybdotoxin, and iberiotoxin. To date, BK_{Ca} channels have been found in neurons, skeletal muscle, smooth muscle, and in epithelial cells, situated on the apical membrane. In addition to being activated by Ca^{2+}, these channels are sensitive to membrane depolarization (Wehner and others 2003). The activation of BK_{Ca} channels during hypotonic stress has been demonstrated in a wide variety of cells. In the A_3 cell line derived from the rabbit medullary thick ascending limb, the probability of open BK_{Ca} channels significantly increases when negative pressure is applied to a patch pipette in addition to a hypotonic stress (Taniguchi and Guggino 1989). This suggests that these channels may be stretch-activated or closely linked to a stretch-activated component that regulates channel function.

The intermediate conductance calcium-activated potassium channels (IK_{Ca}) are also named for their unitary conductance, which ranges from 20-80 pS (at 0 mV). These channels are weakly inwardly rectifying, voltage-independent and activated by nanomolar changes in

concentration of intracellular Ca^{2+}. This sensitivity to Ca^{2+} is mediated by calmodulin binding to the C-terminal domain. IK_{Ca} channels are efficiently blocked by charybdotoxin and clotrimazole, and virtually insensitive to apamin. These channels are typically found in epithelia and endothelium-rich tissues, which generally require an effective osmotic cell homoeostasis to support high rates of vectorial transport (Wehner and others 2003). Several reports indicate the involvement of this type of channel in hypotonically-induced K^+ release such as those in the human epithelial cell line Intestine 407, human T lymphocytes and transformed Madin-Darby canine kidney cells (Wehner and others 2003).

Small-conductance, calcium-activated potassium channels (SK_{Ca}) exhibit an even smaller conductance at around 4-18 pS at 0 mV. These channels are also activated by nanomolar concentrations of Ca^{2+}, are inwardly rectifying, and voltage-independent. Apamin efficiently blocks certain isoforms of SK_{Ca} channels (SK2) while others are blocked less effectively (SK3) or only marginally (SK1 & SK4) (Wehner and others 2003). DL-tubocurarine, however, is known to block all isoforms of SK_{Ca} with a high selectivity. In Mz-ChA-1 cells, hypotonic stress induces an efflux of K^+ contributed to the SK_{Ca} channels, which is partially blocked by apamin and completely blocked by Ba^{2+} suggesting that other K^+ channels are likely to be involved (Wehner and others 2003).

A voltage-gated K^+ channel, Kv1.3, has been identified as a potential mediator of RVD. This channel is a delayed rectifier that is blocked by tetraethylammonium (TEA), 4-aminopyridine, charybdotoxin, and dendrotoxin (Coetzee and others 1999). The most convincing evidence that Kv1.3 is involved in RVD comes from experiments with a murine T-lymphocyte cell line that lacks voltage-dependent K^+ channels and therefore does not exhibit RVD in the face of hypotonic challenge. Transfection of these cells with Kv1.3 allows them a complete volume regulatory response and a detectable voltage-activated K^+ current that can be blocked by charybdotoxin (Deutsch and Chen 1993). The contribution of Kv1.3 to the volume regulatory response has also been evaluated in human T-lymphocytes. In this case, margatoxin, which is highly specific for the Kv1.3, is able to suppress RVD at concentrations in the nanomolar range (Khanna and others 1999).

Like Kv1.3, Kv1.5 is a voltage-gated K^+ channel of the delayed rectifier group. Unlike Kv1.3, though, this channel is blocked by 4-aminopyridine, but not by TEA, charybdotoxin or dendrotoxin (Coetzee and others 1999). Transfection of this channel into the murine fibroblast

cell line Ltk- prevents swelling under hypo-osmotic conditions and generates a voltage-activated outward current of K^+ that can be abolished by the K^+ channel blocker quinidine (Felipe and others 1993). This channel is also involved in the cell volume decrease associated with apoptosis of pulmonary artery smooth muscle cells. In these channels, inhibition of outward K^+ currents with 4-aminopyridine can prevent apoptotic volume decrease and attenuate apoptosis (Ekhterae and others 2001).

The KvLQT1/I_{sK} potassium channel complex, also known as the minK channel, has been found to be significantly involved in RVD of tracheal epithelial cells in mice. These cells are generally insensitive to Ba^{2+}, apamin, and only slightly sensitive to TEA. Tracheal cells from knockout mice lacking the β-subunit (I_{sK}) are unable to regulate their volume during osmotic challenge. In tracheal cells with intact minK channels, clofilium, a blocker of the KvLQT1/I_{sK} complex, potently inhibits RVD (Lock and Valverde 2000).

A member of a more recently discovered family of K^+ channels, TASK2, has been associated with volume regulation in Ehrlich ascites tumor cells (Niemeyer and others 2001). TASK, which stands for TWIK (<u>t</u>andem of P domains in <u>w</u>eak <u>i</u>nward rectifier <u>K</u> channels)-related <u>a</u>cid-<u>s</u>ensitive <u>K</u>+ channel, forms two pores and seems to be involved in the background K^+ conductance detected in many cell types (Wehner and others 2003). These channels are generally insensitive to classic K^+ channel blockers such as TEA, 4-aminopyridine and Ba^{2+}, but are blocked by quinine and with a high potency by clofilium (Lesage and Lazdunski 2000). When TASK2 is expressed in HEK293 cells, the resulting K^+ currents are similar to those in Ehrlich cells and can be blocked by clofilium. Transcripts of TASK2, but not TASK1 and TASK3, have been identified in Ehrlich ascites tumor cells (Niemeyer and others 2001).

Measuring Cell Size

As the focus of this dissertation is the mechanism by which spermatozoa maintain a functional volume, techniques for determining sperm volumes are discussed. A number of methods for measuring changes in cell volume during RVD or RVI have been developed including those based on the principles of dye dilution, light scattering (flow cytometry), dynamic fluorescence quenching (DFQ), fluorescence self-quenching, cell height, morphometry, atomic force microscopy, laser scanning reflection microscopy, scanning conductance microscopy, ion-sensitive electrodes, and electrical sizing (impedance), nuclear magnetic

resonance(NMR) and electronic spin resonance (ESR) (Srinivas and others 2003). For advantages that will be discussed later, flow cytometry was chosen as the method by which to evaluate sperm volume for this research.

Of the listed methods, electronic cell sizing is the most widely used for measuring cell volume. A wide variety of cells have been analyzed using electronic cell sizing machines, usually a Coulter Counter, where the size of the particles is derived from the electrical impedance created when the cell passes between two electrodes and displaces the equivalent volume of electrolyte solution. As examples, the volumes of porcine blood platelets (Tomasiak and others 2005), human airway epithelial cells (Braunstein and others 2004), guinea pig colonic epithelial cells (Manabe and others 2004), newborn rat cardiomyocytes (Taouil and Hannaert 1999), human cervical cancer cells (Shen and others 1996), erythrocytes from a variety of species (Blank and others 1994, Weiser 1985), spermatozoa (see Petrunkina references in Chapter 3), human platelets (Livne and others 1987), and mitochondria from the ciliated protozoon *Tetrahymena pyriformis* (Poole 1983) have been estimated using electronic sizing. Nuclear magnetic resonance (NMR) and electron spin resonance (ESR) have also been widely used on cells such as human erythrocytes (Herrmann and Muller 1986), human spermatozoa (Du and others 1994), rat hippo-campal sections (Buckley and others 1999), frog epithelial cells (Civan and others 1988), and cells of the microalga *Phaeodactylum tricornutum* (Schobert and Marsh 1982).

Flow cytometry

Broadly defined, flow cytometry is a system for measuring and analyzing the signals that result as particles flow in a liquid stream through a beam of laser light (Givan 2001). This technology has a wide range of applications from cell sorting to detection of cell surface proteins that can help in clinical diagnosis of disease. Although flow cytometers vary greatly in the cellular characteristics that they are capable of measuring, all cytometers have three common elements: a light source that can be focused, fluid lines (columns through which fluids including the cell suspension will flow) and controls that direct the fluid through a beam of light, and an electronic network for detecting the light signals resulting from the intersection of the particles with the light and then converting these signals to numbers that are proportional to light intensity (Givan 2001).

Because flow cytometry has developed gradually with the slow convergence of several different areas of scientific study, the date of the first cytometer cannot be defined. Some of the earliest accounts of this technology's being applied to measuring cell size involve the counting and sizing of red blood cells (Coulter 1956, Fulwyler 1965). The machines used in these experiments are the predecessors of modern day flow cytometers, but the basic theory is the same. The types of biological cells that have been studied by flow cytometric techniques are too numerous to list. The important point for this dissertation is that this technique is a valid method for measuring cell size and thus estimating relative cell volume.

The process of measuring cellular characteristics with a flow cytometer begins with a laser beam. Lenses within the cytometer are used to focus the laser and adjust the diameter of the beam at the point of intersection with the fluid suspension of particles. The suspension is a combination of sheath fluid, a buffer that is typically a phosphate-buffered saline, and the cell suspension to be analyzed. These fluids combine within the flow chamber. The cell suspension is injected into the center of the sheath stream after which both streams of fluid are accelerated through a narrowing channel. This hydrodynamic focusing keeps the cell suspension in a central core as it flows within the sheath stream (Fig. 2.5) as it exits the flow chamber.
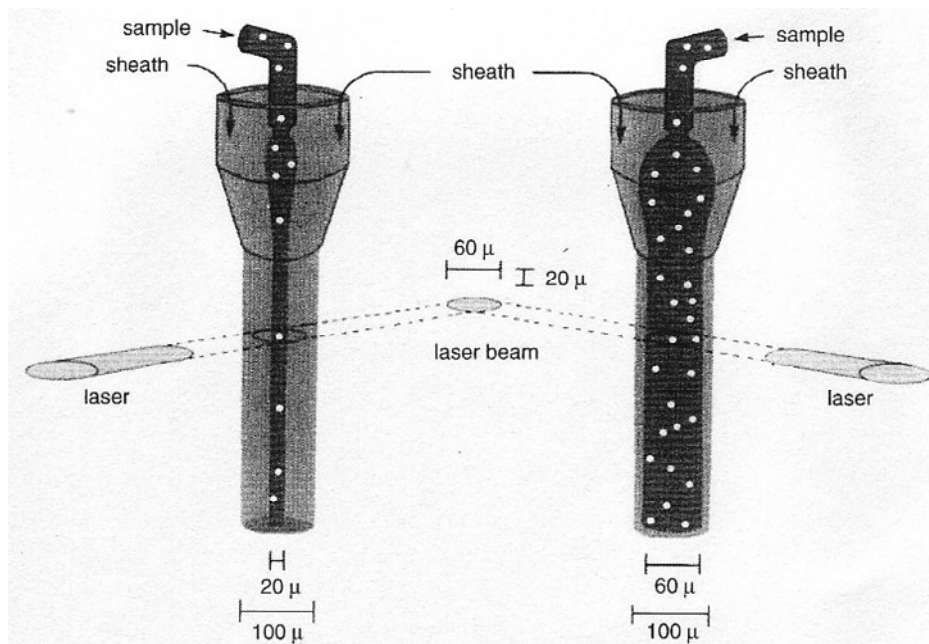
Figure 2.5. The flow of sheath fluid through the analysis point (where the laser meets the core of fluid) when injected at relatively slow (left) or fast (right) speeds. Increased flow rate causes the diameter of the core to increase, potentially catching more than one particle at the analysis point simultaneously, resulting in inaccurate values (from Givan 2001).

The stream of fluid then transects the focused laser beam perpendicularly at a point referred to as the analysis, observation, or interrogation point. The emerging light, or signal, is collected by lenses positioned around the analysis point. These lenses focus the light onto photodetectors, photodiodes or photomultipliers that convert the light signals into electric impulses that are proportional to the intensity of the light received by them. Typically there are four or more photodiodes around the analysis point. This includes one in the direct line of the laser, on the opposite side of the analysis point used to detect forward scattered light, and three or more photomultipliers at right angles from the laser beam that detect side scattered light of the same or different colors from that of the illuminating beam. Specific filters are installed on the photodiodes to ensure that signals are generated only when light of the color of interest is detected.

The term forward scatter light (FSC) can be misleading as it is not light that passes directly through a particle without deflection. Rather, it is light of the same color as the illuminating beam that has been deflected to a small angle from the direction of the original beam (Givan 2001). Placed in front of the forward-angle photodetector is an obscuration bar which prevents undeflected light from reaching the detector. However, if light has been deflected it will bypass the obscuration bar, strike the diode, and generate a signal. This signal is recorded as FSC. These signals are not direct measures of volume or size but are related to the refractive index and cross-sectional area of the particles. Thus, it can distinguish between classes of particles (i.e. larger versus smaller) because larger particles will deflect more light than smaller particles of the same refractive index (Givan 2001).

Light coming out of the analysis point at right angles will be a mixture of colored light that may or may not be of the same wavelength (color) as the illuminating beam. Light that is the same wavelength as the illuminating beam is referred to as side scatter light (SSC). This light is detected when something in the flow stream causes the beam to be deflected to the side. This light is therefore a reflection of particle size, shape, and surface texture as well as internal structure. The rougher or more irregular a particle is, the more it will scatter light to the side. Light that differs in wavelength from SSC may be emitted from a cell if it contains an endogenous fluorescent compound or if it has been intentionally exposed to a fluorescent stain. This fluorescence will be registered by one of the photodetectors equipped with a filter specific for that color.

The electric signals that leave the photodiodes are amplified and then recorded by an analog-to-digital converter (ADC). The ADC evaluates the continuous distribution of signals and groups them into discrete ranges. The ADC comprises channels that represent a specific light intensity range. The number of channels that an ADC has, is determined by the operator who adjusts the photodetector voltage and amplifier gain. Typically there are 1024 channels, although there can be more or fewer. The output data are recorded by the cytometer electronics as light intensities on a scale of 0 to 1023 (for a 1024 channel ADC). These values are analyzed and used to compare the different groups of particles that may be defined by the results of the experiment.

Validity of using flow cytometry to measure cell size

Methods of measuring cell size by flow cytometry have been corroborated against established cell-sizing methods. Bovine corneal endothelial cell size was estimated during a series of osmotic challenges, both hypertonic and hypotonic, by flow cytometry and measurements of cell heights under microscopy. In addition, predicted volume changes caused by exposure to ionophores were consistent between flow cytometry results and cell height measurements. During osmotic challenge and exposure to ionophore, flow cytometric and cell height measurements were in agreement. Flow cytometry also had a higher sensitivity to rapid volume change than the traditional method of measuring cell height (Srinivas and others 2003).

The use of flow cytometry for estimating sperm cell volume has only recently been validated. In the past, methods for measuring sperm cell volume included electronic sizing by a Coulter counter, calculation from spermatocrit (packed cell volume), stereology by electron microscopy, volume exclusion methods using differential radioisotope labeling of intracellular and intercellular spaces, and estimation from the concentration of entrapped fluorophore (Yeung and others 2002). To validate the use of flow cytometry for measuring relative sperm volumes, cauda epididymidal spermatozoa from heterozygous mice were exposed to hypo- and hyper-osmotic conditions with and without the presence of quinine, an agent that prevents volume regulation, and their volume changes gauged by flow cytometry and electronic sizing (Yeung and others 2002). A high linear correlation existed between measurements obtained with these two methods, including distinct subpopulations. In addition, the difference in size between the heterozygous spermatozoa, which can regulate volume, and spermatozoa from c-

ros knockout mice, which cannot, was clearly reflected by forward scatter measurements. This validation was also performed with human spermatozoa, where both flow cytometry and electronic sizing detected comparable decreases in cell size when spermatozoa were treated with Triton, exposure to which causes loss of plasma membrane and cytoplasm (Yeung and others 2003). The strong correlation between the flow cytometric measurements and the volumes estimated by the previously established method of electronic sizing validate the use of this technique for estimating sperm volume.

Advantages of flow cytometry

One of the most important advantages of flow cytometry over electronic sizing is its ability to distinguish between cells with intact and disrupted plasma membranes (an index of their viability) by the use of fluorescent dyes. In a Coulter counter, which measures size from the electrical impedance, the signal is proportional to the volume and electrical characteristics. Intact cells and the nuclei of disrupted cells appear as separate peaks but could be easily confused with debris. For spermatozoa without much cytoplasm, it is often routine to use a separate method for detecting cell viability, with electronic sizing. However, the use of flow cytometry eliminates the need for two methods (one for measuring cell size and the other for viability) as it can assess both parameters for a single spermatozoon simultaneously. In a flow cytometer, cells with leaky plasma membranes have a refractive index closer to that of the surrounding medium and will therefore create dimmer forward scatter signals (Givan 2001), therefore it is usual to introduce a fluorescent membrane-impermeant dye so that non-vital cells are marked and can be eliminated from analysis by gating them out electronically.

Regulation of cellular volume has been well studied in somatic cells. Fortunately, many of the methods used to investigate volume regulation in somatic cells can be modified for spermatozoa. The following chapter will discuss the importance of volume regulation for spermatozoa with specific reference to monkeys, humans, and mice.

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Chapter 3

Regulation of Volume by Spermatozoa

The ability to maintain and regulate cellular volume is one of the most fundamental cell homeostatic mechanisms. It is critical to normal function and survival of all cell types studied. While it has been generally accepted that spermatozoa have mechanisms for adjusting their volume at times of osmotic stress, the physiological significance of volume regulation for sperm function has only recently been appreciated.

Significance of Volume Regulation

While most evidence for the necessity of sperm volume regulation for fertility is gathered from experiments where volume regulation is intentionally inhibited, the earliest evidence was presented in case studies of natural infertility in domestic stock. The observations made on spermatozoa from these infertile animals were recorded but forgotten until similar seminal profiles were observed in transgenic mice. Altogether, the evidence from both natural and induced cases of infertility confirms that volume regulation is essential for fertility, especially during natural mating.

Male infertility in domestic species

Cases of natural infertility can often highlight previously unstudied, yet essential aspects of gamete function. The Dag defect was first reported in a single Jersey bull, after which the syndrome was named. Spermatozoa collected from this bull were sharply angulated at the midpiece/principal piece junction (the site of the cytoplasmic droplet, Blom 1966). Upon further investigation with ultramicrographs, the tail was found to be coiled or rolled up within a single intact plasma membrane. Flagellar angulation and coiling can occur during severe hypo-osmotic events which exceed the volume regulatory capacity of the cell (Drevius 1963).

This defect was later observed in other breeds of cattle, boars, mongrel dogs, and stallions (Cooper and Barfield 2006). For most bulls, increasing the ejaculation frequency significantly reduced the percentage of coiled spermatozoa in semen as the distal caudal sperm reserves were depleted and unaffected spermatozoa from the proximal cauda contributed to the

ejaculate; some other bulls appeared to have more proximally affected spermatozoa (Swanson and Boyd 1962, Hopwood and others 1963, Gustafsson and others 1972, 1974). In addition, fixed spermatozoa from several epididymal regions of bulls and boars with the defect showed that in some males the sperm tails were straight until the spermatozoa entered the distal cauda, whereas in others, tail coiling occurred within segments from the proximal cauda to the distal caput (Cooper and Barfield 2006). This suggested that the defect resulted from an epididymal dysfunction, and while it received some attention at the time, further interest waned until the infertility of the transgenic c-*ros* tyrosine kinase knockout mice was discovered.

Male infertility in transgenic mice

The c-*ros* knockout mice were initially studied because the *ros* gene is an oncogene expressed during organ development.   It was isolated from the oncogene of the chick retrovirus UR2 and a human isolate identified in a transfection tumorgenicity assay (Neckameyer and Wang 1985). The chick and human isolates encode truncated proteins that lack a c-*ros*-specific extracellular domain. The protein is an "orphan" receptor (the ligand is unknown) and is expressed in the Wolffian duct and thus in some of the derivatives of the Wolffian duct, such as the epididymis. Targeted mutations of the c-*ros* gene by homologous recombination in embryonic stem cells were used to generate mice carrying the mutation (Sonnenberg-Riethmacher and others 1996). Female mice were fertile, but the male mice, although healthy, were infertile as a consequence of the failure of the initial segment of the epididymis to develop (Sonnenberg-Riethmacher and others 1996, Yeung and others 1999). Despite their infertility, male knockout mice exhibited normal mating behavior and could be used to stimulate pseudopregnancy in female mice and the cauda epididymidal spermatozoa were able to fertilize oocytes *in vitro* (Sonnenberg-Riethmacher and others 2006). Upon exposure to female tract fluids, however, these spermatozoa swelled and angulated or formed hairpin bends (displayed flagellar angulation of 180°) at the site of the cytoplasmic droplet, and were unable to migrate to the site of fertilization (Yeung and others 2000). By contrast, spermatozoa from wild-type mice, or fertile heterozygous mice, under the same hypo-osmotic challenge perform regulatory volume decrease (RVD) to maintain their volume and a straight flagellum.

For spermatozoa from the c-*ros* tyrosine kinase knockout mice, their journey ends at the uterotubal junction (Yeung and others 2000). Post-coital assessments of sperm motility and

numbers of spermatozoa in the uterus 1 hour after mating were found to be similar between females mated with knockout and wild-type or heterozygous males. Flushing of the oviducts 4 hours after mating revealed that females mated to wild-type or heterozygous mice had high numbers of spermatozoa (>1000 on average) in the oviducts while females mated to knockout mice were devoid of spermatozoa in the oviducts. Histology confirmed the absence of spermatozoa from the uterotubal junction (Yeung and others 2000).

Another transgenic mouse model that produces spermatozoa with swollen morphologies is the GPX5-Tag2 mice (Cooper and others 2004). Glutathione peroxidase 5 (GPX5) is a secretory protein that is strongly expressed in the distal caput region of the epididymis and lightly expressed in the cauda (Lahti and others 2001). The expression of this protein is controlled by androgens, and it has been shown to bind to the acrosome during sperm transit through the epididymis (Jimenez and others 1990, Vernet and others 1997). GPX-Tag2 transgenic mice were created by using the promoter of the GPX5 gene to direct the SV 40 T-antigen to the caput epididymidis in an attempt to disable caput epididymidal function. Epididymal proteins specific to the initial segment (mEP17, CRES, and HE6) were down-regulated in these mice as a result of a change in function of the initial segment. Spermatozoa from these males can neither reach the site of fertilization nor fertilize oocytes *in vitro* (Sipilä and others 2002). When spermatozoa from these mice are released into conventional culture medium, the flagellum displays angulation, as in the c-*ros* KO mice, an indication of swelling (Sipilä and others 2002, Yeung and others 2002). In this model, unlike the c-*ros* knockout mice, the angulation occurs within the epididymis and is maintained upon release (Yeung and others 2002). These examples of infertility, natural and transgenic, emphasize the importance of volume regulation for fertilization.

Volume regulation as a target for contraception

Because volume regulation is a necessary function, it may be a valuable target for male-directed contraception. There are several potential contraceptive approaches (Cooper 1992). At the epithelial level of the epididymis, synthesis and transport of osmolytes that are used by spermatozoa during volume regulatory events could be inhibited. Alternatively, a drug could be designed to disrupt or neutralize the osmolytes in the epididymal lumen thereby preventing the spermatozoa from acquiring them. It may also be possible to target the spermatozoa

themselves by creating a drug that blocks the channels that take up and/or release osmolytes during osmotic stress. Each of these options requires an understanding of the volume regulatory process in spermatozoa, including which channels may be involved and which ions and osmolytes are integral to the osmotic response.

Need for Volume Regulation

Spermatozoa need to perform volume regulation because of the different osmolalities of fluids in the male and female tract. Spermatozoa are subjected to osmotic challenges experimentally in order to examine the mechanisms involved but the source of the spermatozoa and the way they are handled prior to measurement can influence the results obtained.

Osmotic differences in male and female reproductive tract fluids

Volume regulation is necessary for spermatozoa because of the various osmolalities to which they are exposed. While spermatozoa are in the rete testis, the surrounding fluid is approximately 290 mmol/kg (Yeung and others 2006). As the spermatozoa migrate through the epididymis, they are exposed to fluids of increasing osmolality that can reach higher than 400 mmol/kg in the hamster and mouse (Cooper and Yeung 2003). The spermatozoa are held at this high osmolality in the cauda epididymidis until ejaculation. For murine spermatozoa, the difference in extracellular osmolality at ejaculation is approximately 100 mmol/kg, as the female post-coital uterine contents are approximately 330 mmol/kg (Yeung and others 2000). Human spermatozoa face a similar, albeit less severe, decrease in osmolality, as they move from the vas deferens (342 mmol/kg, Hinton and others 1981), through cervical mucus (287 mmol/kg, Rossato and others 1996), uterine fluid (284 mmol/kg, Casslen and Nilsson 1984), and into oviductal fluid (274 mmol/kg, Menezo and others 1982). There are no data for the osmolalities of male and female tract fluids in monkeys. Failure to meet these osmotic challenges can prevent spermatozoa from reaching the site of fertilization.

Experimental hypotonic challenges

The study of volume regulation is complicated by osmotic changes that can occur during the collection of spermatozoa. Of primary importance is minimizing these challenges and ensuring that the spermatozoa are not faced with non-physiological osmotic challenges during

the collection process as this could result in improper volume regulation during the experimental osmotic challenge. Data collected under improperly managed conditions would be difficult if not impossible to interpret. Studies in which spermatozoa are pre-incubated with inhibitory drugs before exposure to hypo-osmolal solutions are limited by the knowledge of the true isotonicity of spermatozoa, as the osmotic events under study may occur during this pre-incubation and thus be missed during the experiment. In this dissertation, spermatozoa were exposed to the inhibitory drugs and a hypo-osmolal challenge simultaneously.

Ideally spermatozoa will not be exposed to a change in their osmotic environment before the experimental osmotic challenge. However, the source of the spermatozoa (epididymal or ejaculated) should influence how spermatozoa are handled prior to experimentation. Studies of murine and monkey epididymal spermatozoa have mimicked nature, in that epididymal contents were transferred immediately into medium of the osmolality of the female tract. This constitues a large osmotic challenge, from 430 mmol/kg in the cauda epididymidis (Yeung and others 1999) to 330 in the uterus (Yeung and others 2000). In other experiments, porcine epididymal spermatozoa (normally bathed in 333 mmol/kg, White and MacLeod 1963) were first transferred to a semen extender (around 300 mmol/kg) and then washed through Percoll (also 300 mmol/klg) before measurements of osmotic response were assessed (Petrunkina and others 2001). The problem with this experimental design is that the spermatozoa may have already responded osmotically before measurements are made when subjected to further hypotonic treatment of another ~150 mmol/kg.

The problem of pre-experimental osmotic challenges is unavoidable when dealing with ejaculated spermatozoa, which are merely epididymal spermatozoa that have already been exposed to a hypotonic insult during ejaculation (see above). For bovine semen (osmolality 340-355 mmol/kg, White and MacLeod 1963, Petrunkina and others 2001) spermatozoa are either subjected to "swim up" in medium around 310 mmol/kg or are diluted in an extender (around 300 mmol/kg) and subsequently centrifuged through Percoll density gradients at around 300 mmol/kg. Sometimes sperm-rich fractions are selected for study, especially for boars (semen osmolality 333 mmol/kg, Petrunkina and others 2001, 2004a) and dogs (semen 317 mmol/kg, Petrunkina and others 2004b). For boars, transfer of spermatozoa to extender and washing through Percoll at 300 mmol/kg is also done, and for dogs only Percoll washing. Again, the

osmotic responses observed may be less marked than those occuring unobserved during sperm preparation.

Several experimental designs for human ejaculated spermatozoa (coming from the vas deferens at 342 mmol/mg, Hinton and others 1981) exist: sperm are washed (Yeung and others 2003) or subjected to density gradient centrifugation through Percoll made up in media of the average osmolality of liquefied seminal plasma (330 mmol/kg, Yeung and Cooper 2001), the individual's own seminal osmolality (Barfield and others 2005, Yeung and others 2005a, Klein and others 2006) or that of the female tract (290 mmol/kg, Fetic and others 2006). In each case, the osmotic status of the spermatozoa at the time of measurement may not have been identical, but control experiments of spermatozoa subjected to the same anisotonic solutions without inhibitors were performed.

Current Knowledge of Sperm Regulatory Volume Decrease

Development of RVD potential

The ability of spermatozoa to undergo RVD is acquired in the epididymis (Yeung and others 1999, 2002b, 2004b). Murine caput spermatozoa appear to have no capacity for RVD, as swelling after a hypotonic challenge is not reversed. Quinine also does not elicit a response from the caput sperm whereas in cauda spermatozoa a dose-dependent increase in size is observed (Yeung and others 2002). Spermatozoa from cynomolgus monkeys also show a maturation of RVD. Cauda spermatozoa are able to recover from swelling after 20 minutes of exposure to hypotonic conditions; corpus spermatozoa show a less extensive recovery, and caput spermatozoa do not exhibit an osmotic response (Yeung and others 2004b). These differences could be due to changes in the plasma membrane lipid composition that is associated with maturation in the epididymis (Jones 1999), changes which are known to alter ion channel activities (Brown and London 1998) as well as osmolyte content of the cell.

Role of potassium ions in sperm volume regulation

Ion channels are extremely important to spermatozoa in that the channels are key elements in the communication between the sperm cell, its environment, and the egg (Darszon and others 1999). Evidence from several studies has suggested the involvement of various ion channels in sperm RVD, especially K^+ channels. It is known that luminal concentrations of K^+

increase along the length of the epididymis, giving spermatozoa the opportunity to accumulate this osmolyte (Turner 2002) and the concentration of intracellular K^+ in spermatozoa is high (120 mM for bulls, Chou and others 1989, Babcock 1983; 80-100 mM for mice, Zeng and others 1995).

The first description of RVD in mammalian spermatozoa was offered by Kulkarni and others (1997). In that report, many inhibitors and ionophores were used to determine which types of channels may be involved in a volume regulatory response by bovine spermatozoa. Quinine was used to prevent the efflux of K^+ during a hypo-osmotic challenge which induced swelling in spermatozoa, and valinomycin, a K^+ ionophore, was used to reverse the hypo-osmotic swelling. Subsequent studies also implicated K^+ as a key osmolyte for sperm RVD. Quinine-sensitive ion channels were detected in human, murine, canine and porcine spermatozoa and confirmed in bovine spermatozoa (Yeung and Cooper 2001, Yeung and others 2002, Petrunkina and others 2001, 2004b).

Measurements of K^+ within and surrounding spermatozoa have been recorded. Luminal K^+ concentrations are high in the epididymis and increase from the caput epididymidis to the cauda epididymidis in the rat, boar, and hamster but decrease in the bull, ram, and man (Turner 2002, Cooper 1986). Intracellular K^+ concentrations of spermatozoa may also change during epididymal transit. In rabbit spermatozoa, K^+ concentrations increase in the sub-acrosomal space from 0.3 mM in the caput to 7.4 mM in the cauda, in the equatorial segment from 0.6 mM to 9.2 mM, in the midpiece from 0.5 mM to 6.8 mM, and in the principal piece from 0.4 mM to 0.5 mM (Oliphant and others 1985). Similarly, ovine spermatozoa contain 49.8 mM K^+ while in the corpus and 64 mM in the distal cauda, and porcine spermatozoa contain 64.1 mM K^+ in the corpus and 78.1 in the distal cauda. In contrast, intracellular K^+ concentrations of bovine spermatozoa decrease from the caput to the distal cauda, dropping from 75 mM to 49 mM, respectively (Cooper 1986). These concentrations are much higher than what is reported for blood plasma (4.6 mM).

Judged by the significant role of potassium in volume regulation by somatic cells and the high concentration of K^+ in spermatozoa, a role for K^+ in regulatory volume decrease of sperm cells is likely. The high concentration of potassium could provide the spermatozoa with sufficient ions for expenditure during physiological osmotic challenges. Indeed, studies in mice (Yeung and others 1999), bulls (Kulkarni and others 1997, Petrunkina and others 2001), boars (Petrunkina and others 2001), humans (Yeung and Cooper 2001, Yeung and others 2003), dogs

(Petrunkina and others 2004b), and monkeys (Yeung and others 2004b) have demonstrated a sustained increase in size when spermatozoa are exposed to hypotonic media in the presence of quinine, a broad-spectrum K^+ channel blocker. The reversal of the effects of quinine by the K^+ ionophore valinomycin demonstrated in bovine (Yeung and others 2003, Petrunkina and others 2001), murine (Yeung and others 2005b), and human (Yeung and Cooper 2001) spermatozoa further supports the involvement of K^+.

Role of cation and anion channels in sperm volume regulation

Potassium channels have been previously studied in spermatozoa as the channels are believed to play a role in the events that prepare a spermatozoon for fertilization, and several have been identified in spermatozoa, spermatogenic cells and testicular tissue (Fig. 3.1). Voltage-gated channels Kv1.1 and Kv1.2 have been localized to the principal piece and head, Kv3.1 to the annulus and Girk1 to the connecting piece of murine epididymal spermatozoa, and the presence of the mRNA of Kv1.1, 1.2 and 3.1 confirmed (Felix and others 2002). Slo3, a potassium channel regulated by pH and membrane voltage, is expressed in seminiferous tubules by developing spermatocytes in humans and mice (Schreiber and others 1998). Large-conductance Ca^{2+}-activated K^+ channels are expressed in germ cells but undergo a down-regulation in post-meiotic germ cells that coincides with an up-regulation of voltage-gated potassium channels (Gong and others 2002). Voltage-gated, delayed, outwardly-rectifying K^+ (probably Kv1.3) channels are also expressed in the cytoplasm of primary spermatocytes and post-meiotic elongating spermatids in rats (Jacob and others 2000) and the inwardly rectifying (Kir) channel in spermatozoa from mice (Munoz-Garay and others 2001). Complete inhibition of these channels by barium (Ba) prevents membrane hyperpolarization which may indicate a role for the Kir channels in capacitation by enhancing K^+ permeability (Munoz-Garay and others 2001). Specifically, Kir5.1 is expressed in the rat testis, and the protein has been detected in the seminiferous tubules, on spermatogonia, primary and secondary spermatocytes, spermatids, and the head and tail of spermatozoa from rats (Salvatore and others 1999).

To maintain electro-neutrality, the loss of cations through channels needs to be balanced by efflux of anions and the involvement of anion channels in spermatozoa has also been reported. In murine and human spermatozoa, RVD can be prevented by broad spectrum anion channel blockers. Although one specific anion channel involved in RVD of somatic cells, ClC-3,

was found in human sperm extracts, it was not detected in murine sperm extracts (Yeung and others 2005a,b). A volume sensitive anion and osmolyte channel (possibly ClC-3) was described for boar spermatozoa (Petrunkina and others 2004a).
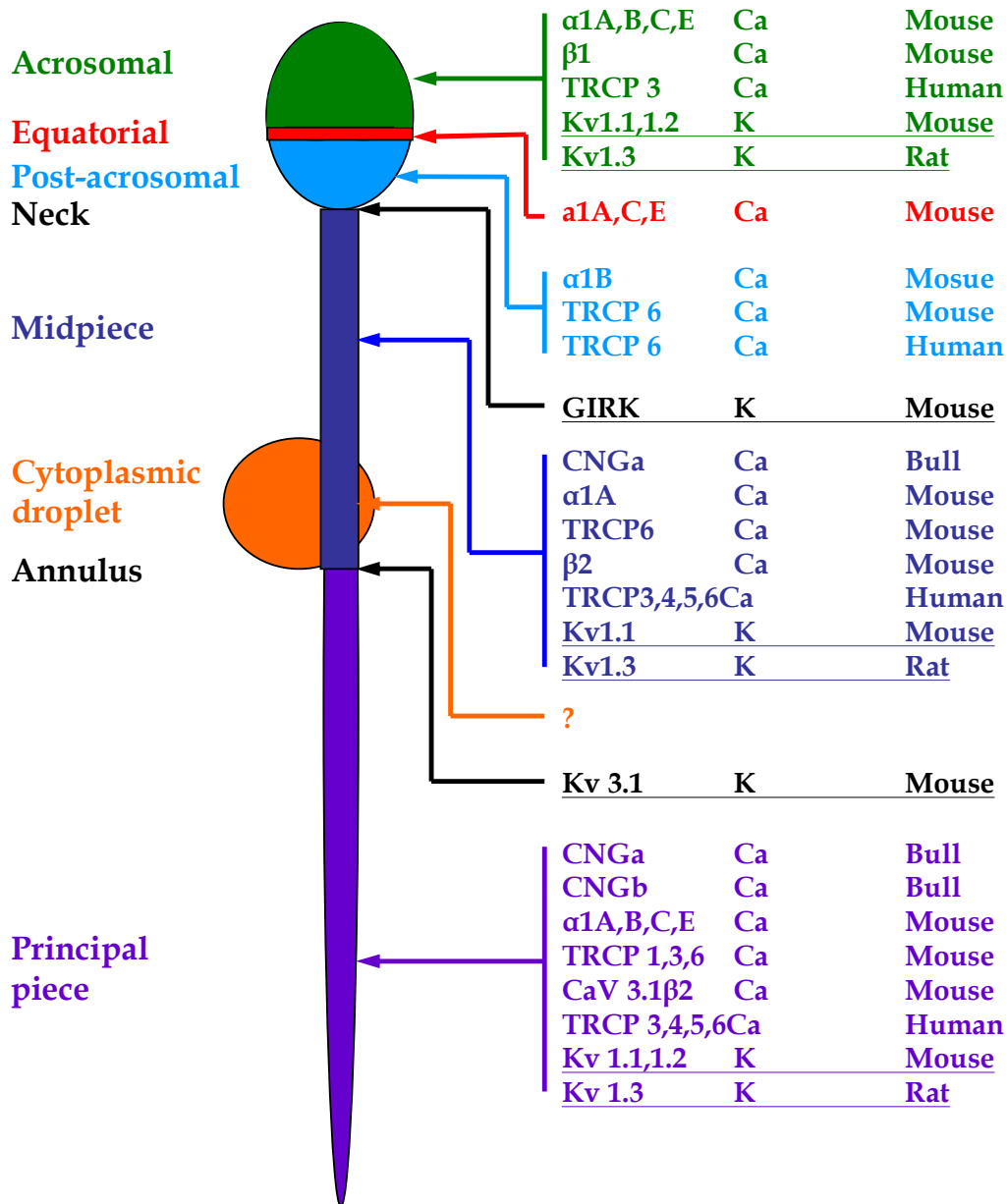
Figure 3.1. Representation of potassium (K) and calcium (Ca) channels that have been idenfied on different regions of spermatozoa from various species. Channels that are underlined have been reported to be involved in volume regulation of somatic cells. CaV, voltage-gated Ca channel; CNG, cyclic nucleotide-gated channel; GIRK, G-protein inwardly rectifying K channel; Kv, voltage-gated potassium channel; TRCP, transient receptor potential cation channel.

As discussed for somatic cells, the role of organic osmolytes in volume regulation of spermatozoa is currently being investigated. As spermatozoa lack the machinery to produce these osmolytes, they must depend on uptake from the environment, most likely in the epididymis. As reviewed by Lang and others (1998), in contrast to the rapid uptake of ions, osmolyte accumulation occurs over a matter of hours or even days. Within the epididymis, there are several low molecular weight, water-soluble compounds present at high concentrations that could act as osmolytes, including L-carnitine, glycerophosphocholine and *myo*-inositol (Cooper, 1986, 1998) and spermatozoa take several days to traverse the epididymis, allowing ample time for these osmolytes to be accumulated. Evidence from murine spermatozoa suggests that the epididymal secretions glutamate, L-carnitine, taurine, sorbitol and *myo*-inositol have the potential to be utilized for volume regulation. The addition of these osmolytes at concentrations considered to be equal to that of intracellular concentrations to hypotonic medium eliminates the concentration gradient that would normally drive the osmolyte efflux response during RVD. Because this gradient was lacking, the osmolytes were unable to leave the cell. Thus, RVD was inhibited resulting in a sustained increase in sperm size (Yeung and others 2004a, 2006).

The accumulation of organic osmolytes for sperm volume regulation may occur through isovolumetric regulation, by which the cells gradually take up osmolytes available to them in the epididymal lumen when they encounter the increasing osmolality generated by the secretion of osmolytes by the epididymis (Cooper and Yeung 2003). This can be a lengthy process taking up to 5 days in the mouse (Dadoune and Alfonsi 1984), which parallels the length of time for somatic cells to acquire organic osmolytes. In this situation, changes in cell volume would be undetectable yet there would be a continual increase in intracellular concentrations of osmolytes. The osmolytes acquired in the epididymis could then be used in the subsequent volume regulation required by the spermatozoa encountering hypotonicity during their transition from the epididymis to the site of fertilization.

Isotonicity and Potential RVD Osmolytes

The response of a spermatozoon to an osmotic challenge depends on its intracellular tonicity. However, there are very few studies that attempt to measure intracellular isotonicity, and it is likely that there are differences even between closely related species. In most studies,

isotonicity is assumed to fall between 280-300 mmol/kg for a wide variety of species (Table 3.1). From a review of literature on osmotic tolerance limits of spermatozoa, the lowest presumed isotonicity is that of frog spermatozoa (220 mmol/kg) and the highest for the domestic turkey (370 mmol/kg). In each paper, the osmolality of the "isotonic" solution is given but without reference to scientific evidence of the intracellular isotonicity of the spermatozoa of that species. If the true osmotic challenges and subsequent volume regulation of spermatozoa are to be understood, the intracellular isotonicity of spermatozoa from each species must be evaluated as there are likely to be significant differences which would affect interpretation of data and potentially cryopreservation success.

Common Name	Scientific Name	Presumed Isotonicity (mmol/kg)	Reference
imperial eagle	*Aquila adalberti*	300	Blanco and others 2000
golden eagle	*Aquila chrysaetos*	300	Blanco and others 2000
domestic cattle	*Bos taurus*	290	Guthrie and others 2002
domestic dog	*Canis lupus familiaris*	290	Songsasen and others 2002
common horse	*Equus caballus*	325	Ball and Vo 2001
peregrine falcon	*Falco peregrinus*	300	Blanco and others 2000
domestic cat	*Felis silvestris*	300	Pukazenthi and others 2002
domestic chicken	*Gallus domesticus*	313	Blanco and others 2000
Bonelli's eagle	*Hieraaetus fasciatus*	300	Blanco and others 2000
human	*Homo sapiens*	286	Gao and others 1993
serval	*Leptailurus serval*	300	Pukazenthi and others 2002
rhesus macaque	*Macaca mulatta*	300	Rutllant and others 2003
		285	Agca and others 2005b
domestic turkey	*Meleagris gallopavo*	371	Blanco and others 2000
clouded leopard	*Neofelis nebulosa*	300	Pukazenthi and others 2002
chimpanzee	*Pan troglodytes*	290	Agca and others 2005a
koala	*Phascolarctos cinereus goldfuss*	300	Johnston and others 2006
leopard frog*	*Rana pipiens*	220	Costanzo and others 1998
wood frog*	*Rana sylvatica*	220	Costanzo and others 1998
boar	*Sus scrofa domesticus*	290	Gilmore and others 1998
common wombat	*Vombatus usrinus shaw*	300	Johnston and others 2006
mice*	*Mus musculus*, various	290	Willoughby and others 1996
	strains	280-300	Walters and others 2005
		290	Koshimoto and Mazur 2002

Asterisks (*) indicate measurements on epididymal rather than ejaculated spermatozoa.

Table 3.1. List of species and the presumed isotonicity of spermatozoa in studies on osmotic tolerance limits.

The osmotic tolerance limits of spermatozoa from the domestic chicken, domestic turkey, golden eagle, Bonelli's eagle, imperial eagle, and peregrine falcon have been compared (Blanco and others 2000). The spermatozoa from raptors were more tolerant of hyperosmolality with 91 to 96.8% "viable" (as determined with the SYBR-14/propidium iodine stain technique) after a 10 minute exposure to a 3000 mmol/kg solution. Peregrine falcon spermatozoa were extremely intolerant of hypo-osmolality (50 mmol/kg) with only 2.8% viability while golden eagle spermatozoa were more tolerant with 76.3% viable. Spermatozoa from Bonelli's eagle were the most tolerant of hyperosmolality with 96.8% viability after 10 minutes at 3000 mmol/kg, which was statistically different from golden eagle spermatozoa with 91% viability. It is clear that even between raptors there are differences in osmotic tolerances which could indicate a difference in sperm isotonicity resulting in varying degrees of osmotic challenge for spermatozoa from different species when exposed to the same hypo- or hyper-osmotic solution.

Osmolyte load and volume regulation

These osmotic tolerance limits may be related to differences in amount or type of organic osmolytes at the cell's disposal. Spermatozoa from the infertile c-*ros* knockout mice have reduced intracellular concentrations of carnitine (19% less), taurine (23%), *myo*-inositol (29%), and glutamate (45%) compared with spermatozoa from wild-type mice (Xu and others 2003, Yeung and others 2004a). Failed volume regulation by these spermatozoa may be the outcome when there are not enough osmolytes within the cell to counter large swelling events. Spermatozoa from GPX5-Tag2 mice, which are also unable to regulate volume, are bathed in a fluid of lower osmolality while in the cauda epididymidis compared with that of wild-type mice of the same strain, which may cause the observed sperm swelling in the epididymal canal. There are also differences in the luminal concentrations of the inorganic osmolyte K^+ in c-*ros* and wild-type mice. A higher concentration of K^+ was the only difference detected in the cauda epididymidal fluid of knockout mice compared to that of wild-type mice. Yeung and others (2004a) speculate that if exposure to higher concentrations of extracellular K^+ leads to K^+ uptake, as occurs in others cells, then this accumulation of K^+ may inhibit the accumulation of other necessary organic osmolytes. The absence or decrease of osmolytes in the epididymal fluid may prevent the sperm from accumulating the necessary amounts of osmolytes for responding to osmotic challenges and thus lower their osmotic tolerance limits.

In somatic cells, the predominant osmolyte used during volume regulatory events varies with cell type and magnitude of the osmotic challenge. Therefore, deficiencies of just one osmolyte may pose problems during volume regulation. For example, rabbit inner medullary collecting duct cells lose betaine when the change in extracellular osmolality is approximately 200 mmol/kg, and lose GPC when that difference is approximately 400 mmol/kg. Inositol, taurine, and sorbitol are released for the osmotic challenges between 200 and 400 mmol/kg (Kinne 1998). Cardiomyocytes release taurine in response to large osmotic challenges and primarily K^+ when the change in osmolality is less severe and more gradual (IVR) (Souza and others 2000). In contrast, hippocampal tissue cells use taurine for IVR and glutamate, taurine, and K^+ during RVD (Franco and others 2000).

Freezability and osmolyte load

If osmolyte imbalance affects RVD response and thus fertility, then it may play a role in the different freezabilities of spermatozoa from different strains of mice. Poor post-thaw fertility as judged by *in vitro* fertilization of C57BL/6N mice oocytes was first published by Tada and others (1990) where the percentage of fertilized eggs was 12.9% compared to other strains (DBA/2N 63.5%, ddY 48%, C3H/HeN and ICR 35%). Subsequently, Songsasen and Leibo (1997) found that simple exposure of spermatozoa to cryoprotectant reduced the fertilization rates in two strains of mice (129/J and C57BL/6J) but not in a third strain (B6D2F1). In cryopreservation experiments, the B6D2F1 spermatozoa also had higher fertilization rates in post-thaw IVF tests (61.2%) compared with the other two strains (17.2% for 129/J and 3.0% for C57BL/6J). Further experiments showed that these differences were mainly attributable to differential sensitivity to the osmotic shocks associated with the addition and removal of cryoprotectant. Because the freezabilities of spermatozoa are different for the different strains (and poorer results were produced with inbred strains of mice), this suggests a genetic basis for osmotic tolerance and post-thaw survival. Other studies that reveal a potential genetic component to freezabilities have been made on spermatozoa from cattle, humans, horses, and pigs (Beatty and others 1976, Kramer and others 1993, Amann and Pickett 1987, Gilmore and others 1996, respectively).

In addition to the differences in freezability of spermatozoa from closely related species or different strains of mice, there are also documented reports of individual differences.

Anecdotal evidence confirms individual differences in dogs, bulls, boars, stallions, and humans (Holt 2000). Applicable research has been done in several species including bulls (Beatty and others 1976), mice (Tada and others 1990, Songsasen and Leibo 1997), dogs (Yu and others 2002), men (Leibo and others 2002), and geese (Lukaszewicz and Kruszynski 2003). Each of these studies provides evidence that particular males produced spermatozoa that were more capable of surviving the cryopreservation procedure.

Individual differences in RVD capabilities have also been reported. Repeated collections from 8 boars indicated that there were "good" and "bad" responders. Good responders were those that had spermatozoa showing an initial swelling followed by a constant or reduced volume after 20 minutes of exposure to hypotonic conditions; bad responders showed no decrease in volume after the initial swelling and, in some cases, an increase in volume during the 20 minute exposure (Petrunkina and others 2004a). A good RVD response was associated with a high fertility index (calculated from data based on >30 inseminations for each animal). Spermatozoa from subfertile bulls display low RVD and poor oviduct binding (Khalil and others 2006). Similar evidence has also linked volume regulatory capability with fertility in bulls (Petrunkina and others 2001).

Spermatozoa from different species vary greatly in many ways, and their osmotic tolerance limits and freezability are likely no exception. Even so, the volume regulatory capabilities of all spermatozoa may depend on mechanisms that have been preserved through evolutionary time, as they have been for somatic cells. These mechanisms may vary in the channels used and the amounts of organic and inorganic osmolytes involved in volume adjustments, but the knowledge gained from the study of spermatozoa from any species is a valuable starting point for this relatively new area of research. In this dissertation, volume regulation by spermatozoa of humans, monkeys, and mice is described.

Value of Studying Volume Regulation of Monkey Spermatozoa

Non-human primate populations have not escaped the globally-recognized threats to biodiversity of habitat destruction, hunting, and genetic isolation. Frequent reports of dwindling population numbers have stimulated research into assisted reproductive technologies for vulnerable primate species, especially in captive breeding programs. Only 50% of threatened primate species are maintained in zoos worldwide, and many of the rare species

do not naturally breed well in captivity (Morrell and Hodges 1998). Assisted reproductive technologies (ART) have the potential to supplement *in situ* conservation efforts by strengthening the genetic integrity of the captive populations. These techiniques can be performed presently with fresh gametes or in the future with cryopreserved gametes.

As explained in previous chapters, availability of viable frozen-thawed spermatozoa is vital for success of many ART techniques, especially where wildlife is involved. Semen has been collected from at least 20 species of non-human primate and cryobiological studies conducted on at least 10 species (Leibo and Songsasen 2002). While sperm survival as judged by progressive motility after cryopreservation is generally acceptable, fertility remains low. Only 13 full-term pregnancies in 5 species have been achieved with cryopreserved spermatozoa (Leibo and Songsasen 2002).

There are many potential reasons for the less than desirable results achieved with frozen-thawed primate spermatozoa, among them being the type of cryoprotectant used. Glycerol is the most commonly used cryoprotectant for primate sperm cryopreservation, but the optimal proportion of glycerol varies with freezing method (Morrell and Hodges 1998). Extended exposure to glycerol has resulted in significant reductions in progressive motility as seen in cynomolgus monkeys (exposure > 25 min, Mahone and Dukelow 1978) and marmosets (exposure > 10 min, Morrell 1997). Because glycerol moves relatively slowly (compared with water) across the plasma membrane, the deleterious effects seen in thawed spermatozoa may be associated with the osmotic shock that occurs during CPA removal (Leibo and Songsasen 2002).

General knowledge on how primate spermatozoa respond to osmotic challenges would be potentially useful for seeking out the root cause of CPA toxicities and improving cryopreservation success. There has been very little research on volume regulation of primate spermatozoa, although there is one report of osmotic tolerance limits and membrane permeability of rhesus macaque spermatozoa (Agca and others 2005b). Published concurrently with the work presented here is evidence that Cl$^-$ channels may be involved in primate sperm RVD (Yeung and others 2004b). Aside from these reports, the mechanisms by which primate spermatozoa control their cellular volume are unknown.

The volume regulatory process can also provide a target for preventing unwanted pregnancies, and any knowledge gained through the study of cynomolgus spermatozoa may have relevance for human studies. The cynomolgus monkey epididymis is a good primate

model for that of the human, since the maturation of spermatozoa within the duct resembles that of man, with respect to acquisition of motility (Yeung and others 1993, 1996), acquisition of ability to undergo the acrosome reaction (Yeung and others 1996, 1997), changes in sperm surface charge (Fain-Maurel and others 1984), modification of sperm antigens (Mahony and others 1994, Yeung and others 1997, 2000, 2001), development of the acrosomal response to second messengers (Mahony and others 1996), condensation of nuclear chromatin (Golan and others 1997) and diminution of sperm head size (Yeung and others 1997, Gago and others 2000, Soler and others 2000). Research in this model may provide clues to the development of a male-directed contraceptive for men as well as captive primates.

There have been many calls within the scientific community for more basic research especially on sperm properties that may be a factor in cryopreservation success (Watson 2000, Morrell and Hodges 1998). With so little information on sperm volume regulation available, these studies are a starting point for further investigation into this phenomenon as it pertains to primates.

Value of Studying Volume Regulation of Murine Spermatozoa

Mice are classic model species for studying a wide range of physiological phenomena. Their short generation time and the ease with which they can be obtained and handled make them a common laboratory species for preliminary experiments with a human health focus. A large amount of research on sperm volume regulation has been performed using murine models. Although there are significant differences between human and murine sperm morphology (i.e. head shape and overall volume), knowledge gained from studies using murine spermatozoa contributes to the general understanding of the relatively unstudied mechanism of volume regulation in spermatozoa, which may involve ion channels that are conserved across species.

As found with monkey spermatozoa, cryopreservation of murine spermatozoa is a valuable tool for scientists. The growing number of transgenic mice used in scientific research has put pressure on laboratories to find adequate housing facilities. For some, maintaining the frozen gametes rather than a live colony of mice is more efficient and cost effective. Cryopreservation of murine spermatozoa is also important for the preservation of valuable genetics in genome resource banks. Although GRBs are often associated with protecting

charismatic endangered species, there are many rare and endangered species in the Muridae family. There are currently 451 Muridae species on the IUCN Red List of Endangered Species excluding those of least concern: 37 classified as critically endangered, 77 as endangered, 106 classified as vulnerable, and the remainder as lower risk (conservation-dependent), near threatened, or data deficient.

Value of Studying Volume Regulation of Human Spermatozoa

Although volume regulation is a relatively unstudied phenomenon in human spermatozoa there is evidence that volume regulation is required for natural fertilization. The association of poor semen quality (lower sperm motility) with reduced expression of sperm aquaporins (Saito and others 2004) suggests that water fluxes are important for fertility. The greater ability of spermatozoa from fertile males to regulate volume than those of infertile patients (Fetic and others 2006) confirms the suspicion that the inability of spermatozoa to regulate their volume may provide one cause of an otherwise idiopathic infertility. As the human population continues to grow, numerous contraceptive options are needed to limit the expansion that, currently unchecked, is fuelling an environmental catastrophe with consequences not only for humanity but for the endangered species it creates. As this burden should be shared by males, new contraceptive options are needed (Barfield and others 2006). Mimicking these cases of natural male infertility, such as impairing sperm volume regulation, may one day be induced as a form of contraception and provide modern humans with the means to control when they reproduce.

Although male contraception *per se* is apparently of little interest to major pharmaceutical companies, there are sources of funding for promoting fertility when conception is not easy. Although clinical trials are usually carried out on animal models before being applied to humans, valuable techniques developed for use in humans can be applied to wildlife (Barfield and others 2006). Therefore, the conservation biologist interested in developing ART for endangered and threatened species should pay close attention to developments in human reproduction.

Research on volume regulation in each of these species has relevance to wildlife conservation in addition to increasing the general reproductive knowledge of animal species.

The following chapter details the experiments carried out on spermatozoa from monkeys, mice, and humans to learn more about the channels and osmolytes involved in volume regulatory events.

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Chapter 4

Materials and Methods

Animals

Adult males of the *Cynomolgus fascicularis* species housed at the University of Muenster, Germany were used in these experiments. All mice were at least 70 days old and of the C57BL6 strain or B6D2F1 strains (Charles River, Sulzfeld, Germany). The experiments using spermatozoa from monkeys and mice were conducted according to the German Federal Law on the Care and Use of Laboratory Animals (license no. G67/2001 for both species). All human semen samples were donated by healthy men between 24 and 55 years of age at the Institute for Reproductive Medicine in Muenster, Germany. Each man signed a written consent form, satisfying the local Ethics Committee, that his semen could be used for research purposes.

Collection of Spermatozoa

Mice

The mice were killed by asphyxiation with CO_2 followed by cervical dislocation. All murine spermatozoa utilized in these experiments were extracted from the epididymis. The testis-epididymis complex was dissected and the cauda epididymidis isolated. The capsule of the cauda epididymidis was gently torn open with fine forceps to expose the tubule. The tubule was then carefully uncoiled so that the entire length of the mid and distal cauda tubule was exposed (Fig. 4.1A). Beginning from the distal end, closest to the vas deferens, sections of the tubule were cut out (Fig. 4.1B) and placed in separate 10 µl drops of Biggers–Whitten–Whittingham (BWW) medium (Biggers and others 1971) with or without inhibitor on a plastic spatula (Fig. 4.1C). The order in which the inhibitors were tested was rotated between experiments to ensure that the location of the spermatozoa within the cauda epididymidis or time before incubation did not confound the results. The contents were then gently teased from the tubule (Figs. 4.1D & E) and the tubule removed from the drop (Fig. 4.1F). The drop was then dispersed in 200 µl of the same medium and the sperm suspensions placed in an incubator (37°C, 5% (v/v) CO_2 in air). After approximately 2 min, sperm suspensions were gently agitated to ensure dispersal of the spermatozoa and the incubation continued for 5 – 75 min.
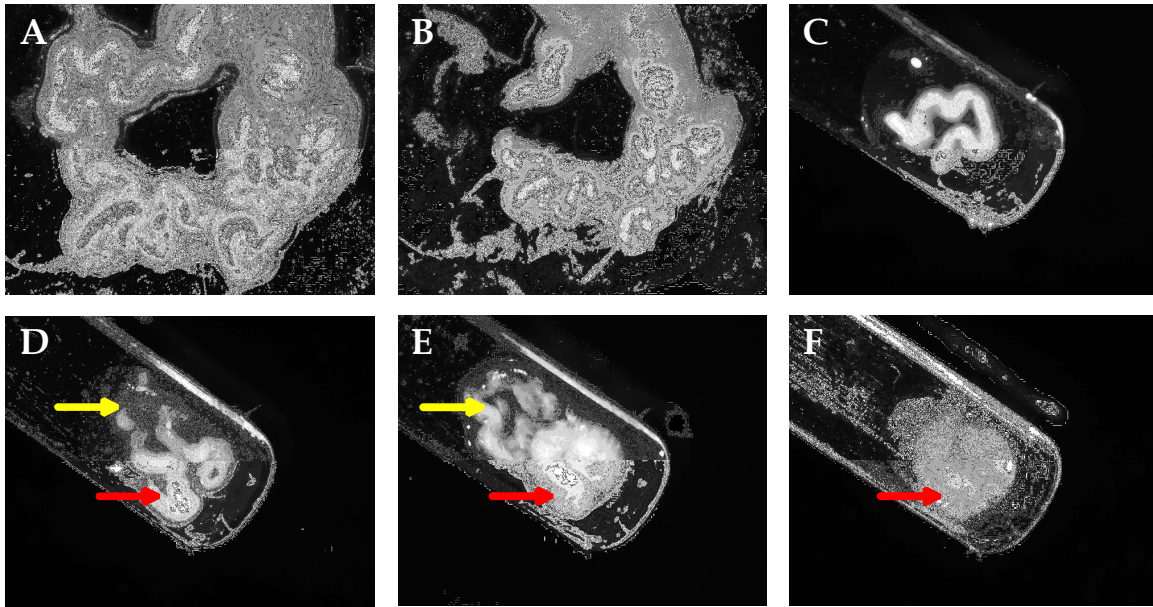
Figure 4.1. Extraction of murine cauda epididymidal spermatozoa as described in the text. Yellow arrows indicate the tubule and red arrows indicate spermatozoa.

For Western blot experiments and immunocytochemical studies, murine epididymal spermatozoa were obtained by cannulation of the cauda epididymidis. The cauda epididymidis was isolated along with a portion of the proximal vas deferens. The vas was cannulated with a 0.5 mm i.d. /0.8 mm o.d. PVC catheter (Dural Plastics, Silverwater BC, NSW, Australia) that had been pulled out over a flame to form a narrow pipette-like tip and tied in place with 5/0 suture thread (Braun Surgical GmbH, Melsungen, Germany). The cannula was attached to a 1 ml tuberculin syringe filled with PBS_{420} (2.7 mM KCl, 5.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4,) with sufficient NaCl to achieve approximately 420 mmol/kg (the osmolality of murine epididymal fluid, Yeung and others 1999). This medium was chosen to limit osmotic changes during sperm collection. A small cut was then made in the epididymal tubule at the proximal cauda region, just beyond the flexure and the contents of the cauda epididymidis were flushed out and collected with a 25 µl positive displacement pipette as it seeped out from the cut. Spermatozoa were dispersed in the PBS_{420} at room temperature and then put on ice until samples from all mice had been collected. Samples were centrifuged at 2000 g for 2 min, the diluted epididymal plasma was removed and the sperm pellet stored at -80°C until use.

Monkeys

Ejaculated spermatozoa were collected from 15 cynomolgus monkeys on several occasions. The monkeys were anaesthetized with Ketamine HCl (Park Davis Ltd., Berlin, Germany, 12 mg/kg) and prepared for rectal-probe electro-ejaculation as described by Weinbauer and others (1994). A homemade device delivering 6 V at 50 Hz in a square wave form through a rectal probe approximately 15 cm long and 1.5 cm in diameter was used to stimulate the monkeys. The current was applied for 2 sec up to 15 times after which, if no ejaculate was produced, the procedure was discontinued. Most monkeys produced an ejaculate after 3 or 4 stimulations. The samples were brought to the laboratory in an incubator at 37°C within 30 min and the exudate was isolated by physically removing the coagulum with forceps. A portion of the exudates was taken to assess sperm concentration and osmolality.

Humans

Human ejaculated spermatozoa were collected by masturbation at the Institute for Reproductive Medicine of the University in Muenster, Germany. Samples were collected in specialized graduated cylinders for easy volume measurement. Samples were received in the laboratory within 5 min from the time of ejaculation. A standard semen analysis according to the World Health Organization (WHO 1999) guidelines was performed by the laboratory technicians on all samples after liquefaction for 30 min at 37°C. The values for specific semen parameters are detailed below for individual experiments.

Routine Measurements of Post-liquefaction Semen Osmolality

All measurements of osmolality were made with a vapor pressure device (Wescor Vapro model 5520, Kreienbaum Messsystem, Langenfeld, Germany). This system was used because dew point depression is not affected by sample viscosity or the presence of suspended particles (Sweeney and Beuchat 1993) and small samples can be measured. The osmometer was calibrated each day with 10 µl of a 290 mmol/kg standard supplied by the manufacturer. When semen was being measured, a delay of 2 min was employed before measurement to ensure chamber saturation and reproducible results. All measurements were made on a 10 µl aliquot of semen. Reproducibility and accuracy, ascertained on 10 measurements of a 290 mmol/kg

standard, established a coefficient of variation (CV) of 1.5% and a mean deviation of 0.7% from the true value. After the 2 min delay, the CV was 0.7%.

Osmolality of Human Semen during Liquefaction

Eighteen semen samples were obtained in the institute by 15 healthy volunteers of median age 28 years (range 24-55) who masturbated after abstinence times of 3-6 days. Ejaculates were measured (mean ± SEM, range) for semen volume (3.8 ± 0.5, 1.3-7.1 ml), sperm concentration (41.2 ± 11.2, 8-127 million/ml), sperm number (160.8 ± 56.3, 17-631 million/ejaculate), percentage motility grades a (36.8 ± 2.5, 25-55), b (13.7 ± 1.6, 7-21), c (7.1 ± 0.9, 1-13), d (42.4 ± 1.5, 35-50) and normal morphology (13.3 ± 1.7, 2-19). Eight of these samples were judged to be normozoospermic by WHO (1999) criteria.

Ejaculates were mixed well in the collecting cylinder with a glass rod upon receipt in the laboratory within 2-5 min of production and the osmolality measured. One aliquot was removed and measured every 5 min during the 30 min liquefaction period at 37°C with 5% (v/v) CO_2 in air.

Incubation Media and Inhibitors

Media

All chemicals were from Sigma (Taufkirchen, Germany) except for clofilium tosylate (Alexis Biochemicals, Grünberg, Germany) and phrixotoxin (Alomone Labs, Jerusalem, Israel). The basal medium used unless otherwise specified was Modified Biggers–Whitten–Whittingham (BWW) medium (Biggers and others 1971). For experiments with murine spermatozoa, the osmolality was adjusted to 330 mmol/kg (BWW_{330}) and for experiments on human and monkey spermatozoa the osmolality was 290 mmol/kg (BWW_{290}). BWW_{330} was chosen as 330 mmol/kg is the osmolality of post-copulatory uterine contents (Yeung and others 2000) whereas BWW_{290} is medium of similar osmolality to that of the human female tract (see Chapter 3). These media are anticipated to be hypotonic to both epididymal and ejaculated spermatozoa which are thus subjected to physiologically relevant osmotic challenges in all species. The details of the medium are listed in Table 4.1. For incubation with the inhibitors, all media were adjusted to pH 7.4, contained 4 mg/ml bovine serum albumin (BSA) and were warmed (37°C) before use. For flow cytometric measurements, BSA-free medium of the same

78

composition containing the vital dye propidium iodide (PI, 0.5 mg/ml water; final concentration 6 µg/µl) was used.

Component	Final concentration (mM) for BWW_{330}
KCl	4.8
$CaCl_2 \cdot 2H_2O$	1.7
$MgSO_4 \cdot 2H_2O$*	1.2
KH_2PO_4	1.2
Na pyruvate	0.25
Glucose	5.5
Na lactate	26
$NaHCO_3$	25
NaCl	108**
Hepes	20

Table 4.1. Composition of BWW medium.
* $MgCl_2$ substituted for $MgSO_4 \cdot 2H_2O$ when testing the effects of $BaCl_2$.
** Concentration of NaCl in BWW_{290} was 80 mM.

Channel blockers

One of the most commonly used inhibitors of potassium channels, and the one used as a control in these experiments, is quinine. Quinine is an alkaloid from the bark of *Chinchona nitida*, and is most well-known for its ability to combat malaria. It was the first chemical compound successfully used against an infectious disease (Encyclopedia Britannica 2007). Quinine has been reported to block a wide variety of K^+ channels including A-type, delayed outward, and Ca^{2+}-dependent K^+ currents. In addition, voltage-dependent Ca^{2+} inward currents and Na^+ outward currents are also inhibited (Kuriyama and others 1995). Quinine is suspected of acting on the external surface of channels, as is the case with maxi-K^+ channels in bovine chromaffin cells, with enhanced blocking action during membrane hyperpolarization (Glavinovic and Tifaro 1988).

Other common inhibitors used to characterize K^+ channels are tetraethylammonium (TEA) and 4-aminopyridine (4AP). TEA was the first, and remains the most frequently used, K^+ channel blocker (Kuriyama and others 1995). Although a specific mechanism of action is yet to determined, TEA blocks most classes of K^+ channel with varying affinities (Cooke and Quast 1990). Four-aminopyridine also blocks many classes of K^+ channel, but is more selective and

more potent than TEA. A wide range of more specific inhibitors such as barium, clofilium, cadmium, glybenclamide, and flecainide is used to identify K^+ channels involved in volume regulation. The channels inhibited by these compounds are listed in Table 4.2.

Naturally occurring toxins are also used to identify potassium channels. Charybdotoxin, a scorpion toxin, is known to block maxi-K^+ channels by direct occlusion of the channel through a specific interaction site on the extracellular side of the membrane (Kuriyama and others 1995). A variety of other channels is reported to be inhibited by charybdotoxin, including Ca^{2+}-dependent, Ca^{2+}-insensitive, and voltage-gated K^+ channels (Kuriyama and others 1995). Apamin, a toxin isolated from the honey bee (*Apis mellifera*), selectively blocks small-conductance K^+ channels by acting on the external surface of the channel (Seagar and others 1984, Blatz and Magleby 1986, and Capiod and Ogden 1989).

The channel blockers used in these studies are involved in the volume regulation of somatic cells and were used at concentrations effective for somatic cells (see Table 4.2).

Inhibitor (abbreviation in Figs.)	Concentrations tested	Function	References
4-aminobutyrate (4AB)*, **	500 μM, 1 mM	Inhibits carnitine transport	Kido and others 2001
4-aminopyridine (4AP)	1 mM, 4 mM	Blocks voltage-gated K^+ channels	Wehner and others 2003 Mathie and others 1998
Apamin (APM)*	200 nM, 1 μM	Potently inhibits SK_{Ca}	Vergara and others 1998
$BaCl_2$ (Ba)	1 mM, 5 mM	Inhibits BK_{ca} TASK3	Wehner and others 2003 Kim and others 2000
Cadmium chloride (Cd)**	0.2 mM, 2 mM	Inhibits Kv4.3	Calmels and others 2001
Charybdotoxin (CTX)*	10 nM, 100 nM	Inhibits Kv1.3 Inhibits IK_{Ca}, BK_{ca} Inhibits maxi-K^+	Deutsch and Chen 1993 Wehner and others 2003 Kuriyama and others 1995
Clofilium tosylate (CLO)	10 μM, 100 μM	Inhibits TASK2 Inhibits minK channels	Niemeyer and others 2001 Lock and Valverde 2000
Dendrotoxin-α*	150 nM, 400 nM	Inhibits Kv1 channels	Gasparini and others 1998
Flecainide (FLE)**	10 μM, 100 μM	Inhibits Kv4.2 Inhibits Kv4.3	Caballero and others 2003 Amberg and others 2002
Gadolinium (Gd)*,**	10 μM, 1 mM	Inhibits stretch activated-ion channels ORCC (VDAC)†	Kraznai and others 2003 Taouil and Hannaert 1999 Thinnes and others 2001
Glybenclamide (GLY)*	10 μM, 100 μM	ATP-dependent K^+ channels	Macho and others 2001
Hydroxyaspartate (HOA)*	20 μM, 1 mM	Glutamate transport inhibitor	Chen and Swanson 2003
Margatoxin (MTX)*	10 nM, 200 nM	Inhibits Kv1.3	Garcia-Calvo and others 1993, Khanna and others 1999
Phlorizin (PHZ)*,**	5 μM, 500 μM	Blocks SGLT1 (SLC5A1) ‡ Inositol transport inhibitor	Loo and others 1999 Higgins and Kane 2003
Phrixotoxin (PTX)**	10 μM, 100 μM	Blocks A-type currents, specifically Kv4.2 and Kv4.3	Diochot and others 1999
Quinine (QUI)	0.3 mM***, 0.8 mM*,**	Broad spectrum channel blocker	Wehner and others 2003
Tetraethylammonium chloride (TEA)	10 mM, 50 mM	Broad spectrum channel blocker, BK_{Ca}	Wehner and others 2003

Table 4.2. List of inhibitors used to identify potential channels involved in RVD of monkey, murine, and human spermatozoa. *Not tested on human spermatozoa, ** not tested on cynomolgus spermatozoa, *** not tested on murine spermatozoa, † Outwardly-rectifying chloride channel (voltage-dependent anion-selective channel), ‡ sodium glucose transporter 1.

Measurement of Cell Size by Flow Cytometry

Changes in sperm cell volume were measured in a flow cytometer (Coulter Epics XL, version 3.0, Krefeld, Germany) according to the method validated against an electronic cell sizer by Yeung and others (2002, 2003). All flow cytometer settings (voltage, gain, signal trigger threshold, analysis windows, etc.) in the flow cytometer protocol used for each experiment were constant throughout the studies. The flow cytometer was calibrated before each experiment with 5 µm diameter standard spherical beads (Coulter Electronics Ltd, Luton, England). The forward and side scatter values generated by the calibration procedure before each experiment were recorded. The size distribution profile of the measured particles was analyzed using Accucomp software provided by the manufacturer. The mean (± SEM) forward scatter of the standard beads (n = 134) was 386.26 ± 0.66 (CV 2.0%) and the side scatter was 507.05 ± 1.52 (CV 3.5%), indicating that the machine was stable and that measurements between experiments were similar.

A minimum of 5000-6000 cells was analyzed in the flow cytometer for PI (emission at 605–635 nm), to eliminate signals from non-viable cells, and for forward and side scatter of laser with an excitation at 488 nm, to gate out debris. Forward scatter signals were analyzed after gating for viable (PI-excluding) spermatozoa. For each individual sperm sample from monkeys, mice and men, a non-treated sample was always included, which served as a control value for comparison for each replicate treatment sample, as well as a positive response sample with quinine. Relative cell sizes were calculated by dividing the mean forward scatter of cells incubated in inhibitor by the mean forward scatter of cells from the same sperm sample incubated without inhibitor at each time point. An example of the data collected from flow cytometry analysis is shown in Fig. 4.2.
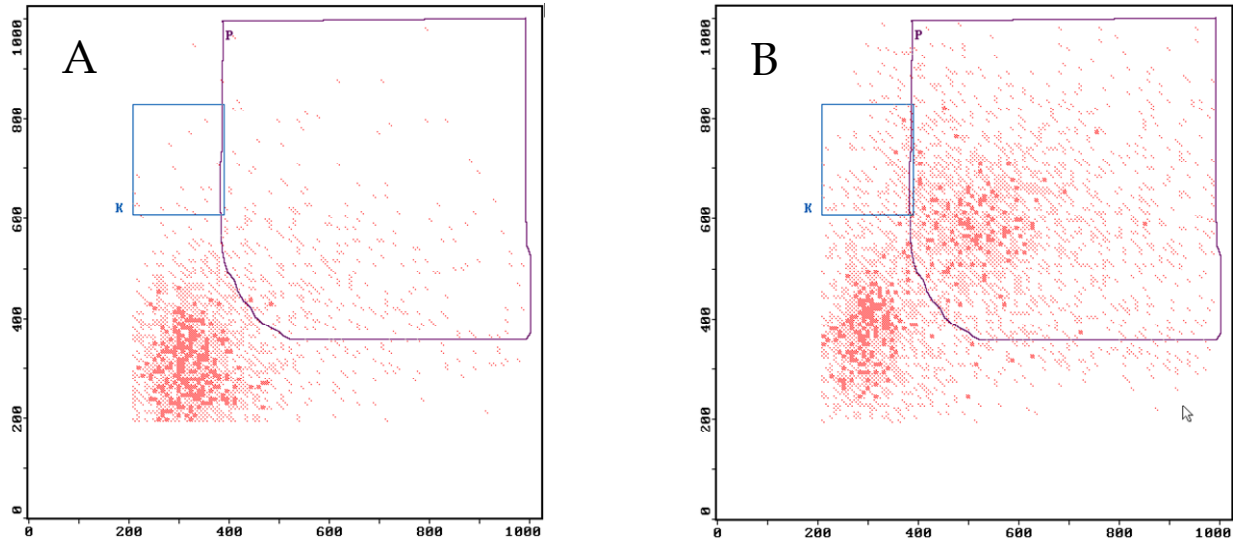
Figure 4.2. Scatter plots produced from the flow cytometer analysis of control murine spermatozoa (A) and spermatozoa incubated with quinine (B) during a hypotonic challenge. The forward scatter (y-axis) is plotted against the side scatter (x-axis) and the small blue box is set to display potential contamination of red blood cells in the sample. The large purple box contains the plots of spermatozoa that have higher forward/side scatter values and are considered swollen. The average forward scatter values for these data are 353 in A and 532 in B.

Monkey spermatozoa

Monkey spermatozoa were dispersed in the appropriate medium for 1–2 min and incubated at 37°C in 5% (v/v) CO_2 in air for 5 and 30 min. For flow cytometric measurement, 10 µl of sperm suspension (approximately 2-10 x 10^6 spermatozoa/ml) were added to 200 µl of the same BSA-free and PI-containing medium. After gentle agitation to mix the sample, flow cytometric measurements were recorded immediately. The methods used for preparing monkey spermatozoa are outlined in Fig. 4.3.
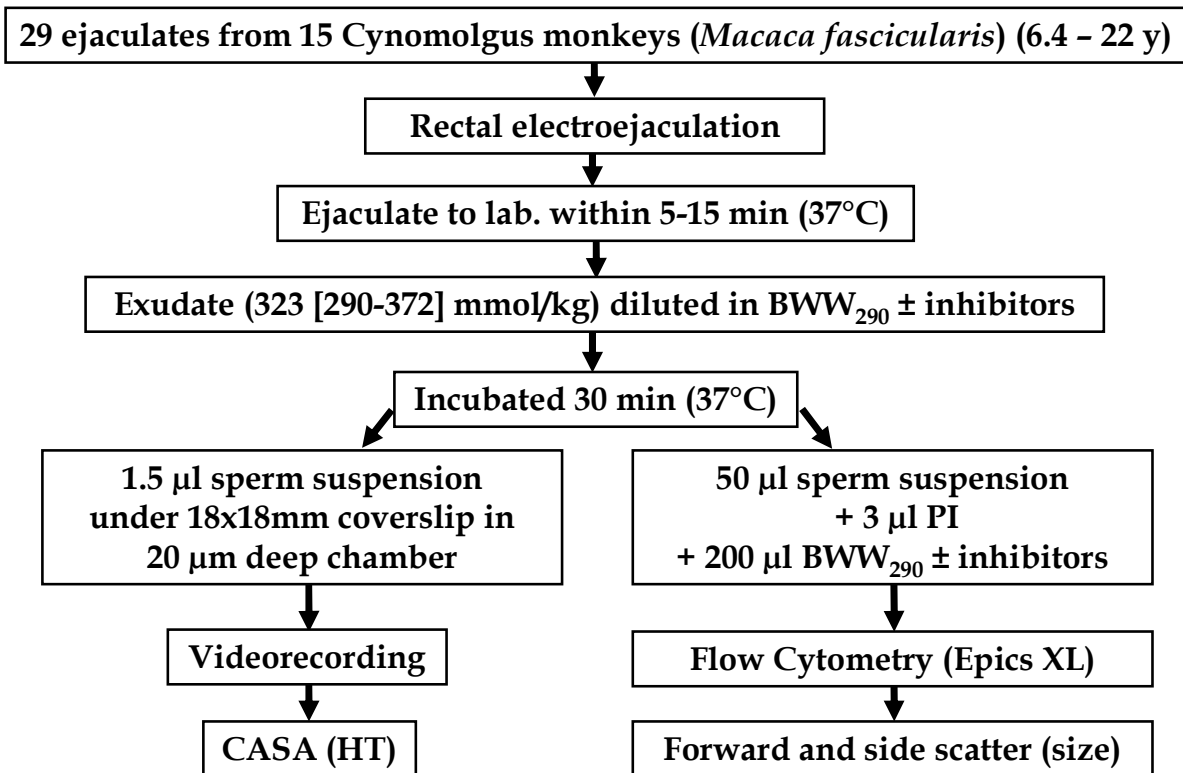
Figure 4.3. Flow diagram of the methods used to prepare monkey ejaculated spermatozoa for analysis of kinematic parameters with CASA and relative size with flow cytometry. Details are given in the text.

Murine spermatozoa

        Murine spermatozoa were incubated in the appropriate medium for 5 and 75 min, at which time approximately 80 µl sperm suspension (approximately $2\text{-}10 \times 10^6$ sperm/ml) were added to 200 µl of the same BSA-free and PI-containing medium. Upon gentle agitation to mix the sample, flow cytometric measurements were recorded immediately.

Human spermatozoa

        The semen samples used in inhibitor experiments were from twenty six ejaculates obtained after abstinence periods of 3.8 ± 0.5 (range 2 -10) days from 20 volunteers (mean ± SEM, range: age 29.6 ± 1.7 y, range 24 – 55). Ejaculates were measured for semen volume (4.0 ± 0.4 ml, 1.3 – 7.8), osmolality (316.8 ± 5.0 mmol/kg, 278 – 383), sperm concentration (41.2 ± 6.8 million/ml, 3.8 -127), sperm number (173.3 ± 36.1 million/ejaculate, 24.7 – 571.5), percentage motility grades a (35.0 ± 2.1, 21 - 55), b (14.2 ± 1.6, 4 - 28), c (8.5 ± 0.9, 1 - 15), d (42.6 ± 1.1, 35 - 53),

and percentage normal morphology (15.0 ± 1.0, 5 - 22). Fifteen of these ejaculates were normozoospermic (WHO 1999).

Upon liquefaction, the osmolality of 10 µl of human semen was measured. Spermatozoa were washed through an 80% (v/v) / 40% (v/v) Percoll gradient (Biosciences, Freiburg, Germany) adjusted to the individual seminal osmolality by combining high and low osmolality Percoll media. Four BWW media were made up for mixture with Percoll (Table 4.3) to create solutions of high and low osmolality for the 80% and 40% levels of the Percoll gradient. The 80% Percoll solutions were prepared 10 ml at a time by mixing 8 ml Percoll with 2 ml BWW High 80% or BWW Low 80% solution (see Table 4.3). The 40% Percoll solutions were prepared by mixing 4 ml Percoll with 6 ml BWW High 40% or BWW Low 40% solution (see Table 4.3). The mean final osmolalities were 393 mmol/kg for High 80% Percoll, 251 mmol/kg for Low 80% Percoll, 364 mmol/kg for High 40% Percoll, and 256 for Low 40% Percoll. The relevant proportions of the low (A) and high (B) osmolality BWW solutions to achieve the osmolality of the individual's seminal plasma (C) were determined by the following formula:

$$C = (A + xB) / (1+x), \text{ where } x = (C-A) / (B-C)$$

	High 80%	Low 80%	High 40%	Low 40%
KCl	24	24	8.01	8.01
$CaCl_2 \cdot 2H_2O$	8.5	8.5	2.8	2.8
$MgSO_4 \cdot 2H_2O$*	6	6	2	2
KH_2PO_4	6	6	2	2
Na pyruvate	1.25	1.25	.42	.42
Glucose	27.5	27.5	9.19	9.19
Na lactate	130	130	43.42	43.42
$NaHCO_3$	125	125	41.75	41.75
NaCl	468	240	194	85
Hepes	100	100	33.4	33.4
Final Osmolality (mmol/kg)	1600	1050	530	350

Table 4.3. Final concentrations (mM) of the components of the modified BWW media used to create Percoll solutions of high and low osmolalities.

Spermatozoa were centrifuged at 500g for 20 min, and the pellet was isolated by removing all but 100 µl of the supernatant. The pelleted spermatozoa were resuspended in the remaining supernatant and placed in an incubator at 37 °C min 5% (v/v) CO_2 in air.

After 5 and 30 min incubation in the appropriate medium ± inhibitor, 40 µl sperm suspension (approximately 2-10 x 10^6 spermatozoa/ml) were added to 200 µl of the same BSA-free and PI-containing medium. Upon gentle agitation to mix the sample, flow cytometric measurements were immediately recorded under the same flow cytometer settings as used for ejaculated monkey spermatozoa. A flow diagram of these methods is represented in Fig. 4.4.
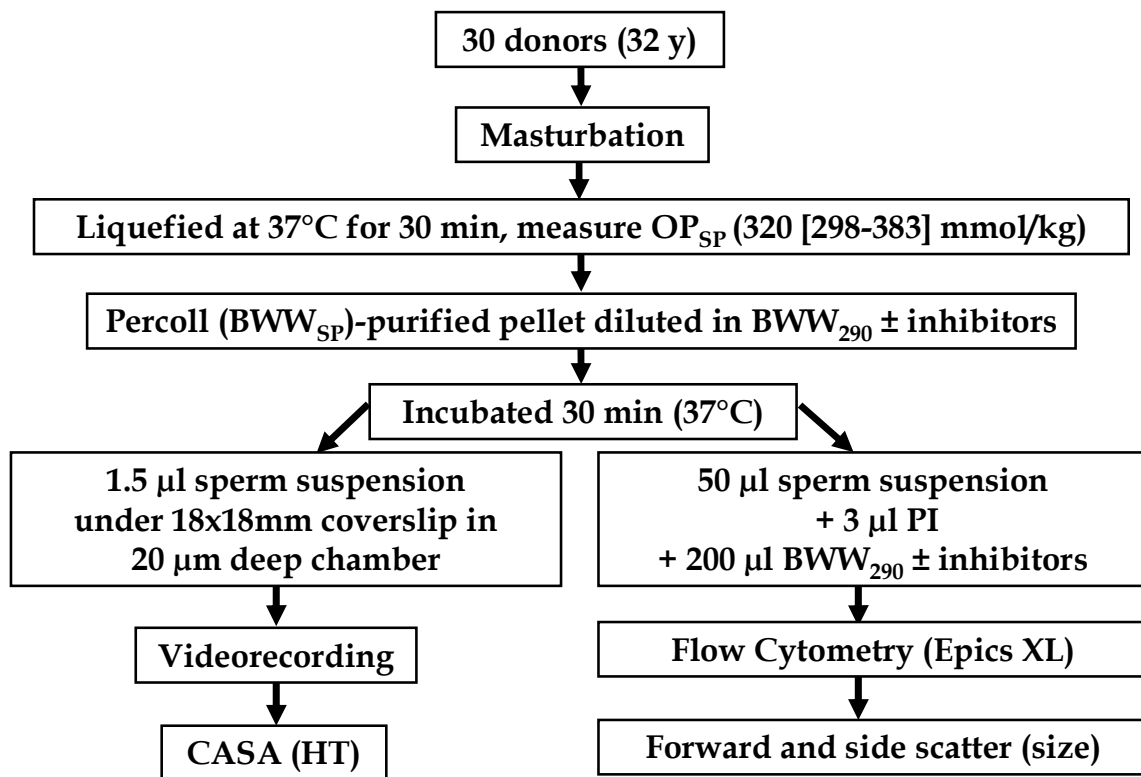
Figure 4.4. Flow diagram of the methods used to prepare human ejaculated spermatozoa for analysis of kinematic parameters with CASA and relative size with flow cytometry. Details are given in the text.

The ability of the Percoll washing step to remove non-sperm cells was evaluated on 23 ejaculates collected from 17 men by expressing the number of non-sperm cells as a percentage of the total number of sperm and non-sperm cells. The percentage of spermatozoa with cytoplasmic droplets (Cooper and others 2004) before and after the Percoll wash was

determined after fixation in the WHO diluent used for assessing concentration (WHO 1999) and examined in a wet preparation by phase contrast microscopy at 40x magnification. The same observer performed all evaluations.

Effect of pH on RVD and Action of Inhibitors

To determine the effect of pH on the RVD response, human spermatozoa were incubated in BWW_{290} at pH 6.3, 7.4, or 8.4 and murine spermatozoa were incubated in BWW_{330} at pH 6.3, 7.4, or 8.4. For media of pH 6.3, Hepes was replaced by Mopso (final concentration 20 mM). Because a pH of 8.4 only just falls within the buffering capabilities of Hepes, spermatozoa were also incubated at pH 8.4 buffered with 20 mM Tris to determine if the buffering system influenced RVD. All media were adjusted to the desired pH with 1M HCl or 1M NaOH. Flow cytometric measurements were taken as described above after incubation times of 5 and 75 min for murine spermatozoa and 5 and 30 min for human spermatozoa.

Measurement of Motility using Computer-aided Semen Analysis

Aliquots (1.5 µl) of human and monkey sperm suspensions were taken after 5 and 30 min incubations and viewed on a pre-warmed (37°C) dual-sided sperm analysis chamber (2X-Cel chamber, 20 µm depth, Hamilton Thorne, Beverly, MA, USA) using a negative phase contrast 10x objective and 3.3x photo-ocular. Several fields of view were video-recorded over approximately 1.5 min for later analysis using the Hamilton Thorne CASA system software, IVOS Version 10.8. For each sample, approximately 200 spermatozoa were tracked and measured for curvilinear velocity (VCL), straight-line velocity (VSL), averaged path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN=VSL/VCL x 100) and straightness (STR=VAP/VCL x 100). Measurements were made on 50 frames at a frame rate of 50 Hz, minimum contrast 60, minimum size 3, minimum of 25 track points, and minimum VAP of 10 µm/sec. The percentage of motile spermatozoa were estimated visually.

Reproducibility of Sperm Responses to Quinine

Human

Thirty semen samples were obtained after abstinence times (mean ± SEM, range) of 3.8 ± 0.4, 2-10 days from 11 healthy volunteers of age 29.7 ± 2.0, 24-55 years. Ejaculates were characterized by semen volume (3.9 ± 0.3, 1.3-7.1 ml), sperm concentration (36.8 ± 5.6, 4-97 million/ml), sperm number (145.7 ± 29.9, 17-631 million/ejaculate), percentage motility grades a (35.6 ± 1.9, 21-55), b (14.2 ± 1.3, 4-28), c (8.5 ± 0.8, 1-16), d (42.1 ± 1.0, 35-53) and normal morphology (14.1 ± 1.0, 2-20). Thirteen of these samples were judged to be normozoospermic by WHO (1999) criteria.

Ejaculates were placed in an incubator with 5% (v/v) CO_2 in air at 37°C during liquefaction. After 30 minutes, seminal osmolality was measured, the spermatozoa Percoll washed, and approximately 5-10 μl of pelleted spermatozoa were added to 200 μl BSA-free BWW_{290} containing 3 μl PI with or without 0.3 mM quinine (control). After a 30 min incubation period, relative size of spermatozoa was gauged using flow cytometry as described above.

Monkey

The size of ejaculated spermatozoa, as reflected in forward scatter, was measured at 5 and 30 min during incubation with control medium (BWW_{290}) and BWW_{290} containing 0.3 mM quinine. Comparisons were made among males (20 samples, 13 males) and among multiple ejaculates produced by 6 males on different occasions.

Mice

The size of epididymal spermatozoa, as reflected in forward scatter, was measured at 5 and 75 min during incubation with control medium (BWW_{330}) and BWW_{330} containing 0.8 mM quinine. Comparisons were made among males (spermatozoa originating from one or two epididymides: 42 samples, 24 males) and between epididymides (spermatozoa that had been collected from both epididymides of the same male: 36 samples, 18 males).

Western Blotting

Murine sperm extracts

For membrane protein extraction, murine epididymal spermatozoa flushed through the vas deferens from the cauda epididymidis were washed in PBS_{420} and centrifuged for 5 min at 500g. Pellets were resuspended in 0.5 ml medium and an aliquot was further diluted (30 μl sperm suspension + 180 μl medium). The sperm concentration was assessed by measuring the absorbance of 100 μl of the diluted aliquot at 405 nm in duplicate in a Spectramax250 spectrophotometer (Molecular Devices, Munich, Germany) against a blank of PBS_{420}. The concentration of the original sperm suspension (million/ml) was calculated using the following formula:

$$C = [(\text{mean absorbance of duplicates} - \text{mean blank})/0.014] \times \text{dilution factor (7)}$$

The sample was then transferred into Eppendorf tubes so that each tube contained 6×10^6 spermatozoa. One ml PBS medium was added to each tube and the spermatozoa centrifuged at 500g for 5 min. The supernatant was removed and the sperm pellets pellets frozen and stored at -20°C.

Human sperm extracts

Preparation of ejaculated human spermatozoa began with Percoll washing of the sample after liquefaction. Fifty μl lysis buffer (below) were added to the pellet of a Percoll-washed sperm sample and intensely vortexed for 3 min. The samples were kept on ice and incubated in the lysis buffer for 1 h and vortexed intermittently. The samples were then centrifuged at 20,000g for 20 min at 4°C (Heraeus Biofuge Stratus, Kendro Lab Products, Langendselbold, Germany). The supernatant was collected and protein was measured using a Lowry protein assay using BSA as standard (BIO-RAD Munich, Germany). Aliquots of 50-60 μg of protein were frozen and stored at –80°C. Although no sonication was performed, the presence of nuclear, mitochondrial and cytosolic proteins cannot be excluded. However, functional potassium channels are membrane proteins that contain transmembrane domains.

Adsorption of antibodies

To prepare the pre-adsorbed control, primary antibody was added to the control peptide antigen (1 µg peptide per 1 µg antibody: TASK2, TASK3, Kv4.3, Kv4.2; 3 µg peptide per 1 µg antibody: Kv1.5, Kv1.4, as recommended by Alomone Labs (Jerusalem, Israel) and incubated overnight at 4°C on a rotating plate. Unadsorbed primary antibody was also rotated overnight at 4°C. Aliquots of the resulting adsorbed solution and primary antibody were frozen and kept at -20°C until use. Westerns blots of all channels potentially present as indicated from the results of the inhibitor experiments were performed except for Kv1.7 because of the unavailability of appropriate antibodies.

Polyacrylamide gel electrophoresis

Thawed pellets of murine spermatozoa were taken up in 50 µl lysis buffer (125 mM NaCl, 25 mM Hepes, 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, and 1% (v/v) Triton-X100 at pH 7.3) containing 10 µl protease inhibitor cocktail (Sigma) and phosphatase inhibitor 1 mM Na_3VO_4 and intensely vortexed for 3 min. The samples were kept on ice for 1 h with frequent vortexing. The suspension was centrifuged for 20 min at 20,000g at 4°C and the supernatant was collected. One aliquot of frozen human protein was thawed and prepared for each lane of the gel. Lysates were heated at 65°C for 10-15 min.

Polyacrylamide gel electrophoretic separation of murine and human proteins was carried out using 4-12% (w/v) NuPAGE Novex Bis-Tris precast gels (8 x 8 cm, 1 mm thick; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with 50 µg protein sample in each lane after heating at 65°C for 10 min in the absence of dithiothreitol (DTT). Separated proteins were transferred onto ECL-Hybond membranes (Hybond ECL, Amersham Pharmacia, Uppsala, Sweden) using 150 mA at 35 V for 3 h and stained with Ponceau Red to check protein loading and visualize the molecular weight markers. After blocking nonspecific binding sites with StartingBlock (Pierce, Perbio Science, Bonn, Germany) for 1 h at room temperature, the membrane was incubated with primary affinity-purified rabbit antibodies against Kv1.4, Kv1.5, Kv4.2, Kv4.3, TASK2 and TASK3 diluted 1:2,500 (Alomone Labs, Jerusalem, Israel) followed by a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody diluted 1:250,000. Peroxidase-bound protein bands were visualized using the ECL-

Plus method (Femto-signal, Pierce). The molecular weights of the signal bands were analyzed using line densitometer software (ChemImage Systems, version 5.5, 15440, Alpha Innotech Corp., San Laendro, California, USA).

Immunocytochemical Localization

Murine epididymal spermatozoa were collected by cannulation of the cauda epididymidis into PBS_{420}. Half of the suspension was then transferred to another Eppendorf tube, centrifuged for 5 min at 500g, and resuspended in 0.5 ml PBS_{420} for comparison between washed and unwashed spermatozoa. Human spermatozoa were washed through Percoll adjusted to 320 mmol/kg as described above and resuspended in 3 ml of PBS_{320}. They were centrifuged for 5 min at 500g and resuspended in 0.5 or 1 ml PBS depending on concentration.

For each species, 5 µl of the sperm suspension were smeared across a polylysine slide (Menzel GmbH and Co KG, Braunschweig, Germany) and air-dried at room temperature for at least 15 min. Slides were placed in a Wheaton staining jar and submerged in 4% (w/v) paraformaldehyde in PBS for 30 min. The paraformaldehyde was poured off and replaced with PBS for washing. The jar was then placed on a rotating plate for 5 min and the PBS was changed twice during this time. A circle with an inner diameter of approximately 1 cm was drawn in the centre of the slide with a grease pen (Dako, Denmark). In some instances the cells were permeabilized by placing a drop of 0.1% (v/v) Triton X-100 in PBS over the cells within the circle for 5 min followed by washing in PBS.

Cells were then blocked by covering them with PBS containing 3% (w/v) BSA (approximately 30 µl) and incubated for 30 min. The slides were washed and a drop of primary antibody (1:60) or adsorbed antibody (adsorbed with 10x more antigen than recommended) was placed over the spermatozoa and incubated overnight at room temperature in a humid chamber. For detection of minK, incubation with secondary antibody only was used as a negative control rather than an adsorbed antibody preparation as insufficient antigen was available. Slides were washed in PBS for 20 min in the dark and a drop of secondary antibody (1:200, anti-rabbit IgG TRITC conjugate, Sigma) was incubated with the spermatozoa for 1 h. Slides were washed in PBS for 20 min, mounted with Vectashield anti-fade medium (Vector Laboratories, Burlingame, CA USA) and observed with a Zeiss Axiovert 200 fluorescence

microscope. Immunolocalization of TASK3 and Kv1.7 was not performed because of lack of appropriate antibodies.

Determination of Sperm Isotonicity

In order to determine if the entry of putative osmolytes and cryoprotectants into spermatozoa were by osmo-sensitive channels, cell swelling (indicating osmotic influx of water following solute entry) was determined under isotonic conditions. Because these conditions have never been defined for spermatozoa, epididymal spermatozoa were incubated in BWW of osmolality extending beyond the values measured for epididymal fluid (400-480 mmol/kg, Cooper and Barfield 2006). Basic BWW was modified with additional NaCl, choline Cl, sucrose, or lactose to obtain the different osmolalities. The final ionic strengths, osmolalities, and concentrations of Na^+ and Cl^- are listed in Table 4.4. Ionic strength (μ) was calculated from the formula, $\mu = \frac{1}{2} \Sigma mz^2$, where m is the molar concentration and z is the charge on each species in solution.

Murine spermatozoa were incubated in the various media for 10 and 75 min. At the appropriate time point, 80 µl sperm suspension (approximately $2\text{-}10 \times 10^6$ spermatozoa/ml) were added to 200 µl of the same medium without BSA and containing PI. Upon gentle agitation to mix the sample, flow cytometric measurements were recorded as detailed above.

The method of null-point volume change was employed in which spermatozoa from the murine cauda epididymidis were incubated in a range of osmolalities in the absence and presence of 0.8 mM quinine. In hypotonic media, spermatozoa should swell and initiate RVD; in the presence of quinine, this is inhibited and the cells remain swollen (Yeung and others 2002). In hypertonic media, spermatozoa will shrink; quinine can partially prevent regulatory volume increase (RVI) in lymphocytes (Grinstein and others 1983) and may prevent reversal of shrinkage with spermatozoa remaining shrunken. Isotonicity was interpreted as the osmolality at which there was no change in sperm cell volume in response to quinine. The mean isotonic osmolality was obtained by linear regression.

	NaCl	Sucrose	Lactose	Choline Cl
BWW 340				
Ionic strength (μ)	0.189	0.177	0.177	0.179
Measured Osm. (mmol/kg)	335	336	336	338
$[Na^+]$ mM	171	159	159	159
$[Cl^-]$ mM	128	116	116	121
BWW 380				
Ionic strength	0.207	0.177	0.177	0.192
Measured Osm. (mmol/kg)	360	377	377	377
$[Na^+]$ mM	189	159	159	159
$[Cl^-]$ mM	146	116	116	146
BWW 420				
Ionic strength	0.237	0.177	0.177	0.205
Measured Osm. (mmol/kg)	423	413	413	420
$[Na^+]$ mM	219	159	159	159
$[Cl^-]$ mM	176	116	116	173
BWW 460				
Ionic strength	0.267	0.177	0.177	0.219
Measured Osm. (mmol/kg)	487	455	455	458
$[Na^+]$ mM	249	159	159	159
$[Cl^-]$ mM	206	116	116	200
BWW 500				
Ionic strength	0.291	0.177	0.177	0.230
Measured Osm. (mmol/kg)	528	492	492	496
$[Na^+]$ mM	273	159	159	159
$[Cl^-]$ mM	230	116	116	223

Table 4.4. The various media used in isotonicity experiments. Osm, osmolality.

Permeability of the Plasma Membrane to Osmolytes

To examine the spermatozoon's permeability to various potential osmolytes, compounds were added to BWW made up to 280 mmol/kg by decreasing the concentration of NaCl from 108 mM (as in BWW_{330}) to 83 mM. Only one osmolyte, at a concentration of 250 mM, was present in the media in each experiment. The mean (± SD) osmolality of these solutions was 533.7 ± 11.0 mmol/kg (n=39), an osmolality chosen based on the results of the isotonicity experiments.

The following is a list of the osmolytes tested (abbreviation in Figs., see Chapter 5) [formula weight (d)/density (g/ml)]: acetamide (Ace) [59], adonitol (Ado) [152], L-arabitol (LAr) and D-arabitol (Dar) [152], D-arabinose (Ara) [150], betaine (Bet) [154], L-carnitine HCl (Car) [161], dimethyl sulfoxide (DMS) [78/1.1], D-dulcitol (Dul) [182], *meso*-erythritol (mEr)

[122], ethyl(hydroxymethyl)propane (EHM) [134], ethylene glycol (EG) [62/1.11], formamide (For) [45/1.13], fructose (Fru) [180], glucose (Glc) [180], Na and K glutamate (Glt) [147], glutamine (Gln) [146], Cd-free glycerophosphocholine (GPC) [257], glycerol (GLY) [92/1.26], glycine HCl (Gly) [75], *myo*-inositol (mIn) [180], lactose (Lac) [360], L-lysine (Lys) [146], maltose (Mal) [360], D-mannitol (Man) [182], melibiose (Mel) [342], L-proline (Pro) [115], (S)-propane-1,2-diol (PD2) [76/1.01], propane-1,3-diol (PD3) [76/1.05], raffinose (Raf) [595], sarcosine (Sar) [89], serine (Ser) [105], D-sorbitol (Sor) [182], sucrose (Suc) [342], taurine (Tau) [125], trehalose (Tre) [378], tris(hydroxymethyl)ethane (THM) [120], thiourea (Thi) [76], and urea (Ure) [60].

Spermatozoa were incubated at 37°C in 5% (v/v) CO_2 for 75 min in the absence and presence of 0.8 mM quinine HCl. Swelling under isotonic conditions indicates the diffusion of a permeant compound drawing in osmotically-obligated water, and quinine-sensitivity of the channels mediating influx would be revealed by different cell sizes in the presence and absence of this inhibitor.

Osmotic Responses of Spermatozoa from Different Strains of Mice

Spermatozoa from two strains of mice, C57BL6 and B6D2F1, were exposed to media of varying degrees of hypotonicity. The basal BWW medium (see above) was modified with NaCl or sucrose to create solutions with osmolalities between 160 and 530 mmol/kg (see Table 4.5). To evaluate the effect of Cl, the NaCl content of the basal BWW (330 mmol/kg) was replaced with Na gluconate. The Na^+ and Cl^- concentrations and ionic strength of these media are shown in Table 5. Volume responses of PI-intact cells were also monitored with time for spermatozoa from the two strains of mice of differing post-thaw fertility, B6D2F1 having a high fertility and C57BL6 having low fertility (Songsasen and Leibo 1997).

Epididymal spermatozoa were collected as described in the inhibitor experiments and were incubated in the appropriate media with flow cytometric measurements (same settings as in the inhibitor experiments) being recorded every 15 min for 2 h to create an osmotic response time profile.

Osmolality mmol/kg	[solute] mM	[Na$^+$] mM	[Cl$^-$] mM	Ionic Strength
	NaCl			
530	227	278	235	0.296
330	108	159	116	0.177
245	6.25	58	14	0.075
160	0.1	51	8	0.069
	Sucrose			
530	322	65	22	0.083
330	172	65	22	0.083
245	93	65	22	0.083
160	2.5	65	22	0.083
	Na gluconate			
330	0	159	116	0.177
330	58	159	58	0.177
330	87	159	29	0.177
330	101	159	15	0.177

Table 4.5. The various media used to profile the osmotic response of C57BL6 and B6D2F1 mice.

Statistics

Comparisons between the forward scatter measurements from spermatozoa incubated in different inhibitors were performed using a one-way repeated measures analysis of variance (ANOVA) followed by Dunnett's *post hoc* test against the control. For data that were not normally distributed, one-way ANOVA on ranks was followed by Dunnett's *post hoc* test against the control. Comparisons of the non-sperm cells and spermatozoa with cytoplasmic droplets before and after Percoll centrifugation were made using the Mann-Whitney Rank sum test. Values were considered significantly different with P < 0.05.

The change in osmolality during liquefaction of human semen was examined for each sample by linear regression. One-way ANOVA on ranks was performed to determine differences with time for all the samples. Differences were accepted with P< 0.05.

For evaluation of the repeatability of the response to quinine for a given monkey, mouse, or man, one-way repeated measures ANOVA was performed. The mean squares (MS) values generated in this analysis were used to calculate the intraclass correlation coefficient (an

index of reliability of a given parameter of a single man). The following is the applicable formula (Sokal and Rolf 1995):

Intraclass correlation coefficient (r_i)
= [(MS between - MS within)/2] / {[(MS between – MS within)/2] + MS within}

Differences in sperm kinetic data for monkey and human spermatozoa were compared by one way ANOVA for repeated measures, followed by Dunnett's post-hoc test. Volume changes by various treatments were expressed as a ratio of each control value by one way ANOVA followed by a Dunnett's post-hoc test or for non-normal distributions by the nonparametric Kruskal–Wallis one way ANOVA on ranks followed by Dunn's test.

Null points for isotonicity experiments were determined by linear regression analysis. Effects of quinine on penetration and the size differences at different osmolalities were examined by paired t-tests. Significant changes in size with time were tested with one way ANOVA (performed on Ranks when not normally distributed or with unequal variance). Comparisons between mouse strains with time were made with two way ANOVA (SigmaStat, Version 2.03, SPSS Inc., Erkrath, Germany). Differences were considered to be significant when $P<0.05$.

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Chapter 5

Results

Volume Regulation of Spermatozoa

Volume regulation was studied by subjecting epididymal and ejaculated spermatozoa to physiologically relevant osmotic challenges and measuring their volume (forward scatter) by flow cytometry in the absence and presence of putative inhibitors of potassium channels that operate to control volume regulation of cells. As spermatozoa should not need to regulate their volume under isotonic conditions, regardless of the presence of quinine (Kulkarni and others 1997, Petrunkina and others 2001), persistent cell swelling in the presence of quinine during hypotonic stress was taken to indicate the inhibition of active volume regulation for monkey, murine, and human spermatozoa. BWW_{330} with 0.8 mM quinine was used as the positive control for murine spermatozoa on the basis of previous studies showing that these cells remain swollen when exposed simultaneously to quinine at this concentration and a hypotonic challenge (Yeung and others 2002). Human and monkey spermatozoa were exposed to BWW_{290} and 0.3 mM quinine under hypotonic conditions as a positive control. This concentration of quinine was based on previous studies that showed no toxic effects at this concentration on human spermatozoa (Yeung and Cooper 2001, Yeung and others 2003).

Volume Regulation of Monkey Ejaculated Spermatozoa

Effects of channel inhibitors on volume and kinematic parameters

The osmolality of the exudate of ejaculates from 15 monkeys was 333 ± 6 mmol/kg (mean ± SEM). The size of ejaculated spermatozoa at 5 min incubation in BWW_{290} was no different from that examined after 30 min (1.0045 ± 0.0047, n = 26, expressed as ratios of the 5 min values) indicating that the spermatozoa were effectively volume regulating in media of 290 mmol/kg. Of the range of channel inhibitors tested in this medium at both time points, only quinine (0.3 mM) and 4-aminopyridine (0.4 mM) increased the sperm size examined at 30 min (Fig. 5.1). None of the specific K^+ channel inhibitors (margatoxin, charybdotoxin, dendrotoxin or clofilium) nor the less specific TEA or $BaCl_2$ affected volume.

The viability of ejaculated spermatozoa (% membrane intact) was not affected by any of the 22 different treatments at the various doses of tested blockers. The highest mean viability was achieved by the quinine treatment group (87 and 85% at 5 and 30 min, respectively), the lowest by the dendrotoxin group (70 and 71%) compared with control values maintained at 83 and 79%.

Figure 5.1. The effect of channel inhibitors on the size (expressed as ratio of non drug-treated control value, mean + SEM, ordinate) of monkey ejaculated spermatozoa incubated for 30 min in quinine (QUI), 4-aminopyridine (4AP), tetraethylammonium (TEA), $BaCl_2$ (Ba), margatoxin (MTX), charybdotoxin (CTX), dendrotoxin-α (DTX), apamin (APM), glybenclamide (GLY), and clofilium (CLO). Inhibitors and concentrations given on the the abscissa. * significantly different from drug-free controls ($p < 0.05$).

In addition to causing an increase in cell volume, quinine and 4AP altered sperm kinematic parameters (Table 5.1). Neither inhibitor had a significant effect on sperm vigor (curvilinear velocity, VCL) but both depressed straightness (STR), linearity (LIN), and beat cross frequency (BCF) and increased the amplitude of lateral head displacement (ALH), indicative of reduced forward progression. Quinine additionally depressed average path velocity (VAP) and straight line velocity (VSL). With the other blockers tested, no effects on sperm kinematic parameters could be detected (data not shown).

	Control	QUI (0.3 mM)	4AP (4 mM)
VAP (μm/s)	102.6 ± 6.6	85.4 ± 4.1*	85.9 ± 6.2
VSL (μm/s)	86.4 ± 5.6	53.5 ± 3.2*	61.4 ± 6.3
VCL (μm/s)	128.8 ± 6.1	137.4 ± 5.2	121.7 ± 5.8
ALH (μm/s)	5.1 ± 0.2	8.9 ± 0.4*	6.8 ± 0.4*
BCF (Hz)	31.9 ± 0.4	23.9 ± 0.6*	29.0 ± 1.3*
STR	91.5 ± 0.5	71.1 ± 1.6*	76.1 ± 4.2*
LIN	72.5 ± 1.7	40.1 ± 1.8*	51.9 ± 4.7*
Motility (%)	48.5 ± 6.4	60.3 ± 4.8	57.2 ± 5.9

Table 5.1. Effects of channel inhibitors on kinematic parameters of monkey ejaculated spermatozoa; mean ± SEM, n=25 (QUI), n=7 (4AP), * $p < 0.05$ compared to control.

Repeatability of monkey ejaculated sperm responses to quinine

The intra-class correlation coefficients calculated on the repeated measurements of relative cell size for control and quinine-treated cells for a given monkey were generated using the average forward scatter value of cells with intact plasma membranes as indicated by propidium iodide fluorescence. The coefficient for control (0.494) and quinine spermatozoa (0.262) indicate that there were large differences between ejaculates from the same individual in terms of the volume of the spermatozoa and that these differences were exaggerated when the cells were exposed to quinine. Figure 5.2 displays the forward scatter values recorded on replicate semen samples from the same monkey. The differences between collections from a given monkey are apparent (i.e. approximately 40 channel numbers difference in control measurements for monkey 3 and for measurements in the presence of quinine for monkey 1).
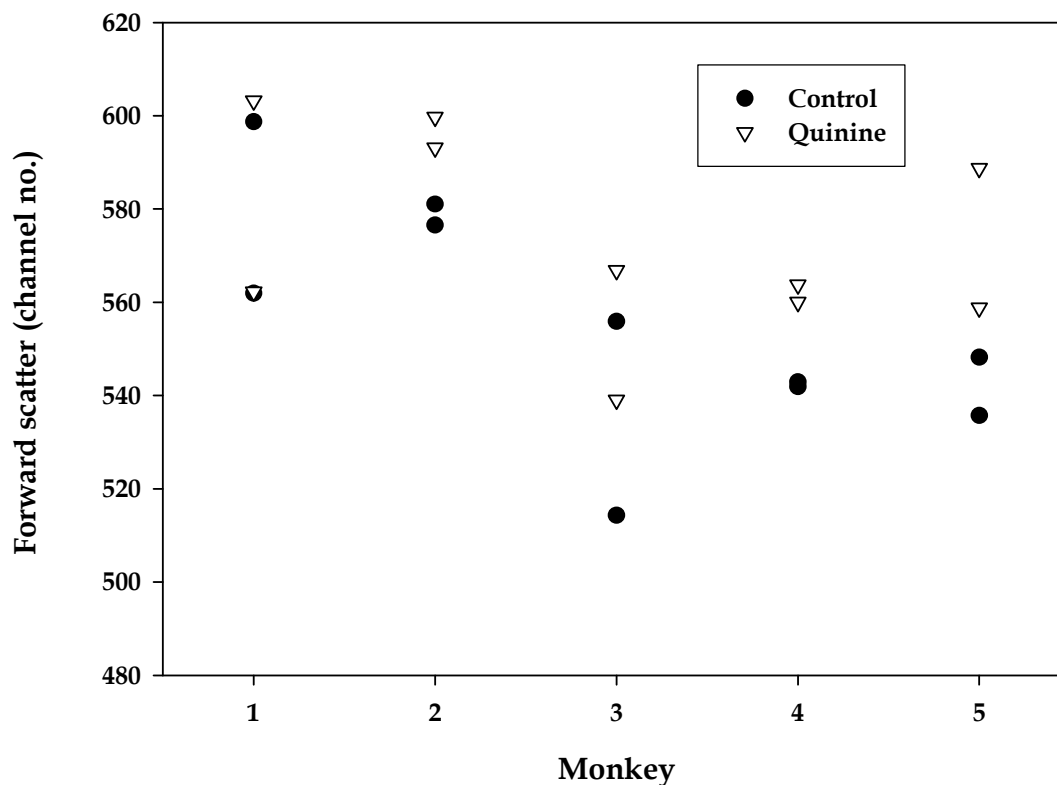
Figure 5.2. Repeated forward scatter measurements (y-axis) for ejaculated spermatozoa incubated in control (●) medium or in the presence of quinine (∇) for 5 monkeys (x-axis). Coefficients of variation for control and quinine treated samples were 3.9% and 3.0%, respectively.

Western blotting

Although no specific channels involved in RVD by monkey spermatozoa could be identified, western blotting was performed on monkey sperm proteins for the channels potentially involved in murine and human spermatozoa. No specific bands were detected for any of the channels tested (see methods for western blotting of murine and human spermatozoa).

Volume Regulation of Murine Epididymal Spermatozoa

Effects of channel inhibitors on volume

Preliminary time course experiments indicated that changes in the volume of control- and quinine-treated spermatozoa after incubation in hypotonic medium appeared to stabilize after approximately 75 min (data not shown); thus the secondary incubation time point of 75 min was chosen for examining the effects of channel blockers. After 5 min incubation two inhibitors caused a significant difference in forward scatter. Murine spermatozoa incubated in quinine (0.8 mM) and 4-aminopyridine (4 mM) were significantly larger than the controls (Fig. 5.3).

Several inhibitors induced swelling of murine spermatozoa at 75 min, including quinine (0.8 mM), flecainide (100 µM), 4-aminopyridine (4 mM), cadmium (0.2 mM), barium (1 mM), phrixotoxin (100 nM), and clofilium (10 µM). Spermatozoa incubated in phlorizin (500 µM) produced significantly lower forward scatter values than those of the control (Fig. 5.4), whereas other inhibitors had no effect.

At 5 min there were no significant differences in the percentage of membrane intact spermatozoa between any of the inhibitors tested, as indicated by PI staining. Only flecainide (100 µM) and cadmium (0.2 mM) had significantly lower percentages of PI negative cells after 75 min incubation (control, 63.5 ± 1.8%; flecainide 45.5 ± 3.6%; cadmium 48.0% ± 4.0).

Repeatability of murine epididymal sperm responses to quinine

The intra-class correlation coefficients calculated on the repeated measurements of relative cell size for control and quinine-treated cells for a given mouse were generated using the average forward scatter value of cells with intact plasma membranes as indicated by PI fluorescence. These values, 0.551 and 0.538 respectively, indicate that there were large

differences between spermatozoa collected from different epididymides from the same mouse in terms of the volume of spermatozoa whether treated with quinine or not. Forward scatter values recorded for spermatozoa from both epididymides of the same mouse are presented in Figure 5.5.
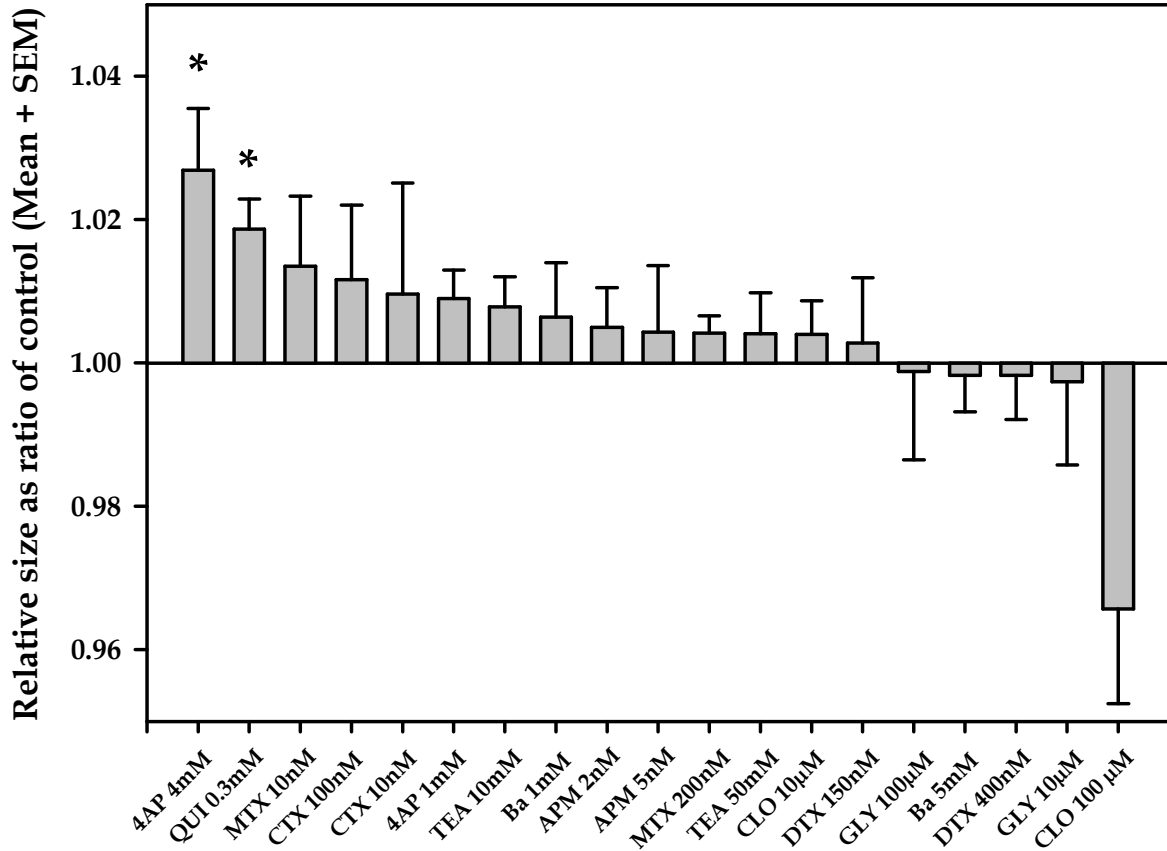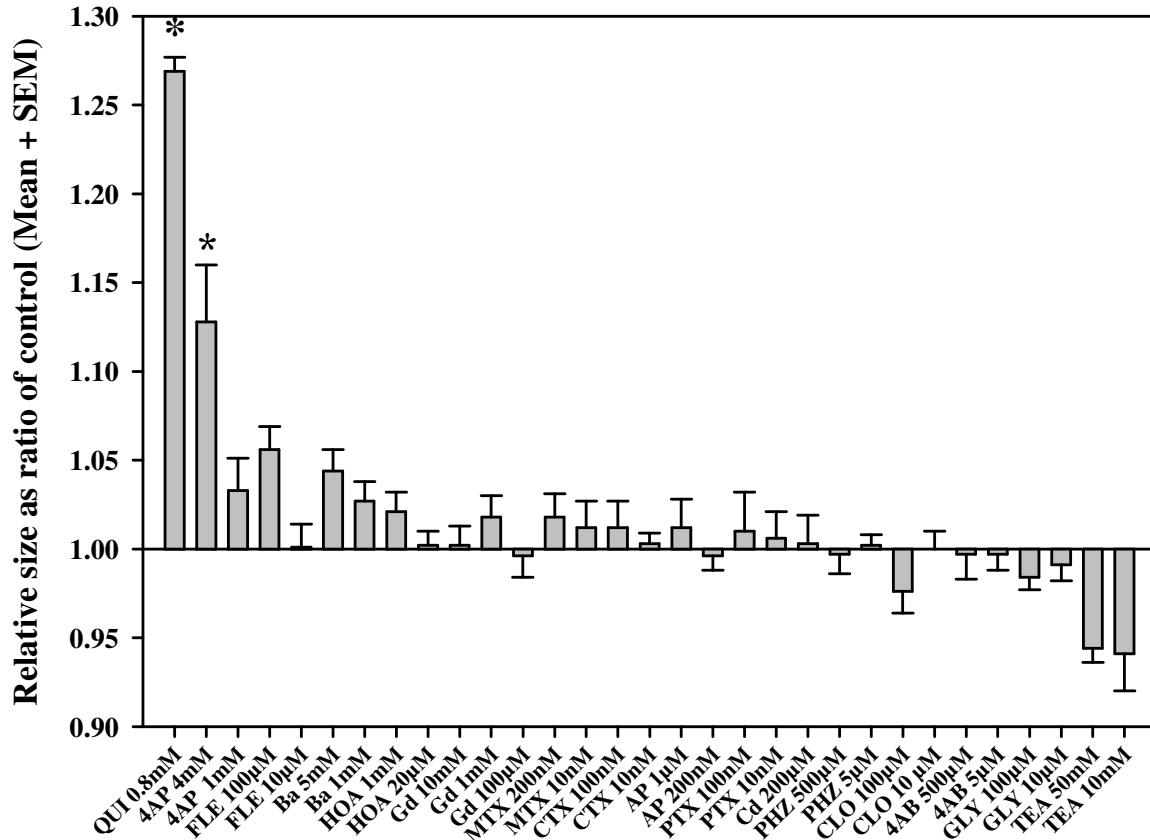

Figure 5.3. The effect of channel inhibitors on the size (expressed as ratio of non drug-treated control value, mean + SEM, ordinate) of murine epididymal spermatozoa incubated for 5 min in quinine (QUI), 4-aminopyridine (4AP), flecainide (FLE), cadmium (Cd), barium (Ba), clofilium (CLO), 4-aminobutyrate (4AB), phrixotoxin (PTX), threo-β-hydroxyaspartate (HOA), gadolinium (Gd), margatoxin (MTX), charybdotoxin (CTX), apamin (AP), phlorizin (PHZ), glybenclamide (GLY), and tetraethylammonium (TEA). Inhibitors and concentrations given on the abscissa. N = 56 for quinine, 4-10 for other inhibitors. *, significantly different from drug-free controls (p < 0.05). Spermatozoa were incubated in hypotonic solutions for 5 min in the presence of one of the listed inhibitors and then size measured by flow cytometry.
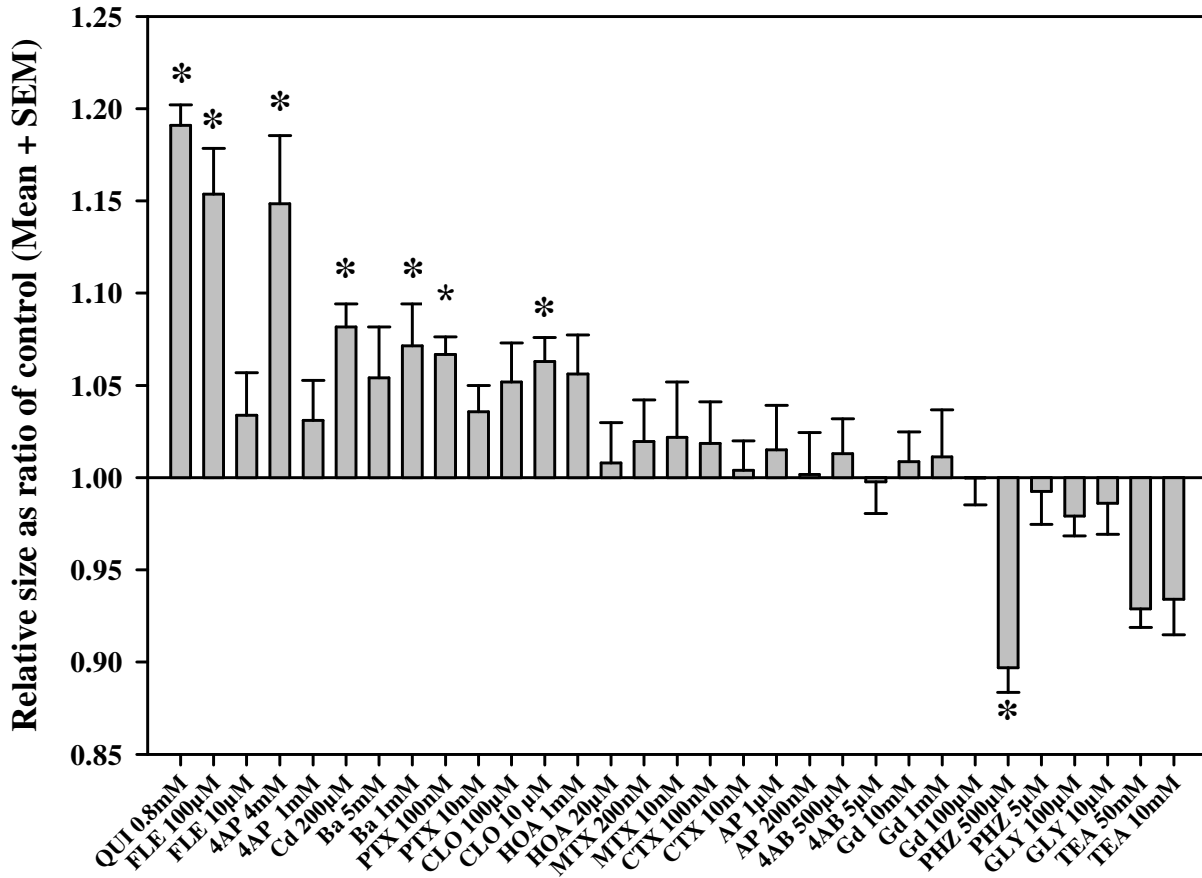
Figure 5.4. The effect of channel inhibitors on the size (expressed as ratio of non drug-treated control, mean + SEM, ordinate) of murine epididymal spermatozoa. See Figure 5.3 for abbreviations of inhibitors. Concentrations given on the abscissa. N = 56 for quinine, 4-10 for other inhibitors. *, significantly different from drug-free controls (p < 0.05). Spermatozoa were incubated in hypotonic solutions for 75 min in the presence of one of the listed inhibitors and then size measured by flow cytometry.
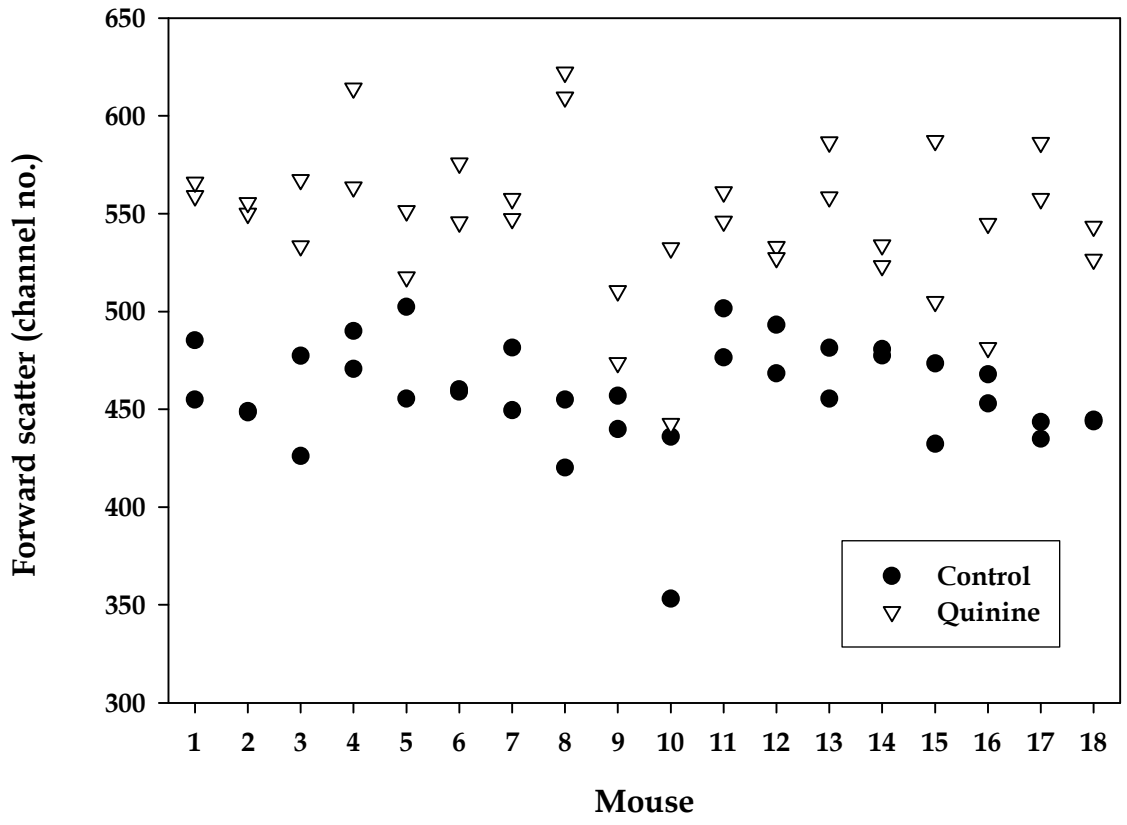
Figure 5.5. Repeated forward scatter measurements for spermatozoa taken from each epididymis of 18 different mice and incubated in control (●) medium or in the presence of quinine (∇). Coefficient of variation for control spermatozoa was 4.9% and for quinine-treated samples 5.8%.

Effects of pH on murine sperm RVD

        After 5 min incubation, murine spermatozoa incubated in drug-free BWW_{330} at pH 6.3 or at pH 8.4 in Hepes buffer were the same size as those at pH 7.4, but those incubated at pH 8.4 in Tris were significantly larger. Results were the same after 75 min incubation (Table 5.2). The viability of spermatozoa was not significantly different at different pH values at either time point.

pH	Buffer	5 min	75 min	n
6.3	Mopso	1.01 ± 0.00	1.10 ± 0.03	7
7.4	Hepes	1.00 ± 0.00	1.00 ± 0.00	11
8.4	Hepes	1.00 ± 0.01	0.99 ± 0.04	4
8.4	Tris	1.11 ± 0.01*	1.33 ± 0.04*	11

Table 5.2. Relative volumes of murine spermatozoa at 330 mmol/kg at different pH (expressed as ratio of pH 7.4 control) after 5 and 75 min incubation. Values are mean ± SEM. *, $P < 0.05$ compared to pH 7.4 at each time point.

Western blotting

        Two specific bands detected by the antibody against Kv1.5 protein were visible and estimated to be of sizes 85 and 76 kDa but were not present when the antibody was adsorbed with antigen (Fig. 5.6, lanes 1-4). Incubation of sperm membrane proteins with a TASK2 antibody also yielded two specific bands, at 113 and 99 kDa, which were not present on membranes incubated with the pre-adsorbed antibody (Fig. 5.6, lanes 5-8). When exposed to minK antibody, three bands appeared at 23, 20, and 12 kDa. However, the band at 12 kDa was not completely absent in the presence of the adsorbed antibodies despite the increased antigen concentration (Fig. 5.6, lanes 10, 12). No specific bands were observed when membranes were incubated with antibodies against Kv1.4, Kv4.2, Kv4.3 and TASK3 (data not shown).
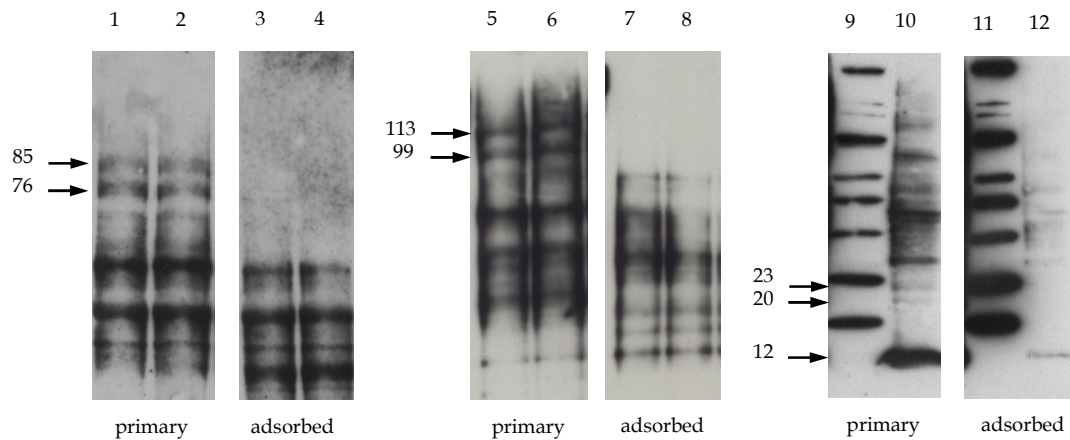
Figure 5.6. Identification of Kv1.5, TASK2, and minK channels in murine epididymidal spermatozoa by Western blotting. Lanes 1 and 2 were incubated with anti-Kv1.5, lanes 3 and 4 with adsorbed anti-Kv1.5 (pre-incubated with the control peptide antigen), lanes 5 and 6 with anti-TASK2, and lanes 7 and 8 with adsorbed anti-TASK2. Lanes 9 and 11 were loaded with molecular weight markers. Lane 10 represents proteins exposed to anti-minK and lane 12 to adsorbed anti-minK. Specific bands are indicated by arrows and the determined molecular weight (kDa).

Immunolocalization

Kv1.5, TASK2, and minK proteins were detected by immunocytochemistry. Washed and unwashed spermatozoa exposed to anti-Kv1.5 antibodies displayed similar patterns of fluorescence (Fig. 5.7). The signal was strongest at the connecting piece and faint, patchy fluorescence characterized the length of the flagellum. Adsorption of Kv1.5 antibodies resulted in loss of fluorescence at the connecting piece on washed and unwashed spermatozoa.

There was also no difference between washed and unwashed spermatozoa exposed to TASK2 antibodies. These cells exhibited a faint fluorescence along the length of the cell with a brighter signal on the midpiece, and a bright, concentrated signal from the connecting piece, and droplet when present (Fig. 5.8).

Washed spermatozoa incubated with anti-minK antibody exhibited a light fluorescence along the length of the flagellum, patchy fluorescence over the midpiece, and a bright fluorescence at the connecting piece (Fig. 5.9). Unwashed spermatozoa displayed a similar pattern but with strong fluorescence on the cytoplasmic droplet and connecting piece. When exposed to adsorbed minK antibodies, fluorescence of the droplet and connecting piece was lost and a faint glow was seen along the length of the cell for both washed and unwashed cells.
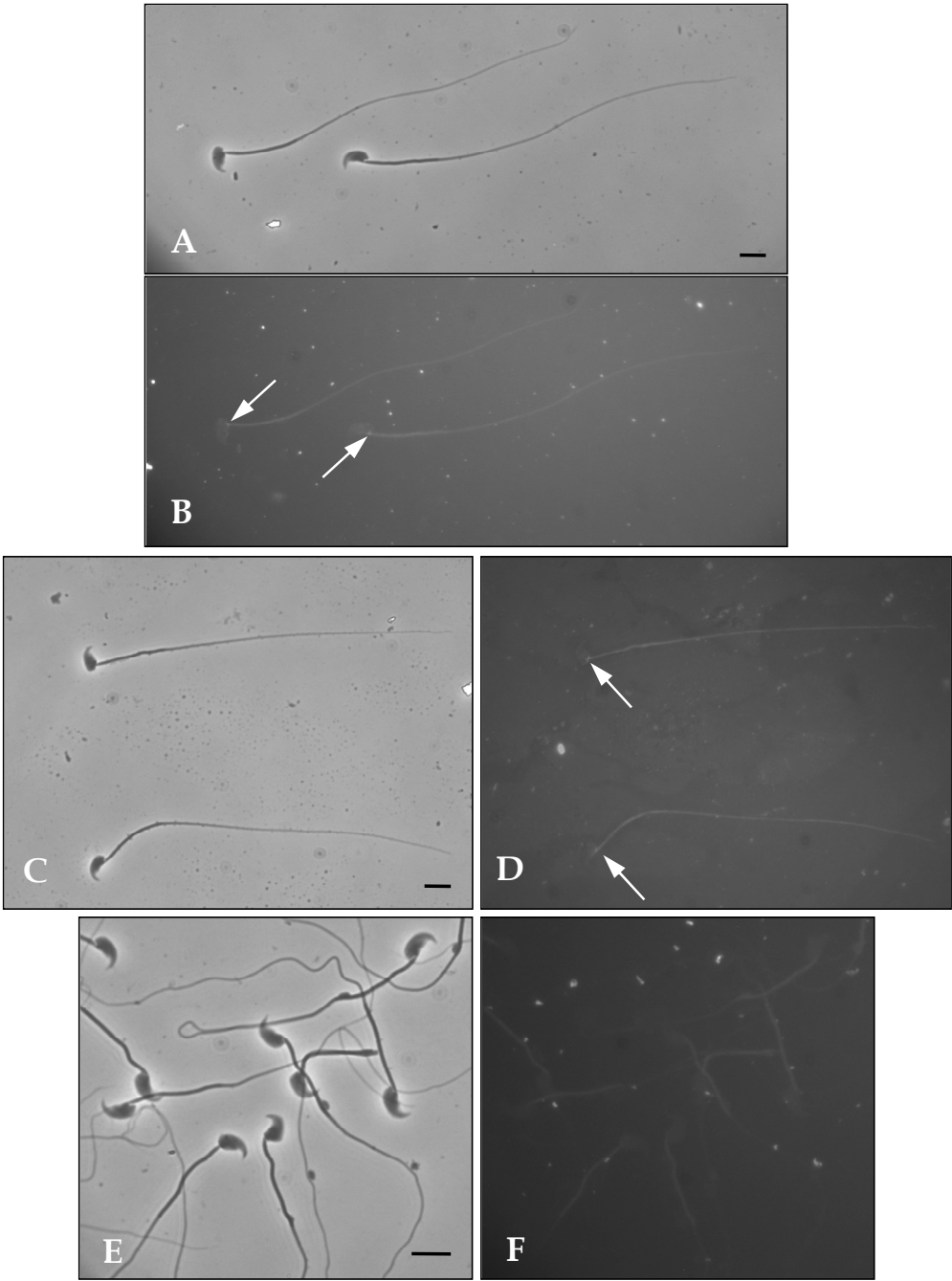
Figure 5.7. Immunolocalization of Kv1.5 proteins on murine epididymal spermatozoa. Frames A, C, and E are phase contrast images of the corresponding fluorescence images B, D, and F. Images A and B are of washed spermatozoa incubated with anti-Kv1.5, while images C and D are of unwashed spermatozoa. Images E and F are of washed spermatozoa exposed to adsorbed Kv1.5 antibodies. Arrows in B and D, connecting piece. Scale bar = 10 μm.
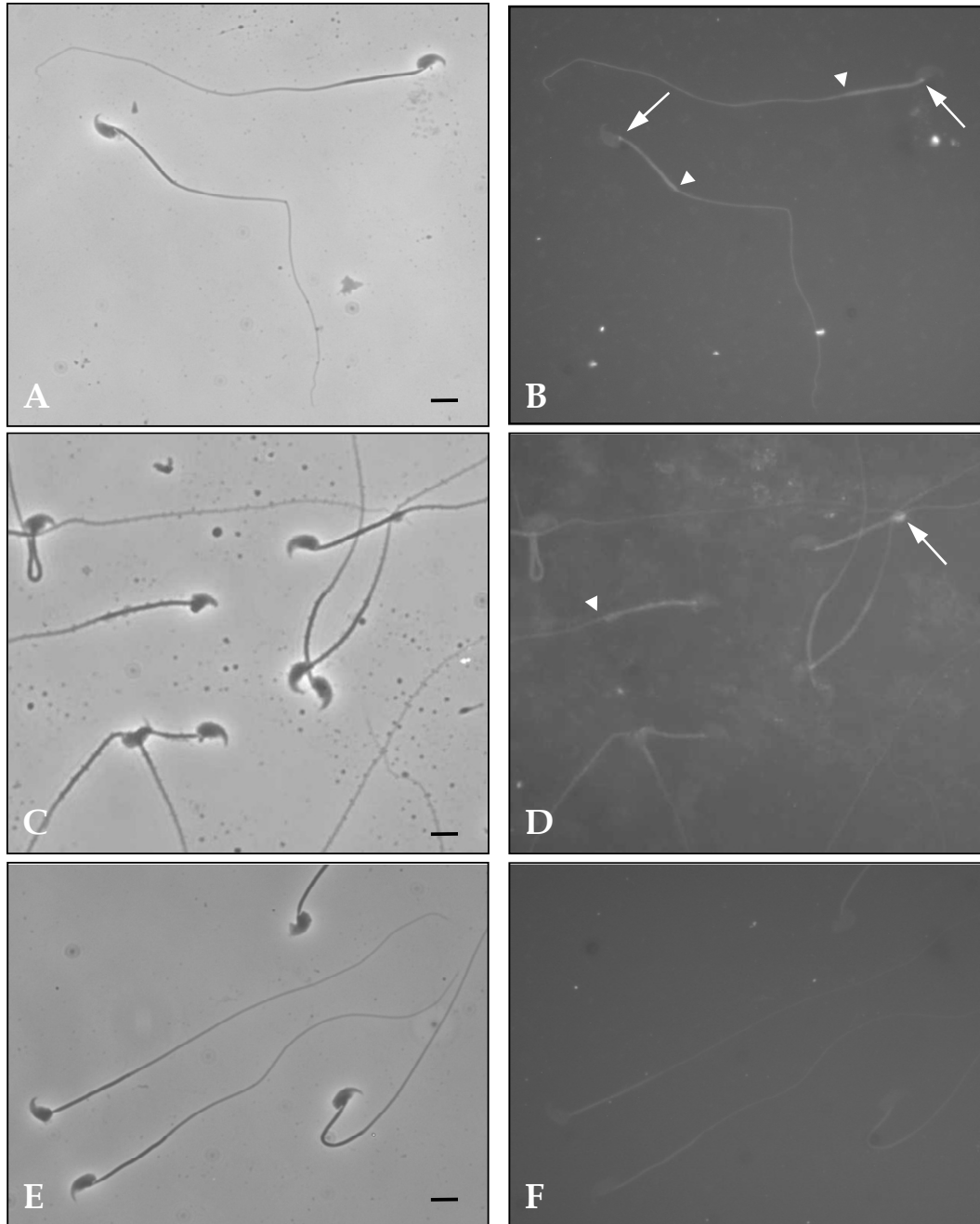
Figure 5.8. Immunolocalization of TASK2 proteins on murine epididymal spermatozoa. Frames A, C, and E are phase contrast images of the corresponding fluorescence images B, D, and F. Images A and B are of washed spermatozoa incubated with anti-TASK2, while images C and D are of unwashed spermatozoa. Images E and F are of washed spermatozoa exposed to adsorbed TASK2 antibodies. Arrows in B, connecting piece; arrow in D, cytoplasmic droplet; arrowheads in B and D, end of midpiece. Scale bar = 10 μm.
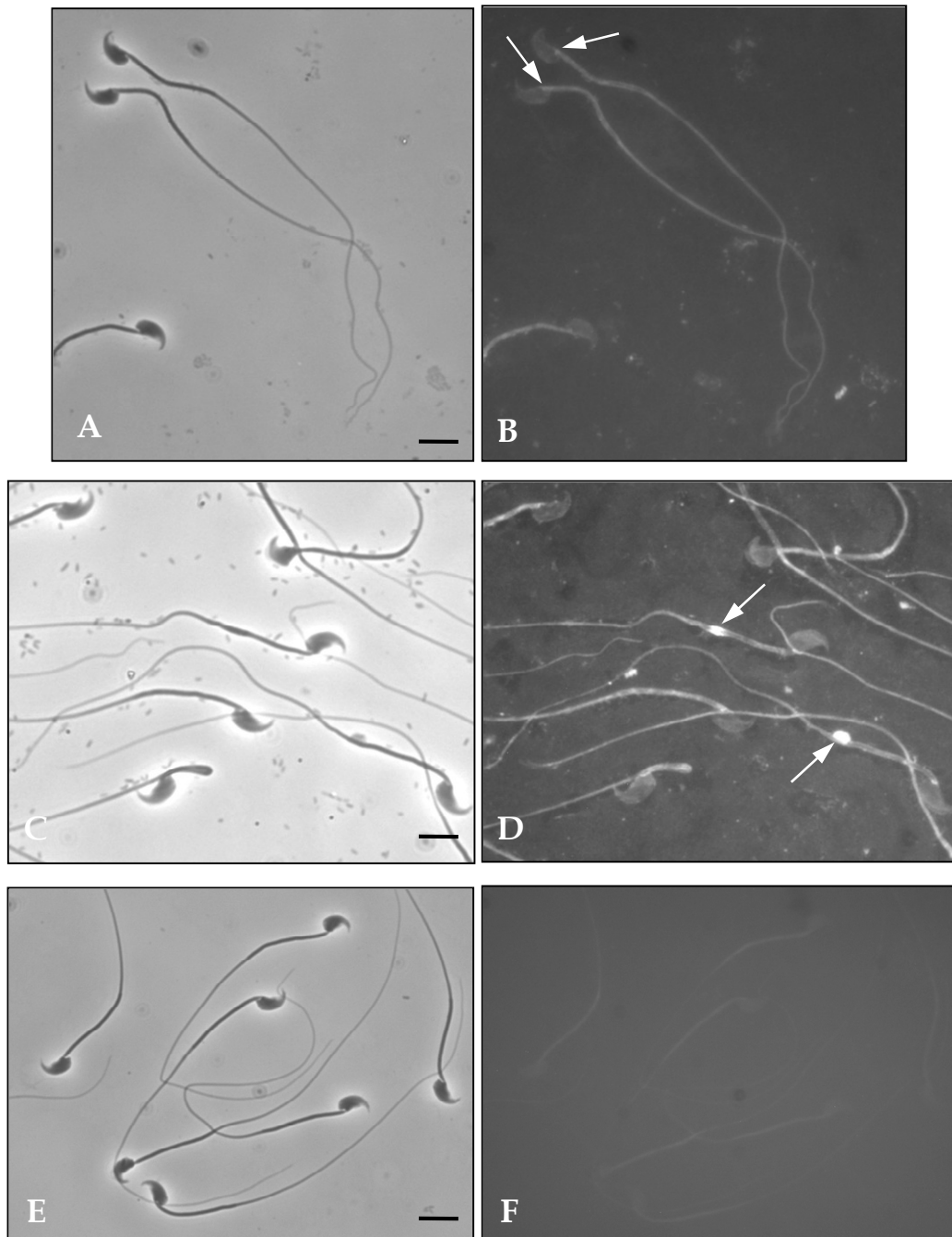
Figure 5.9. Immunolocalization of minK proteins on murine epididymal spermatozoa. Frames A, C, and E are phase contrast images of the corresponding fluorescence images B, D, and F. Images A and B are of washed spermatozoa incubated with anti-minK, while images C and D are of unwashed spermatozoa. Images E and F are of washed spermatozoa exposed to adsorbed minK antibodies. Arrows in B, connecting piece; arrows in D, cytoplasmic droplets. Scale bar = 10 μm.

Volume Regulation of Human Spermatozoa

Measurement of seminal osmolality during liquefaction

Within 5 min of being ejaculated, the osmolality of human semen was low (mean ± SEM, 294 ± 4 mmol/kg, range 269-311) and increased during the time of liquefaction (Fig. 5.10). There were statistically significant increases in osmolality with time for 14 of the 18 semen samples. By the time of complete liquefaction (30 min), the osmolality (312 ± 5 mmol/kg, range 280-311) was in the range reported previously post-liquefaction (Yeung and others 2003). The osmolality was significantly lower at 5 min than that obtained at 25 and 30 min. Two men provided two ejaculates the osmolality of which increased significantly during liquefaction on both occasions, and one man provided two samples but the osmolality of only one increased significantly with time.
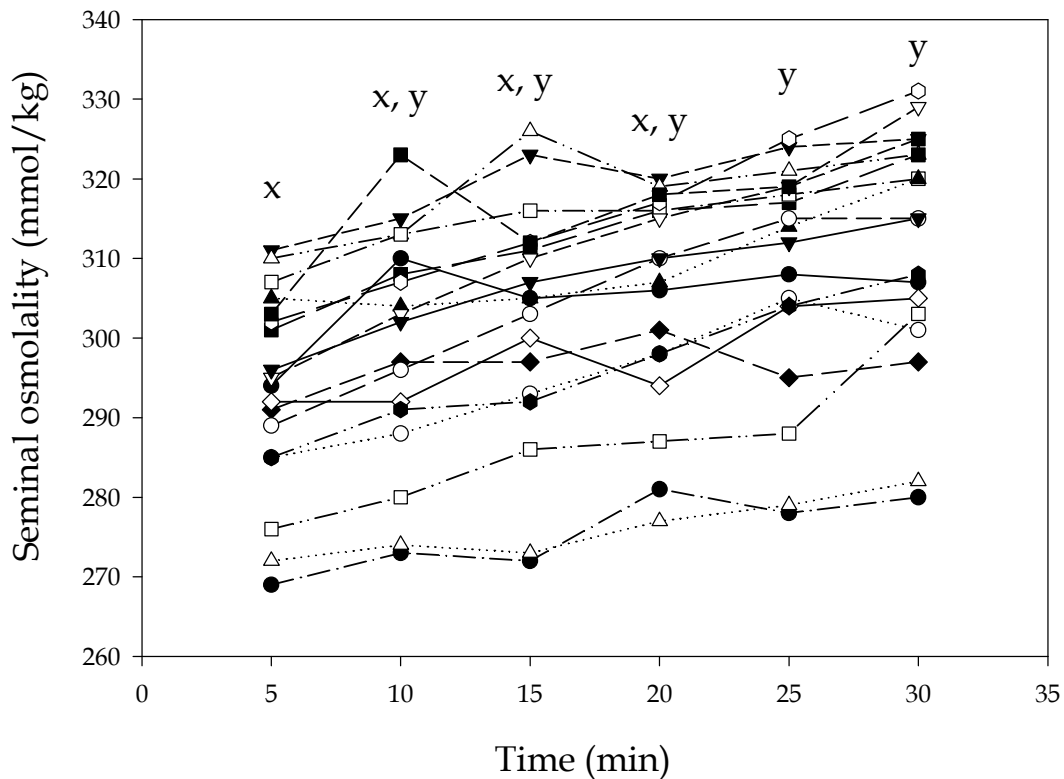
Figure 5.10. The osmolality of liquefying human semen (ordinate) measured within minutes of production and during liquefaction at 37°C (abscissa). Each line and symbol combination represents an ejaculate from one individual. Significant differences between the average osmolality calculated for all ejaculates are distinguished with x and y superscripts (p < 0.05). Mean seminal osmolality is significantly different at time points that do not share a common superscript.

Purification of sperm samples by Percoll gradient centrifugation

All semen samples were washed through an 80% / 40% (v/v) Percoll gradient adjusted to seminal osmolality. Washed samples had a statistically significant decrease of non-sperm particles (before, $15.0 \pm 2.1\%$; after, $1.4 \pm 0.2\%$; $p < 0.05$). In the same samples, there was also a significant reduction in the percentage of spermatozoa bearing a cytoplasmic droplet before and after Percoll gradient centrifugation (before, 34 ± 3; after 7 ± 1; $p < 0.05$).

Effects of channel inhibitors on volume and kinematic parameters

Spermatozoa from all men in these experiments were capable of volume regulation as indicated by the statistically significant (paired t-test, $p < 0.05$) increase in their forward scatter in the presence of quinine compared to the control (mean difference in channel number from the control $\pm$ SEM, 16 ± 2.8).

Human spermatozoa had significantly higher forward scatter values when incubated in 4-aminopyridine (4AP; 4 mM), clofilium tosylate (10 µM), and quinine (0.3 mM) after 30 minutes compared with control (Fig. 5.11), but there were no significant differences at 5 min (data not shown). There were also no significant differences in the percentage of spermatozoa with intact plasma membranes in the presence of the different inhibitors, as indicated by the exclusion of propidium iodide at either time point.

There were no significant differences in the percentage of motile spermatozoa incubated in the various inhibitors with the exception of cadmium (Table 5.3). Incubation in quinine or 4AP induced a hyperactivation-like motility in the spermatozoa. Quinine significantly increased VCL and both drugs significantly decreased VSL, STR, and LIN (Table 5.3). Clofilium also decreased VSL and STR. In addition, cadmium decreased VSL, VAP, and VCL, and barium significantly increased VAP without affecting the percentage of membrane-intact cells.
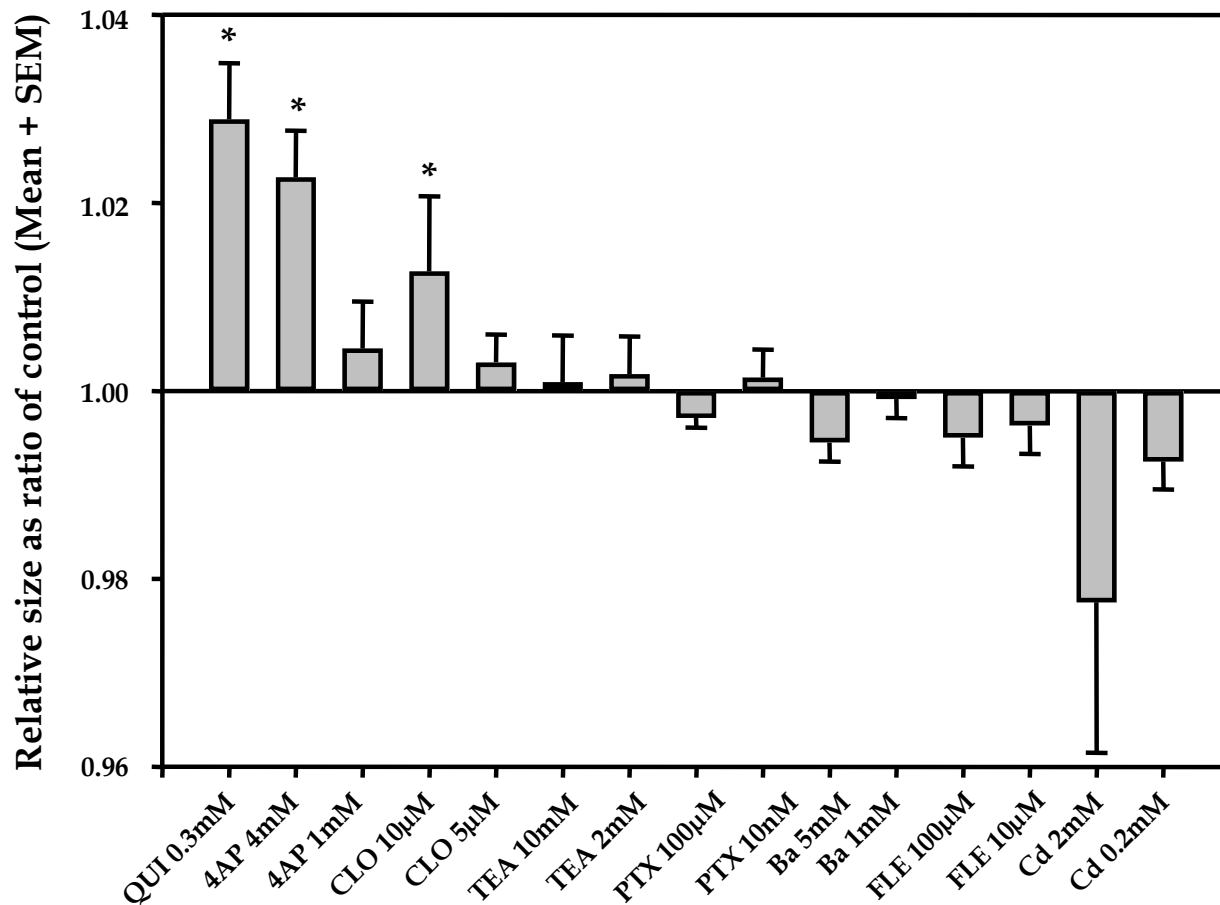
Figure 5.11. The effect of channel inhibitors on the size (expressed as ratio of non drug-treated control, mean + SEM, ordinate) of human ejaculated spermatozoa incubated for 30 min. See Figure 5.3 for abbreviations of inhibitors. Concentrations given on the abscissa. N = 19 for quinine, 7-11 for other inhibitors. *, significantly different from drug-free controls ($p < 0.05$).

	Control	QUI (0.3 mM)	4AP (4 mM)	Ba (5 mM)	CLO (10 μM)	Cd (2mM)
VAP (μm/s)	56.3 ± 1.8	56.6 ± 1.7	61.3 ± 2.8	62.6 ± 3.7*	56.6 ± 2.4	35.0 ± 2.5*
VSL (μm/s)	43.1 ± 2.0	23.2 ± 1.6*	38.3 ± 3.1*	46.6 ± 4.1	39.9 ± 3.0*	24.5 ± 1.5*
VCL (μm/s)	88.7 ± 2.4	106.0 ± 3.6*	102.7 ± 4.8	99.7 ± 5.9	94.7 ± 4.7	59.4 ± 3.3*
ALH (μm/s)	5.6 ± 0.3	7.6 ± 0.3*	7.2 ± 0.4	6.4 ± 0.5	6.3 ± 0.5	4.1 ± 0.5
BCF (Hz)	22.7 ± 0.9	20.0 ± 1.2*	21.9 ± 1.3	21.2 ± 0.9	21.0 ± 1.2	22.8 ± 1.1
STR	82.6 ± 2.0	46.7 ± 3.5*	71.0 ± 3.2*	81.3 ± 2.6	78.0 ± 3.5*	82.5 ± 2.6
LIN	49.4 ± 2.7	22.9 ± 1.3*	37.6 ± 3.1*	47.9 ± 3.7	44.8 ± 4.9	42.0 ± 3.5
Motility (%)	86.2 ± 2.0	86.0 ± 3.1	85.3 ± 3.4	88.3 ± 3.0	86.0 ± 2.9	37.1 ± 12.6*

Table 5.3. Effects of channel inhibitors on kinematic parameters of ejaculated human spermatozoa, mean ± SEM, n = 19 (control and QUI), n = 11 (4AP and Ba), n = 10 (CLO), n = 5 (Cd).

Repeatability of human ejaculated sperm responses to quinine

There was high repeatability of the forward scatter measurements of spermatozoa in separate ejaculates from the same individual. This was shown by the intra-class correlation coefficients generated using the average forward scatter value of cells with intact plasma membranes as indicated by PI fluorescence. These values were 0.946 for cells exposed to quinine and 0.952 for control cells. Forward scatter measurements recorded for spermatozoa from repeated semen samples of 11 men and incubated with and without quinine are presented in Figure 5.12.
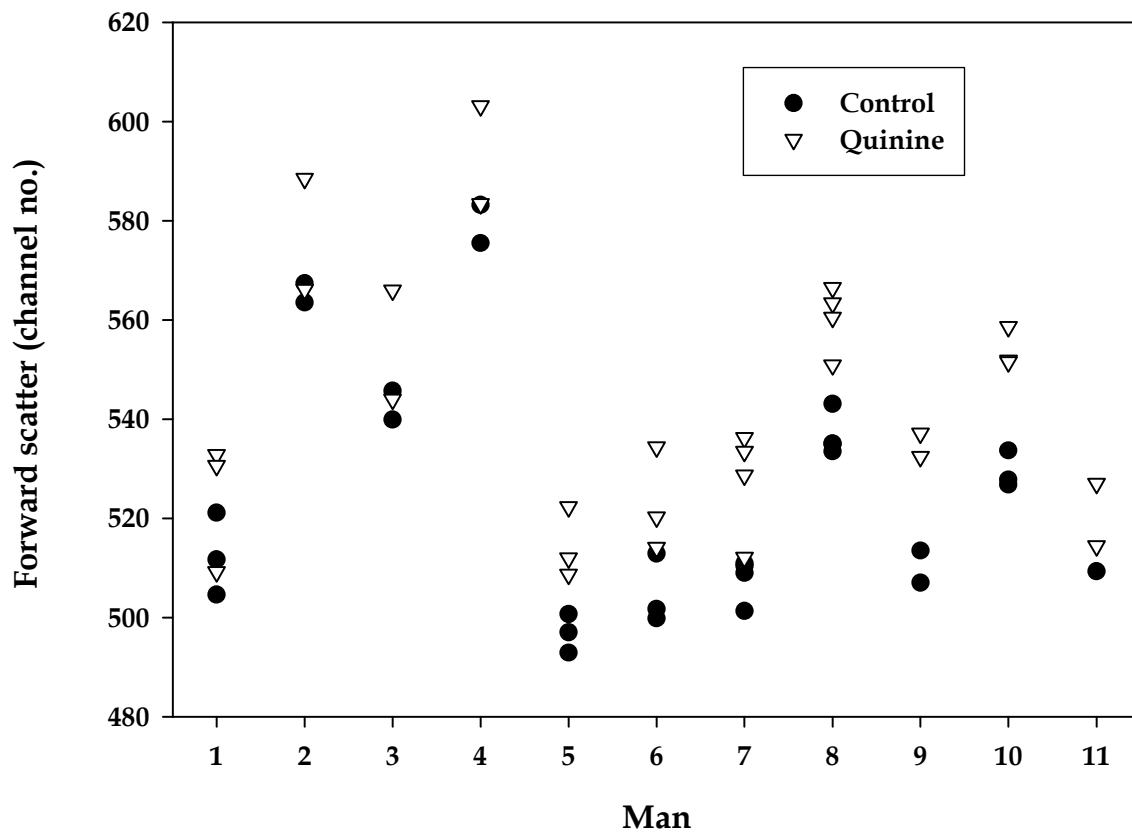
Figure 5.12. Repeated forward scatter measurements for spermatozoa taken from two to four ejaculates from 11 men and incubated in control (●) medium or in the presence of quinine (∇). Coefficients of variation for control and quinine-treated spermatozoa were 5.3% and 4.7%, respectively.

Effects of pH on volume regulatory decrease

After 30 min incubation, spermatozoa incubated in BWW$_{290}$ at acidic or alkaline pH without inhibitors were not significantly different in size from the control (cells in BWW$_{290}$ pH 7.4 without inhibitors) although cells in pH 8.4 tended to be larger (Table 5.4).

pH	Buffer	5 min	30 min	n
6.3	Mopso	0.998 ± 0.002	0.992 ± 0.003	7
7.4	Hepes	1.00 ± 0.00	1.00 ± 0.00	7
8.4	Hepes	1.009 ± 0.004	1.007 ± 0.007	5
8.4	Tris	1.008 ± 0.004	1.012 ± 0.0002	7

Table 5.4. Relative volumes of human spermatozoa in 290 mmol/kg media of different pH (expressed as ratio of pH 7.4 Hepes control) after 5 and 30 min incubation. Values are mean ± SEM.

Western blotting

Specific protein bands were defined as those not present on membranes incubated with the antibody that had been pre-adsorbed with that particular channel antigen. One specific band detected using an antibody against Kv1.5 protein was estimated to be of size 76 kDa (Fig. 5.13, lane 1). Antibodies against TASK2 also revealed specific bands determined to be 55 and 72 kDa (Fig. 5.13, lane 3). Exposure to anti-minK antibodies created a specific band at 14 kDa (Fig. 5.13, lane 5). Incubation of sperm membrane proteins with TASK3 antibodies yielded 2 specific bands of 49 and 75 kDa (Fig. 5.13, lane 7). No specific bands were observed when membranes were incubated with antibodies against Kv1.4, Kv4.2 or Kv4.3 (data not shown).
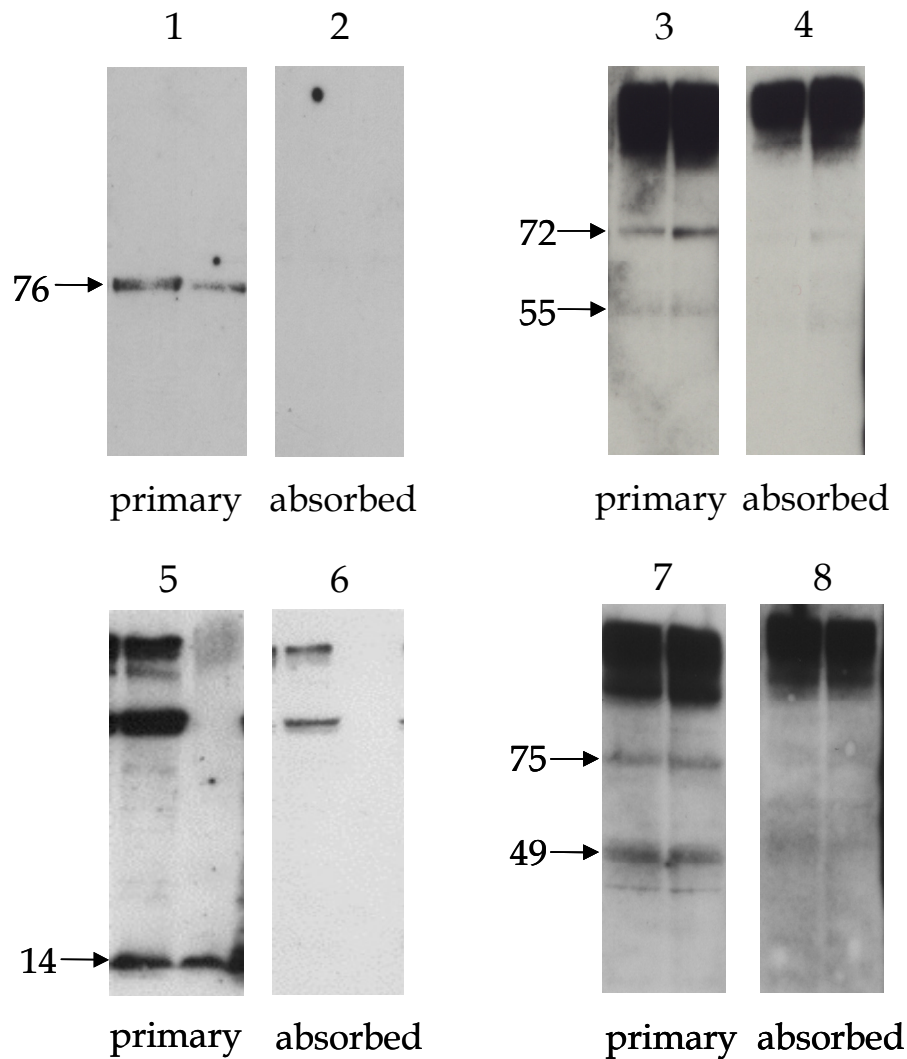
Figure 5.13. Identification of Kv1.5, TASK2, minK, and TASK3 channels in human ejaculated spermatozoa by Western blotting. Duplicate lanes under 1 were incubated with anti-Kv1.5, lanes under 2 with adsorbed anti-Kv1.5 (pre-incubated with the control peptide antigen), lanes under 3 with anti-TASK2, and lanes under 4 with adsorbed anti-TASK2. Lanes under 5 were incubated with anti-minK, lanes under 6 with adsorbed anti-minK, lanes under 7 with anti-TASK3, and lanes under 8 with adsorbed anti-TASK3. Specific bands are indicated by arrows and the determined molecular weight (kDa).

Immunolocalization

Kv1.5 was localized to the midpiece and posterior post-acrosomal region of the sperm head (Fig. 5.14). In the case that a cytoplasmic droplet was still present, there was an intense fluorescence at that site (inset in Fig. 5.14A). TASK2 appeared to be localized to the posterior post-acrosomal region and neck and possibly along the tail although this staining was very weak (Fig. 5.15). Some non-specific staining on the sperm head was still detected on sperm incubated with the adsorbed preparation. The protein minK was localized to the posterior post-acrosomal region of the head and also on the neck of the spermatozoon (Fig. 5.16). However, staining was weaker along the midpiece and became more intense again along the principal piece of the flagellum.
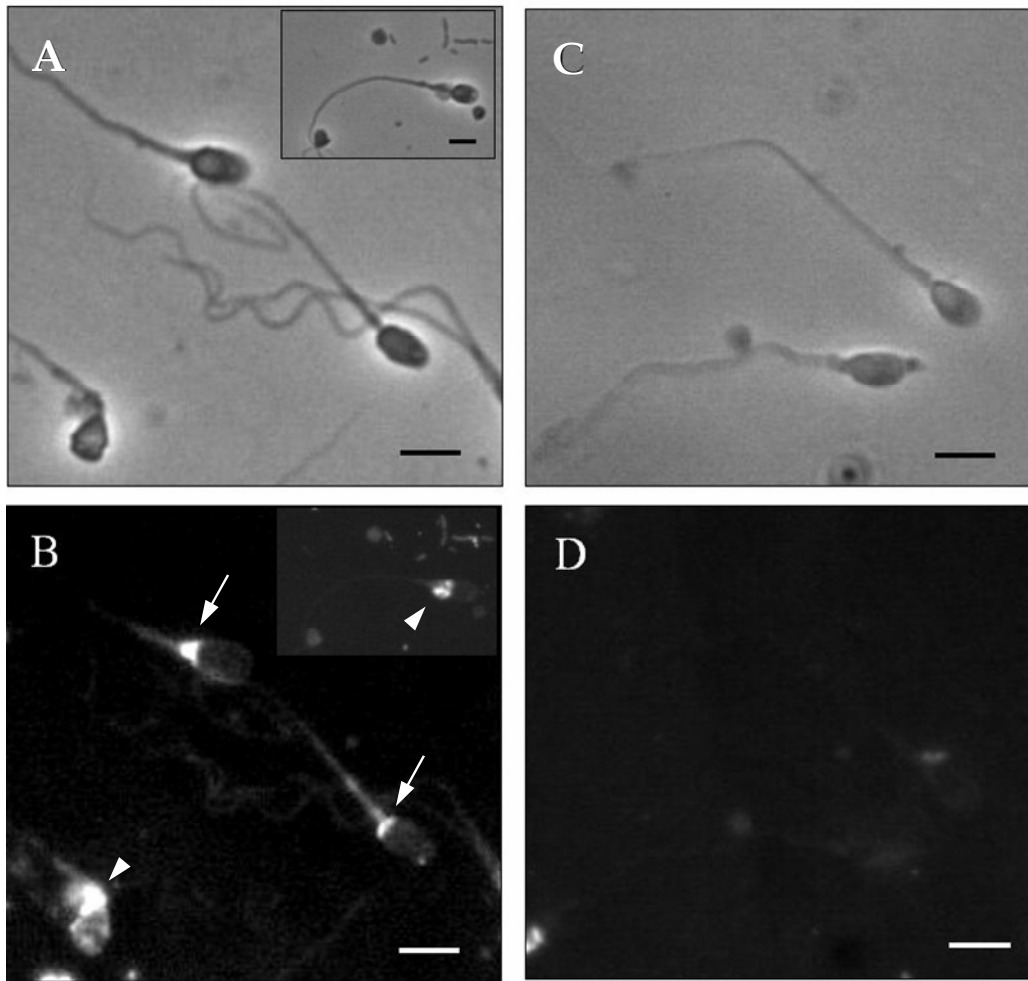
Figure 5.14. Immunolocalization of Kv1.5 on human ejaculated spermatozoa. Phase contrast (A and C) and fluorescence (B and D) images of spermatozoa incubated in primary (A and B) or adsorbed (C and D) antibody preparations. Arrows in B, post-acrosomal and neck regions; arrowheads in B, cytoplasmic droplet. Inset in frames A and B are the phase and fluorescence images of a spermatozoon with an intact cytoplasmic droplet. Scale bar = 5 μm.
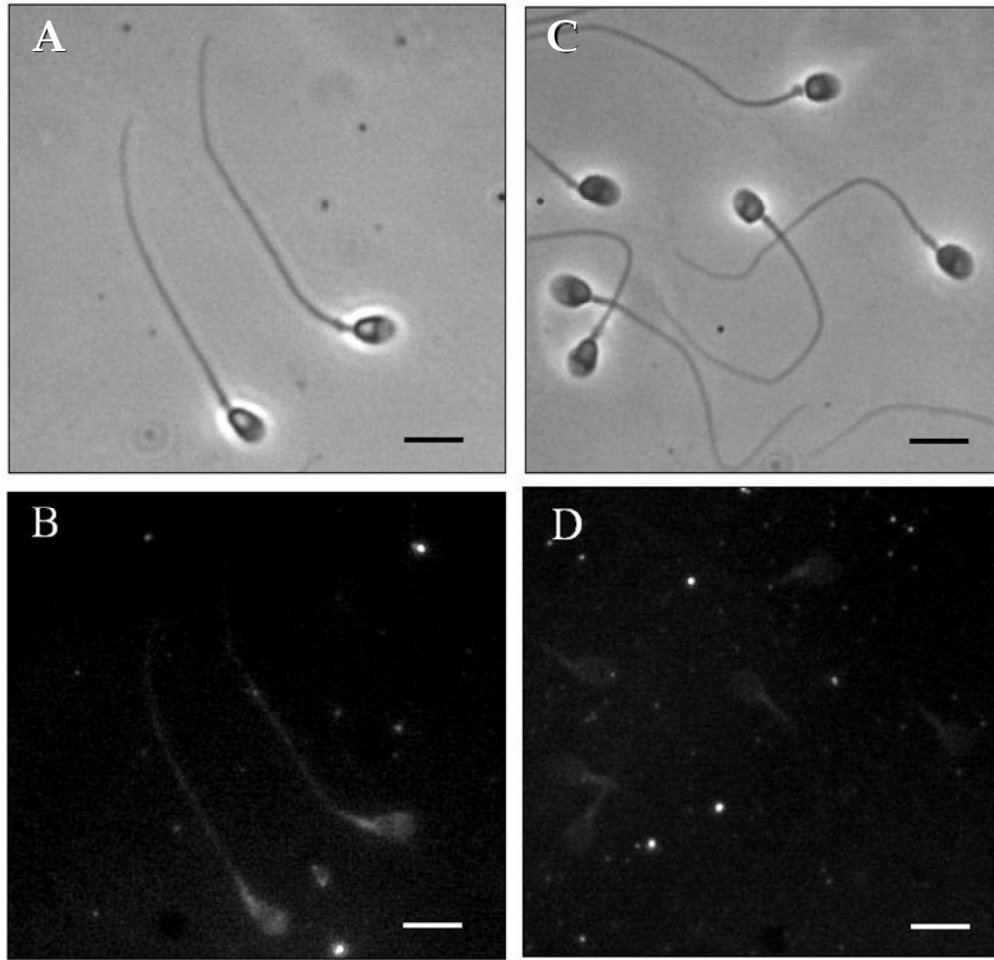
Figure 5.15. Immunolocalization of TASK2 on human ejaculated spermatozoa. Phase contrast (A and C) and fluorescence (B and D) images of spermatozoa incubated in primary (A and B) or adsorbed (C and D) antibody preparations. Scale bar = 5 µm.
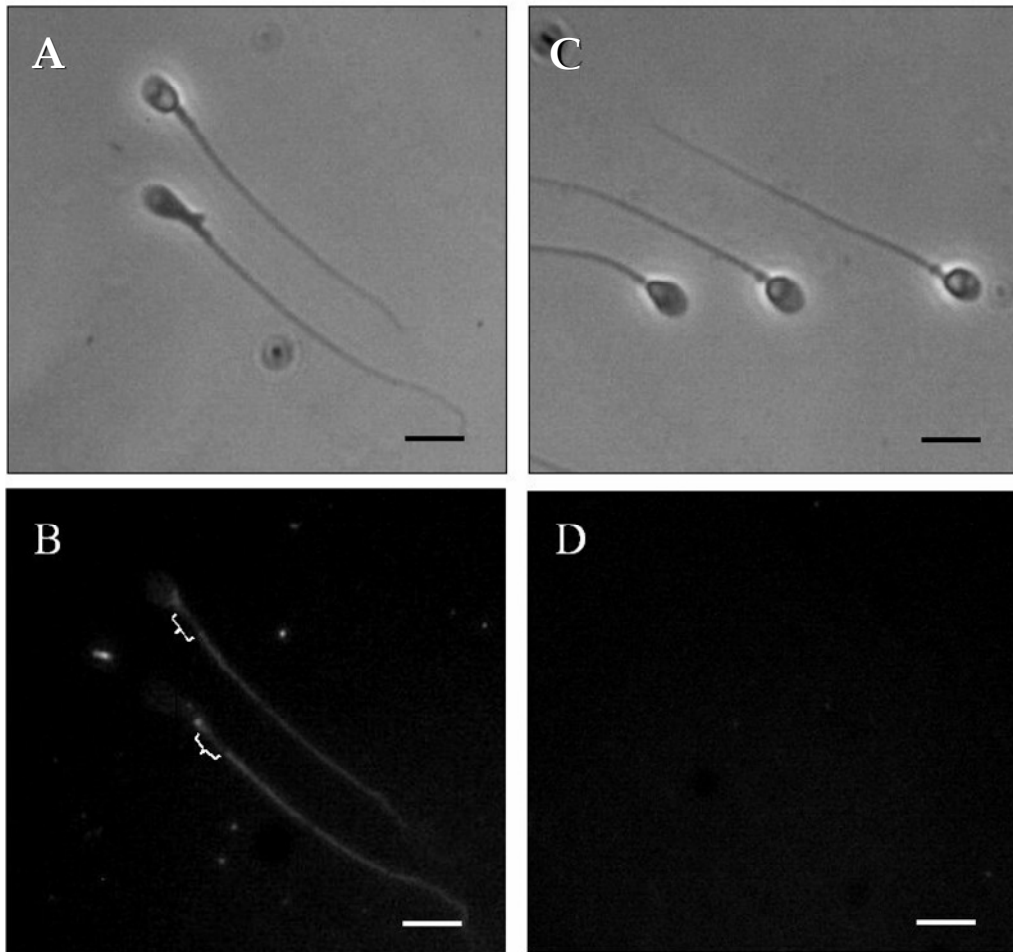
Figure 5.16. Immunolocalization of minK on human ejaculated spermatozoa. Phase contrast (A and C) and fluorescence (B and D) images of spermatozoa incubated in primary (A and B) or with secondary antibody only (C and D) preparations. Braces (B) indicate stain-free midpiece compared with head and principal piece. Scale bar = 5 μm.

Determination of Isotonicity of Murine Epididymal Spermatozoa

When spermatozoa swell or shrink osmotically, they lose or gain osmolytes through channels activated by RVD or RVI. However, cryoprotectants may not need to utilise channels but permeate directly through the membrane. This could be recorded if osmotic movement of water is prevented by studying cells at isotonicity. These experiments were designed to determine the tonicity of murine cauda epididymidal spermatozoa.

Because refractive index can alter forward scatter signals (Shapiro 1995), the forward scatter of standard beads was measured in every medium used and compared with that obtained in standard BWW_{330} to provide correction factors for all values generated from spermatozoa. There was little effect of refractive index on the measurements with a mean ($\pm$ SD) correction factor of 0.988 $\pm$ 0.020).

At 75 min, the extent of swelling induced by quinine in hypotonic medium depended on the nature of the medium and was always higher in the presence of NaCl than in low ionic strength media (sucrose, lactose) as well as high ionic strength medium containing choline Cl. As the osmolality increased, the difference in size between quinine-treated and –untreated spermatozoa (reflecting the extent of volume regulation) decreased for all major medium components (Fig. 5.17). There was no significant difference between this measure of volume regulation and zero (i.e. no volume regulation) when the osmolality reached 528 mmol/kg for NaCl, 462 mmol/kg for choline Cl, 458 mmol/kg for lactose but was far lower (377 mmol/kg) for sucrose. Linear regression analysis revealed the null point to be 527 mmol/kg for both NaCl and choline Cl, 477 mmol/kg for lactose, and 423 mmol/kg for sucrose. These values indicate a range of osmolalities within which isotonicity of murine epididymal spermatozoa may be achieved.
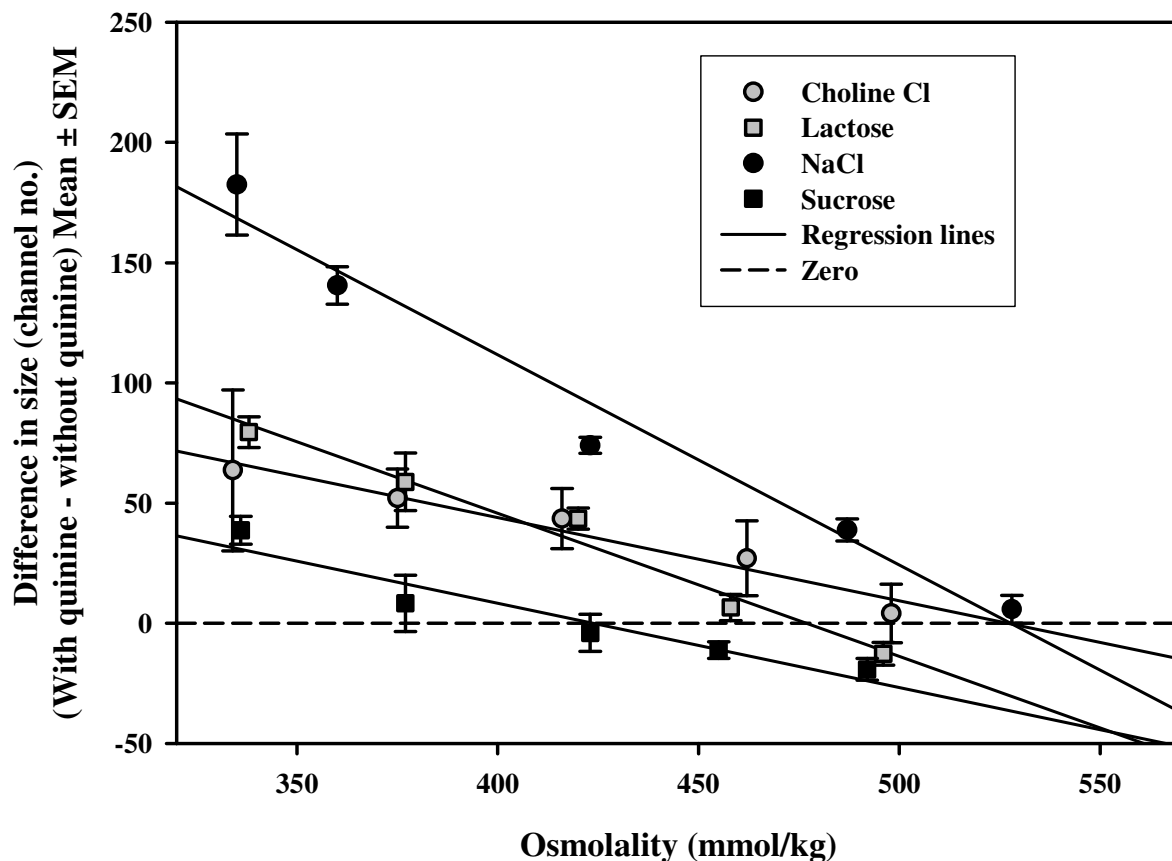
Figure 5.17. Regression lines for the difference in size of murine epididymal spermatozoa incubated with and without quinine (ordinate) in media of various osmolality (abscissa) composed of high (NaCl, choline Cl) and low (lactose and sucrose) ionic strength.

Osmolyte Permeability

As the null point was dependent on the charge of the major fluid component (ionic strength), and the osmolytes to be tested for permeability were both uncharged polyols and charged amino acids, the higher tonicity medium (530 mmol/kg) was taken as isotonic for these studies. By creating isotonic media with this osmolality the possibility that osmotic swelling, and the concomitant opening of volume–sensitive channels, would complicate the study of permeability was reduced. The impermeant trisaccharide raffinose (C_{18}) and disaccharides (C_{12}) sucrose, trehalose, lactose, melibiose and maltose (>340 d) at 250 mM did not cause spermatozoa to increase size above a mean channel number (CN) of 308 in isotonic medium, and the upper 95% confidence limit (mean + 2 SD) of the size of spermatozoa in all six

126

impermeant sugars (342 CN) was taken as the size threshold below which compounds were considered to be non-penetrating (Fig. 5.18).

In the absence of extracellular osmotic differences, no bulk water movement can occur, but osmotic entry of water can follow penetration of permeant solutes into the cell. Most compounds < 200 dalton were thus considered penetrating because they induced cell volume increase above this threshold in cells held at isotonicity. The extent of entry was independent of the nature of the compound as both uncharged and charged polyols, sugars, and amines all caused swelling. Overall, the polyols penetrated to a greater extent than the amines (Fig. 5.18). Several polar compounds, including the major epididymal secretions glycerophosphocholine (GPC), *myo*-inositol, L-carnitine, glutamate, as well as serine and lysine, were considered impermeant by this criterion (Fig. 5.18). Of the common cryoprotective agents relative order of penetrability was DMS>EG>PD2>PD3≥GLY>THM=EHM for spermatozoa from C57BL6 mice (Fig. 5.18).

There was no relationship of swelling to the number of hydroxyl groups or the polarity as defined by the C/OH ratio (Storey and others 1998) or the mean molecular cylindrical radius, as measured from molecular modeling (Strange and Jackson 1995) or formula (Tsukaguchi and others 1998, data not shown). The presence of quinine did not reduce the entry of any permeant compound into spermatozoa; on the contrary, significantly larger volumes were achieved in the presence of quinine for all compounds tested (Fig. 5.19). Overall, the greater the cell size induced by permeating solutes, the greater the effect of quinine (Fig. 5.20).
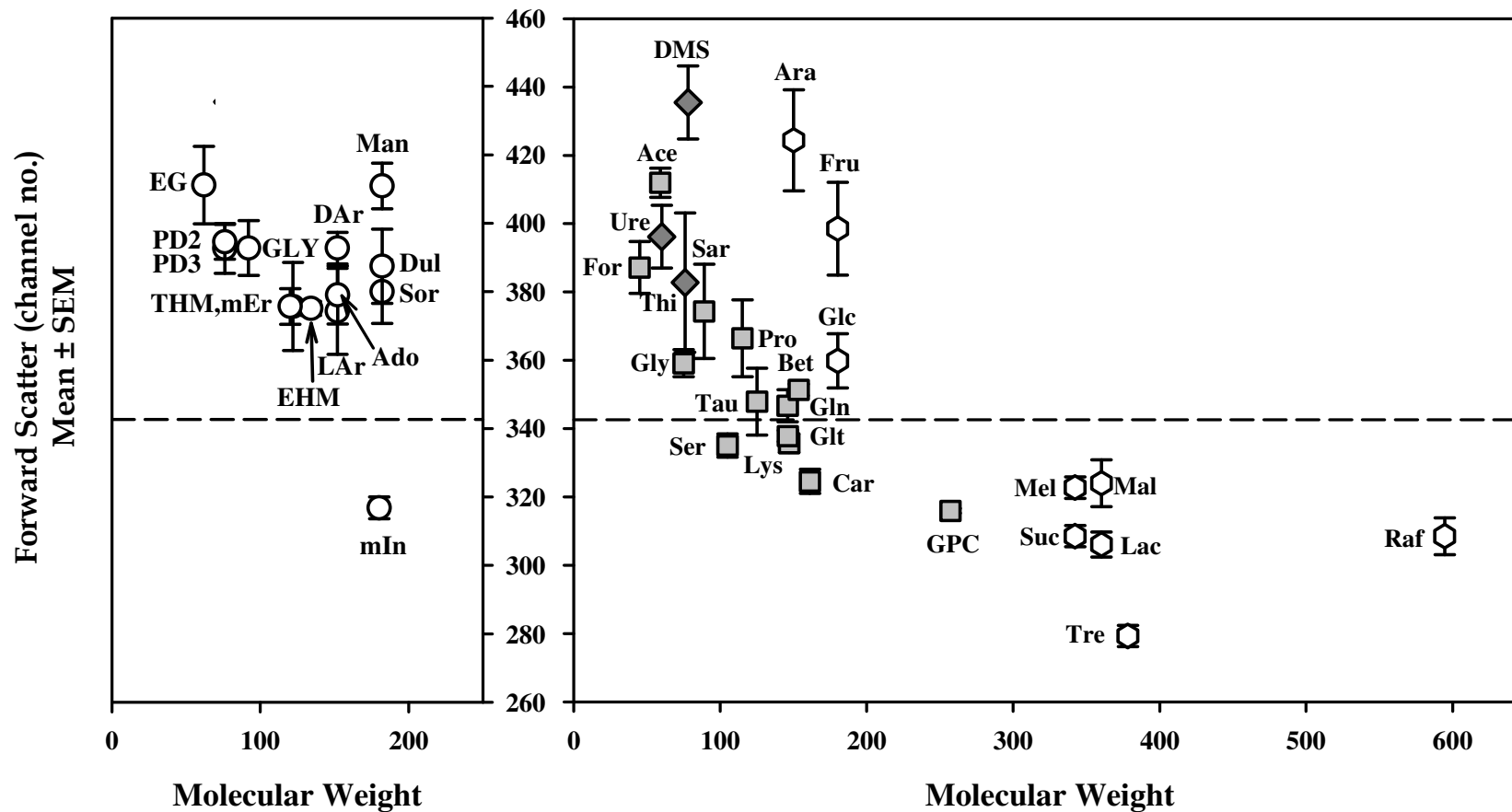
Figure 5.18. Mean forward scatter (± SEM) of epididymal spermatozoa (ordinate) from C57BL6 mice in isotonic medium (530 mmol/kg) containing 250 mM of osmolyte (circles, alcohols; hexagons, sugars; squares, amines; diamonds, others) of various molecular weights (abscissa). The dashed line represents the threshold below which osmolytes were considered to be impermeable; open circles represent polyols, open hexagons represent sugars, shaded squares represent amines, and shaded diamonds represent osmolytes that do not fit into the other categories. Left panel, polyols; Right panel, sugars, amines, others.

128
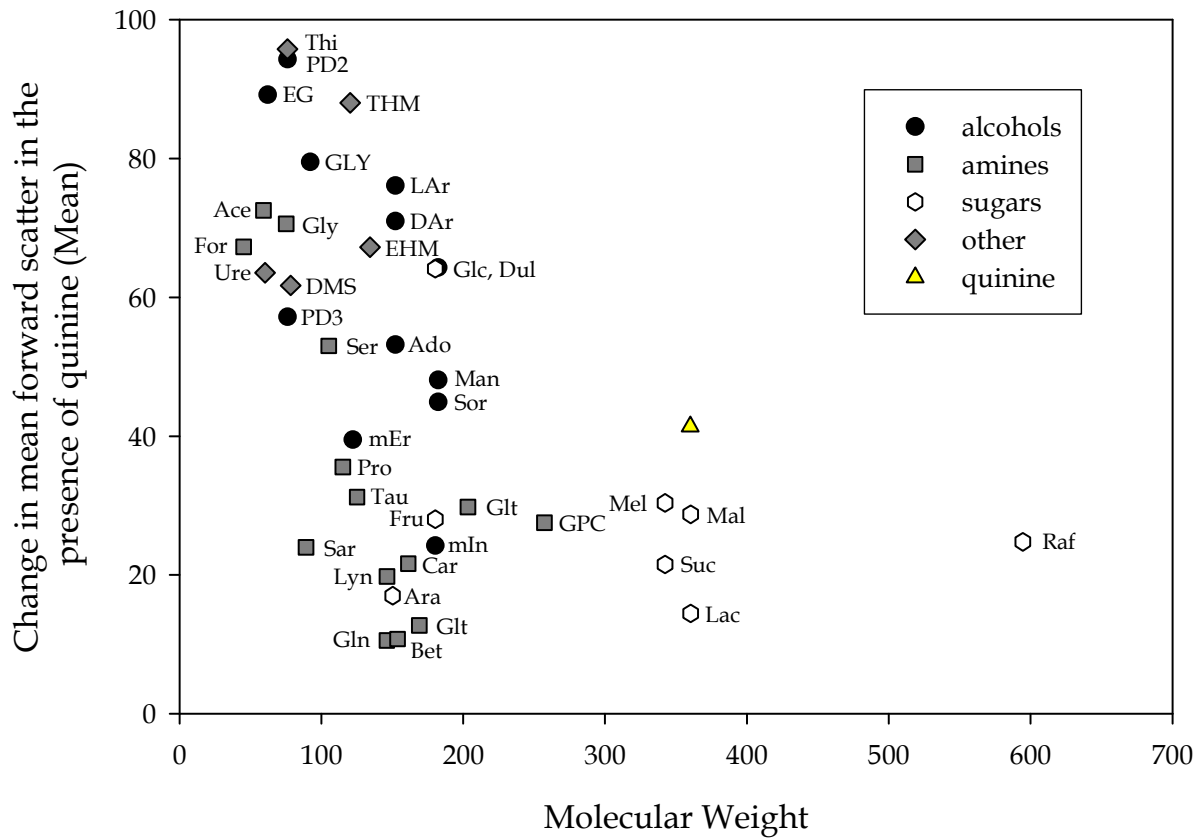
Figure 5.19. Mean difference in forward scatter (FS in presence of quinine – FS without quinine) of epididymal spermatozoa from C57BL6 mice (ordinate) in isotonic medium (530 mmol/kg) containing 0.25 M osmolytes (see Chapter 4, Methods, for abbreviations) of various molecular weights (Da, abscissa).
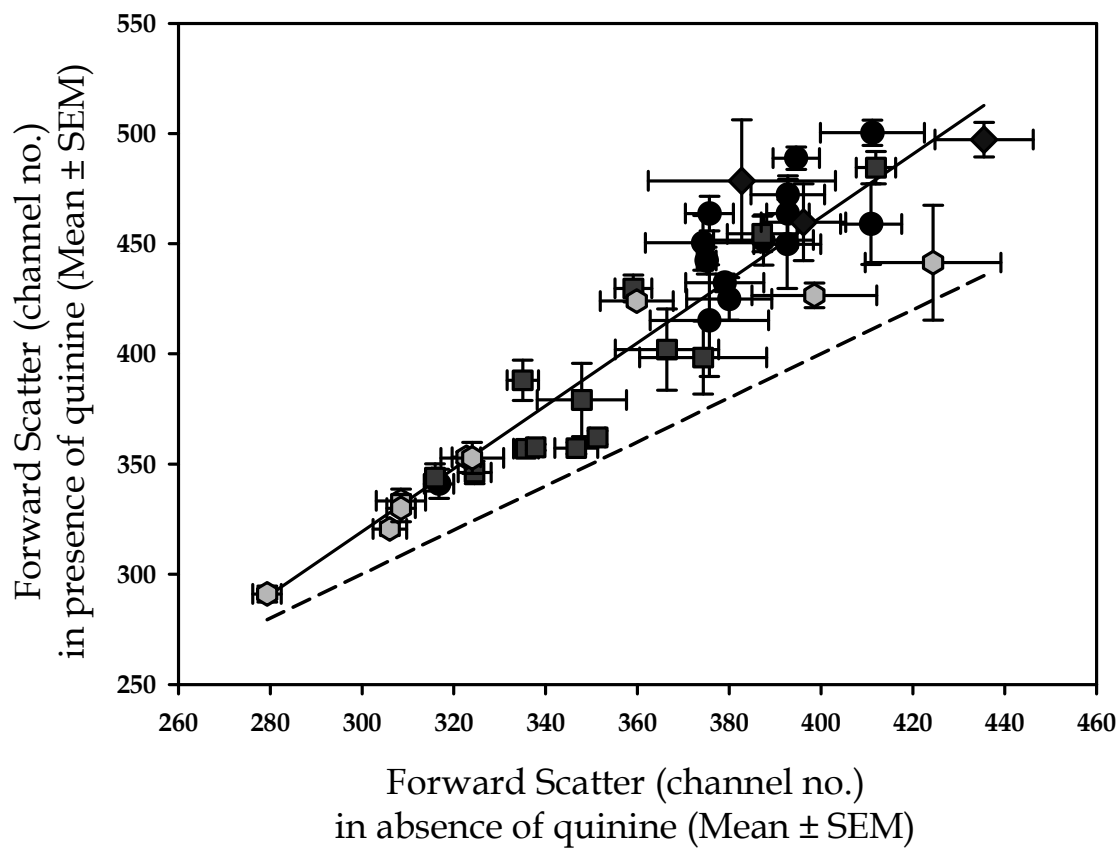
Figure 5.20. Mean forward scatter (± SEM) of epididymal spermatozoa from C57BL6 mice in isotonic medium (530 mmol/kg) with (ordinate) or without (abscissa) 0.8 mM quinine and containing 250 mM osmolytes of various molecular weights (see Chapter 4, Methods, for abbreviations). Dotted line is the line of identity.

Osmotic Responses of Spermatozoa from Two Strains of Mice

NaCl medium

Because isotonicity in NaCl medium was measured to be 527 mmol/kg (Fig. 5.17), the medium of 530 mmol/kg was assumed to be isotonic. At this osmolality there were significant differences in the relative volume of the spermatozoa from the two strains of mice at all time points except 45 min and after 105 min of incubation (Fig. 5.21, paired t-test, $p < 0.05$). Spermatozoa from the C57BL6 mice were larger at earlier time points than the B6D2F1 spermatozoa, yet spermatozoa from both strains reached a similar volume at the end of the 2 hour incubation period. In medium of 330 mmol/kg there were significant differences in relative volume at the 30, 45, 75, and 90 min time-points. Throughout the incubation period spermatozoa from C57BL6 mice were larger than B6D2F1 mice in 330 mmol/kg medium, with the largest difference observed at the 30 min time-point where spermatozoa from the C57BL6 mice were approximately 50 channel numbers larger. Spermatozoa incubated in medium of 245 mmol/kg were not significantly different between strains, and there was only one significant difference between strains in 160 mmol/kg medium at the 30 min time-point, although the C57BL6 spermatozoa tended to be larger.

Thus, the spermatozoa from the two strains at isotonicity (530 mmol/kg) were not different in volume but a difference between strains became evident as the osmolality was reduced, with spermatozoa from the B6D2F1 strain coping better at maintaining volume with the osmotic challenge. Surprisingly, the lowest osmolality (160 mmol/kg) did not produce the largest cells by 120 min, which may reflect the low chloride ion concentration of these media.
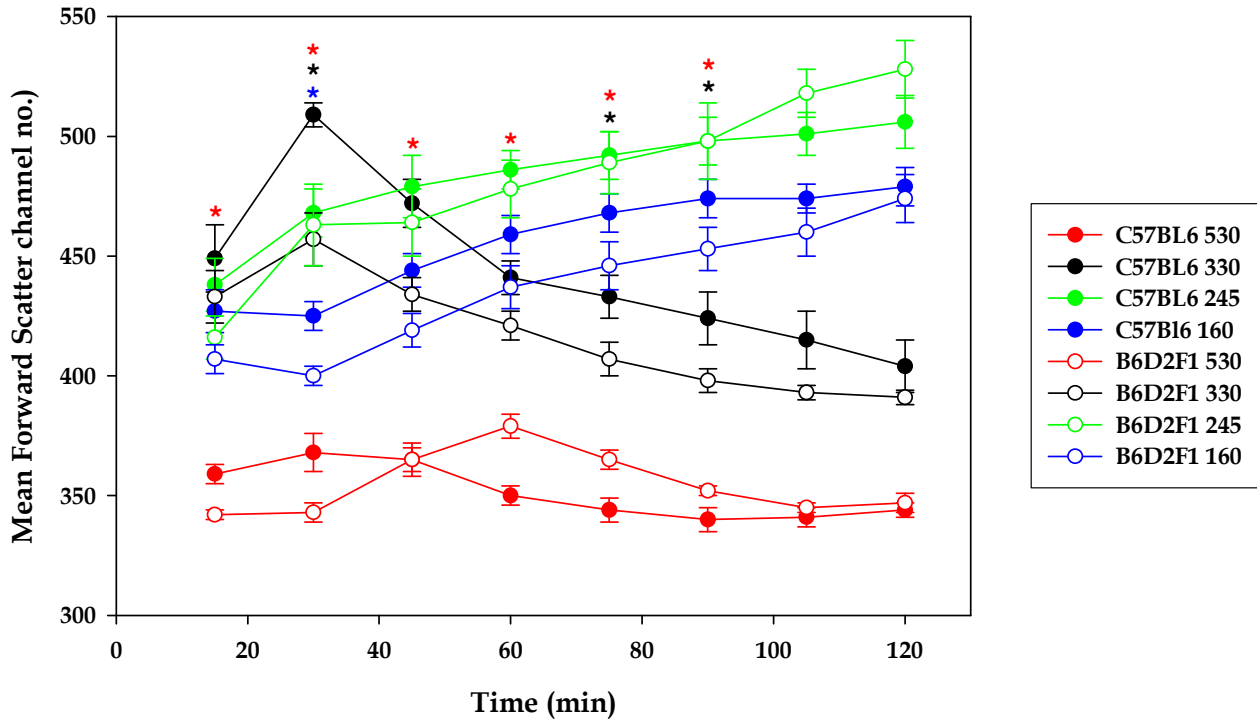
Figure 5.21. Mean forward scatter measurements ± SEM (ordinate) of epididymal spermatozoa from C57BL6 mice (solid circles) and B6D2F1 mice (open circles) over a 2 hour incubation period (abscissa) in media of various osmolalities. See legend for color scheme of osmolalities. Asterisks indicate a significant difference ($p < 0.05$) in size of spermatozoa from the two strains of mice at the osmolality indicated by the color of the asterisk.

Na gluconate medium

As the lowest osmolality of NaCl medium tested was obtained at the expense of lower Cl^-, Na^+, and ionic strength, other factors may be influencing osmotic response at such low osmolality. If chloride needs to efflux from the cell with K^+ to maintain electroneutrality, the lower Cl^- content of the 160 mmol/kg medium may promote greater Cl^- (and hence K^+) efflux with better volume regulation. In order to check this possibility, media of 330 mmol/kg and constant Na^+ and ionic strength were made by replacing Cl^- with gluconate. Cell volumes were indeed smaller with lower concentrations of Cl^- (indicative of better volume regulation) and generally there was less fluctuation in volume in response to the different osmotic challenges when compared with the spermatozoa in media with differing levels of Cl^- (Fig. 5.22). In 15 mM Cl^- at 15 and 30 min and in 29 mM at 30 min spermatozoa from the B6D2F1 strain were significantly smaller than those from the C57BL6 strain. However, in 116 mM Cl^- at 45 min

spermatozoa from the C57BL6 strain were significantly smaller than those from the B6D2F1 strain.
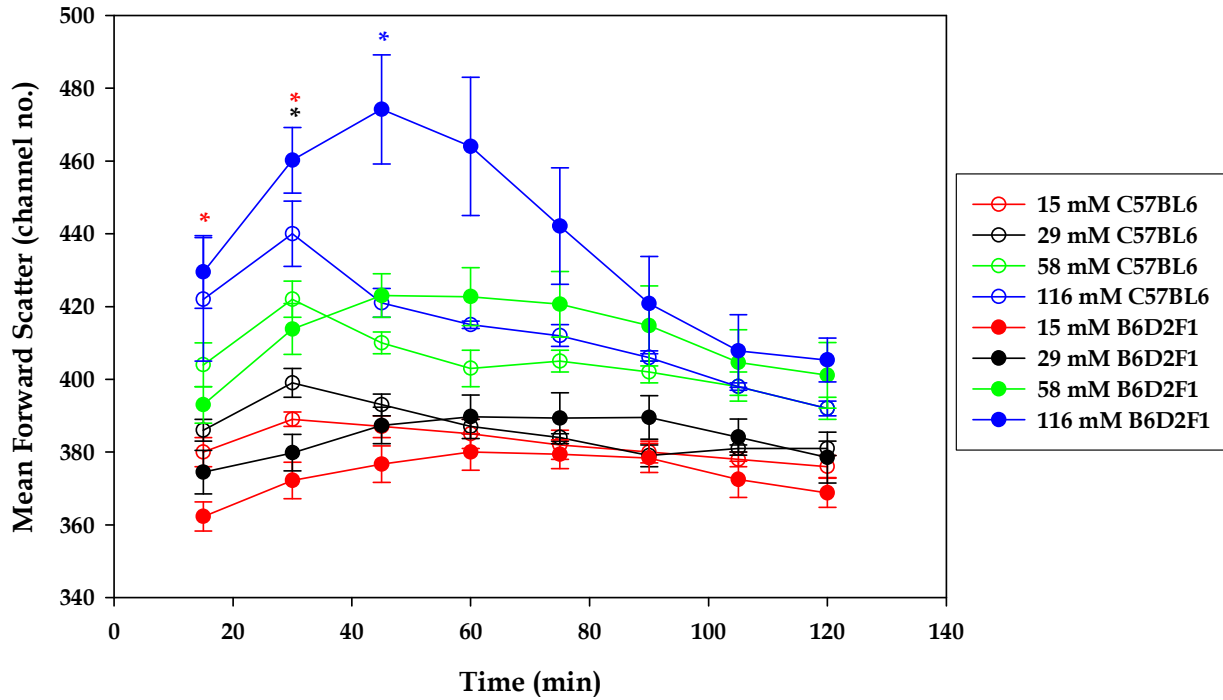
Figure 5.22. Mean forward scatter measurements ± SEM (ordinate) of epididymal spermatozoa from C57BL6 mice (open circles) and B6D2F1 mice (solid circles) over a 2 hour incubation period (abscissa) in media of 330 mmol/kg and containing various concentrations of Cl⁻. See legend for color scheme of Cl⁻ concentrations. Asterisks indicate a significant difference (p < 0.05) in size of spermatozoa from the two strains of mice at the Cl⁻ concentration indicated by the color of the asterisk.

Sucrose medium

When sucrose was used to raise the osmolality, the forward scatter measured in 530 mmol/kg was far lower than that obtained in medium adjusted with NaCl, and spermatozoa from the B6D2F1 mice were significantly smaller than spermatozoa from C57BL6 mice despite very little change in volume for either strain over time (Fig. 5.23). This may reflect the overall lower Cl⁻ concentration in the low ionic strength medium. Sperm sizes at the other osmolalities were also below those determined in NaCl medium but increased with decreasing osmolality. As found at 530 mmol/kg, there was little volume change in spermatozoa from either strain observed in medium of 330 mmol/kg although there were significant differences during the first hour of incubation. When osmolality was lowered to 245 mmol/kg, volume gradually

increased over 60 min after which the C57BL6 spermatozoa were smaller than B6D2F1 spermatozoa (significantly at 90 and 105 min). At 160 mmol/kg, spermatozoa from neither strain were able to maintain a constant volume. Spermatozoa from the B6D2F1 strain were significantly smaller than those from the C57BL6 strain before this capacity was lost and a plateau value of approximately 510 channel number was reached (Fig. 5.23). With sucrose the lowest osmolality led to the largest sperm volume by 120 min.
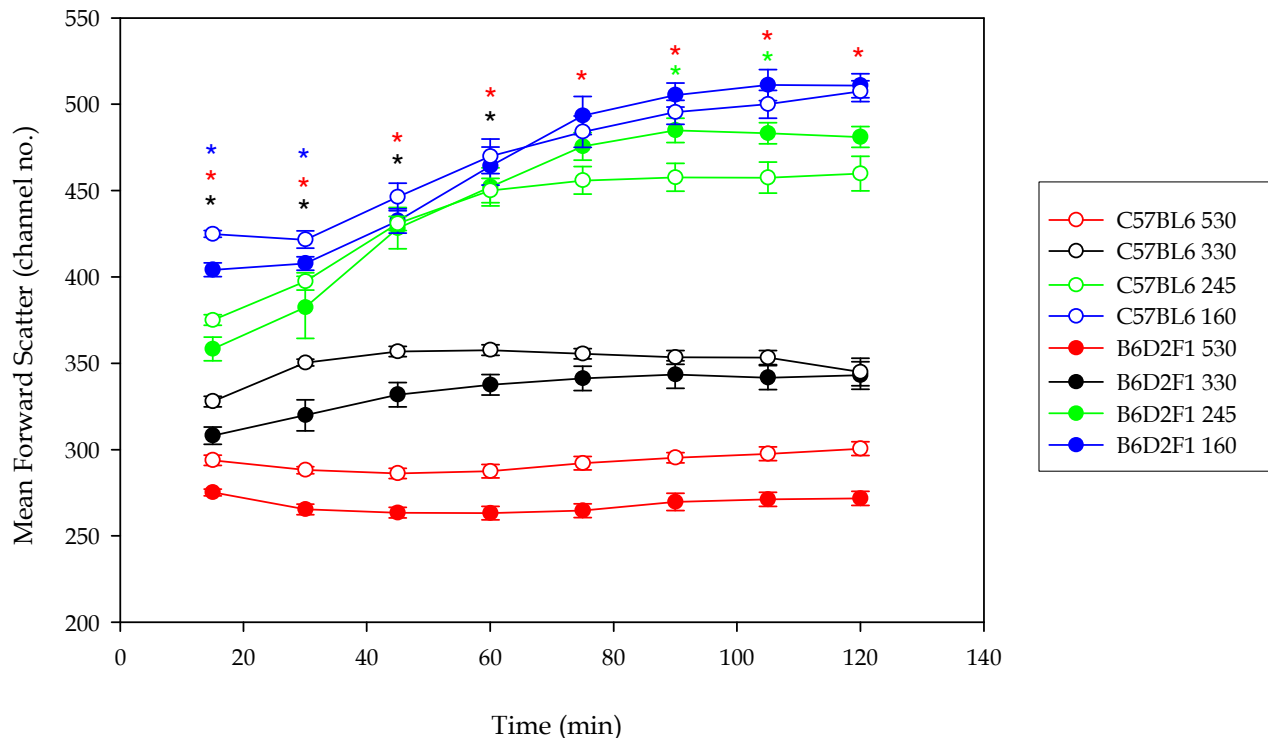
Figure 5.23. Mean forward scatter measurements ± SEM (ordinate) of epididymal spermatozoa from C57BL6 mice (open circles) and B6D2F1 mice (solid circles) over a 2 hour incubation period (abscissa) in media of various osmolalities adjusted with sucrose. See legend for color scheme of Cl⁻ concentrations. Asterisks indicate a significant difference ($p < 0.05$) in size of spermatozoa from the two strains of mice at the osmolality indicated by the color of the asterisk.

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Chapter 6

Discussion and Conclusions

Volume regulation is an extremely important process for spermatozoa, that if disrupted leads to infertility. The mechanisms underlying volume regulatory responses of spermatozoa from various species are just beginning to be revealed. The experiments described in this dissertation contribute to the growing knowledge on sperm volume regulation.

The first part of this dissertation was dedicated to characterizing the channels, in particular the K^+ channels, involved in the regulatory volume decrease (RVD) of monkey, murine, and human spermatozoa. Candidate channels were identified by comparing volume responses of spermatozoa to blockers of channels that prevent volume regulation of somatic cells. The potential channels identified here have pharmacological sensitivities that correspond to those found in somatic cells. For each species investigated, potential channels were identified from the cell's response to inhibitors, and the experiments that followed were conducted to characterize further these channels on spermatozoa.

The second part of this dissertation was designed to investigate potential osmolytes that could be utilized by spermatozoa during osmotic events. These experiments involved first a determination of the isotonicity of spermatozoa, followed by a test of permeability of spermatozoa at isotonicity towards a wide range of molecules, including some that are commonly used as cryoprotectants and others that spermatozoa are exposed to in high concentrations in the epididymis.

The third part of this dissertation evaluated the osmotic responses of spermatozoa from two different strains of mice, one of which has a relatively higher post-thaw fertility rate than the other. The reason for the difference in the freezability of spermatozoa from these two strains of mice is unknown. These experiments were conducted to determine if this difference in freezability could be related to the volume regulatory ability of the spermatozoa.

This discussion will first address each of these topics, followed by a more general discussion on the potential applications of this work in the fields of reproductive and conservation biology, as well as future directions of this research area.

Volume Regulation by Spermatozoa

Volume regulation is a complicated process, and it is likely that RVD involves more than one channel working in concert with others to reduce volume. Owing to the lack of absolutely specific inhibitors, swelling due to blockage of a single channel can not be distinguished from that caused by blockage of multiple channels. To screen for possible channels involved, inhibitors and doses were chosen for their reported ability to block K^+ currents in somatic cells.

The pharmacological profiles of all channels were compared with the channel blockers that did and did not cause swelling of spermatozoa. A Table was constructed for each species (see below) to elucidate the logic used in identifying potential RVD channels (those that can be inhibited by at least one of the tested blockers). Channels were not excluded if no information about the effects of an inhibitor on that channel were available (empty box in the Table), or if the channels were insensitive to a tested inhibitor (X in box).

Exposure to Channel Inhibitors

Monkey

Cynomolgus monkey ejaculated spermatozoa were evaluated for their response to potassium channel inhibitors, including quinine as positive control. Results from employing a wide range of inhibitors of channels involved in the volume regulation of somatic cells indicated that Kv4.1, 4.2, and 4.3 may be involved in the RVD response because of the cell's sensitivity to 4AP, but not the other inhibitors tested (Table 6.1). By the same logic, the other channels listed in Table 1 are most likely not involved in RVD of monkey spermatozoa as incubation in these inhibitors in hypotonic conditions did not cause an increase in cell volume.

The response of monkey epididymal spermatozoa to quinine has also been examined (Yeung and others 2004b). Caudal spermatozoa swell in hypotonic medium followed by a reduction in volume (RVD). RVD is blocked by quinine and caudal sperm motility is affected similarly to ejaculated spermatozoa with decreases in VSL and LIN. Corpus epididymidal spermatozoa also swell in hypotonic medium but are unable to reduce their volume to the extent of caudal spermatozoa. Caput spermatozoa showed no reaction to the same osmotic challenges. Unfortunately, the large numbers of epididymal spermatozoa needed to test the range of inhibitors used on ejaculated sperm were unavailable for comparison.

Mouse

Murine epididymal spermatozoa were sensitive to several inhibitors and this permitted several potential channels involved in RVD to be identified. These channels are listed in Table 6.2 along with their pharmacological profiles that could be demonstrated on murine spermatozoa.

Four-aminopyridine (4AP) is a quaternary ammonium blocker routinely used in electrophysiological studies to identify voltage-gated (Kv) channels (Mathie and others 1998). The significant increase in sperm size after 75 min incubation in hypotonic medium with 4AP thus suggests that voltage-gated potassium channels are involved in volume regulation (Table 6.2). However, the insignificant results from incubation in TEA, charybdotoxin and dendrotoxin exclude the involvement of the majority of Kv channels except Kv1.4 and Kv1.5 (see Table 6.2). Voltage-gated potassium channel 1.5 (Kv1.5) has previously been implicated in RVD of electrically unexcitable cells (Felipe and others 1993), but there is currently no evidence for the involvement of Kv1.4 in volume regulation of other cell types. Recently, a high-conductance, 4AP-sensitive channel has been identified in human spermatozoa from patch-clamping experiments (Gu and others 2004). In addition, 4AP also inhibits RVD of human (Yeung and Cooper 2001) and cynomolgus monkey spermatozoa (Yeung and others 2004b).

The sensitivity of murine spermatozoa to flecainide, cadmium, phrixotoxin, and 4AP suggests that the Kv4 potassium channels may have a role in RVD of murine spermatozoa, as in monkey spermatozoa. Murine spermatozoa were sensitive to flecainide at 100 μM but not 10 μM, which is a similar response to that of COS7 cells transfected with Kv4.2 where there was a dose-dependent inhibition by flecainide in the μM range (Caballero and others 2003). Murine colonic myocytes contain all three Kv4 channels but Kv4.3 transcripts are relatively more abundant and extremely sensitive to micromolar concentrations of flecainide (Amberg and others 2002). Kv4.3 channels, which mediate the transient outward potassium current in the human heart, when transfected into HEK293 cells, are inhibited by cadmium chloride with an EC50 of 0.110 μM (Calmels and others 2001). Phrixotoxin specifically inhibit Kv4.2 and Kv4.3 by altering their gating properties (Diochot and others 1999). Western blotting did not reveal specific bands for Kv4.2 or Kv4.3. The reactivity of the antibodies used in these experiments had not been confirmed in mice but had been in the rat, and the sequences are homologous for 15 of 16 residues with murine Kv4.2 and for 16 of 17 residues with murine Kv4.3.

Spermatozoa were significantly larger than controls when incubated in hypotonic medium containing clofilium, another quaternary ammonium derivative, and barium chloride. The family of potassium channels known to have two pore regions and four transmembrane helices (2P-4TM) are blocked by clofilium. These proteins are thought to channel the leak or background conductances that maintain the passive properties of the cell (Niemeyer and others 2001), including volume regulation. The TWIK-related, acid-sensitive potassium channel 2 (TASK2) in Ehrlich cells is blocked by clofilium with an IC_{50} of 25 µM and may participate in RVD of these cells (Niemeyer and others 2001). The 2P-4TM channels are also characterized by an insensitivity or low sensitivity to a range of conventional K^+ channel blockers such as 4AP, TEA and barium chloride (Niemeyer and others 2001). However, barium was found to inhibit TASK3 transfected into COS-7 cells (Kim and others 2000).

The present results also suggest channels that are unlikely to be involved in volume regulatory responses of murine spermatozoa. That glybenclamide did not affect volume regulation argues against the utilization of inwardly rectifying (Kir) channels 6.1 and 6.2. By similar reasoning the Ca^{2+}-activated K^+ channels Slo1 and SK4 (CTX-sensitive) and SK2 and SK3 (apamin-sensitive) are unlikely to mediate sperm RVD (Coetzee and others 1999, Vergara and others 1998). Of the Kv channels, Kv1.2, Kv1.3 and Kv1.6 are inhibited by CTX, and Kv1.3, Kv1.6 and Kv1.7 are inhibited by MTX. Tetraethylammonium (TEA) inhibits numerous Kv channels (see Table 2, Coetzee and others 1999, Wehner and others 2003), thus ruling out their operation in sperm RVD as the process was not affected by these inhibitors.

Phloridzin, 4-aminobutyrate and threo-β-hydroxyaspartate are inhibitors of channels or transporters used by organic osmolytes (*myo*-inositol [Higgins and Kane 2003]; carnitine, [Kido and others 2001]; glutamate [Chen and Swanson 2003], respectively). Judging by the lack of swelling upon incubation in these inhibitors, there was no indication of significant involvement of such channels or transporters in the volume regulation of spermatozoa at this osmolality during the observed time periods. Other inhibitors are known to block channels used by organic osmolytes that may inhibit RVD of sperm cells, and could be used in future experiments for a more thorough investigation of the role of organic osmolytes in RVD.

Phloridzin is a sodium-dependent hexose transport inhibitor that blocks *myo*-inositol transport in murine oocytes (Higgins and Kane 2003). However, this non-specific blocker also inhibits water transport through the sodium-glucose co-transporter (SGLT1) expressed in

Xenopus laevis (Loo and others 1999) and fluid absorption by renal proximal tubules of the teleost *Carassius auratus* (Kanli and Terreros 1997). Thus, the finding that murine spermatozoa incubated in phloridzin were significantly smaller than the control may be due to the inability of water to enter the cell. The time-course experiment confirmed that control spermatozoa underwent initial swelling and did not return to their initial volume by the end of the incubation period. The difference between the initial volume, represented by the size of cells incubated in phloridzin (in which hypo-osmotic swelling is blocked), and the volume of the control cells at 75 min may reflect more the swelling of the control cells (incomplete RVD) rather than an RVD effect induced by phloridzin.

Several potassium channels seem to be involved in volume regulation of murine spermatozoa, as judged from the effects of specific channel blockers on this process. After excluding K^+ channels not likely to be involved in RVD, Kv1.4, Kv1.5, Kv4.2 Kv4.3, and minK remain as putative channels, suggestions consistent with the inhibitory effects of PTX and flacainide (which inhibits both Kv4.2 and Kv4.3) and Cd^{2+} (which inhibits Kv4.3). The sensitivity to clofilium also suggests an involvement of the TASK2 channel. Western blots confirmed the presence of Kv1.5 and TASK2 proteins in sperm membranes but did not provide evidence for Kv4.2, Kv4.3, or Kv1.4. These channels, which may have overlapping ranges of activation, could work in concert to produce successful volume regulation. While K^+ channels appear to have a prominent role in RVD of murine spermatozoa, it is possible that other channels are also involved. Current research has determined that various anion and organic osmolyte channels and co-transporters contribute to the volume regulatory responses of murine spermatozoa (Yeung and others 2005b; Klein and others 2006).

Human

Candidate channels for regulating volume in human spermatozoa were identified if the channels were inhibited by 4AP and/or CLO, which inhibited RVD of human spermatozoa, but not inhibited by TEA, PTX, FLC, Ba^{2+} or Cd^{2+} as these inhibitors did not prevent RVD (Table 6.3). According to this response, it is possible that Kv1.4, Kv1.5, Kv1.7, minK and TASK2 are involved in volume regulation of human spermatozoa. Of these channels, Kv1.5, minK and TASK2 have been previously implicated in RVD of somatic cells, as discussed for murine spermatozoa.

In summary, several K$^+$ channels may be involved in the volume regulation of human spermatozoa, including those in the voltage-gated and acid-sensitive K$^+$ channel families. These channels, which may have overlapping ranges of activation, could work in concert to produce successful volume regulation. Whereas K$^+$ channels appear to have a prominent role in RVD of human spermatozoa, it is possible that other channels are involved. These experiments do not rule out the participation of other types of channels blocked by the inhibitors tested here. Thus future research should determine if various anion and organic osmolyte channels contribute to the volume regulatory responses of human spermatozoa. Evidence for the involvement of Cl- channels in human sperm RVD has been presented (Yeung and others 2005a).

Voltage-gated K channels seem to mediate much of the RVD of spermatozoa but these channels differ between species: Kv4.1, 4.2, 4.3 in the monkey and mouse but not in man; Kv1.4, Kv1.5, minK, TASK1, and TASK2 in man and mouse but not in monkey. The overlap in channels potentially involved in RVD of spermatozoa from different species may reflect the presence of primitive channels (Sitaraman & Sauna 2000) that evolved to prevent stresses in spermatozoa encountered during maturation in the epididymis and upon ejaculation into the female tract.

Western Blots
Monkey

The voltage-gated Kv4 channels (Kv4.1, Kv4.2 and Kv4.3) are involved in A-type currents (rapidly inactivating, 4AP-sensitive, K$^+$ currents: Amberg and others 2002) and can be distinguished by flecainide, cadmium and phrixotoxin. However, flecainide, cadmium, and phrixotoxin were not tested on monkey spermatozoa, because of the difficulty obtaining ejaculates with sufficient normal spermatozoa. Since the Kv4 channels were also identified as candidates in murine sperm RVD, monkey spermatozoa were used alongside the murine spermatozoa in western blots, rather than in inhibitor experiments that require larger numbers of spermatozoa. Western blotting did not reveal any specific bands for Kv4.2 and Kv4.3, and appropriate antibodies for Kv4.1 were not available. The failure to find specific bands during western blotting made immunocytochemistry for monkey spermatozoa unjustifiable. It remains possible that Kv4.1 is involved in RVD of monkey spermatozoa.

Mouse

Western blotting of proteins from several potential potassium channels (Kv1.5, Kv1.4, Kv4.2, Kv4.3, TASK2, TASK3, and minK), revealed specific bands for Kv1.5, TASK2, and minK in murine spermatozoa. The specific bands found for Kv1.5 (85 and 76 kDa) were similar to, but not identical with, those reported for Schwann cells (90 and 65 kDa, Sobko and others 1998) and rat atrium (75 and 60 kDa, Yamashita and others 2000). The TASK2 specific bands at 113 and 99 kDa were different from the reported value of 45 kDa found in rat kidney by the antibody distributor (Alomone Labs, Israel). MinK proteins were previously found to be 16 kDa in canine Purkinje fibers (Han and others 2002), 26, 22, and 17 kDa in Sf9 cells (Alomone Labs, Israel), and 23 and 17 kDa in rat heart (Alomone Labs, Israel). These are similar to the bands detected in this study at 23, 20, and 12 kDa. Any differences between the values found here and those reported in the literature may represent the presence of sperm-specific isoforms of these channels in murine spermatozoa.

Human

Western blot experiments on human spermatozoa revealed two specific bands for TASK2 determined to be 55 and 72 kDa. This is in contrast to the reported value of 45 kDa in rat kidney membranes (Alomone Labs, Israel) and rat taste receptor cells (Lin and others 2004). Immunolocalization revealed a distribution over the principal piece of the flagellum and detection at the connecting piece. The presence of TASK2 mRNA has previously been detected in post-meiotic germ cells of the mouse (Schultz and others 2003).

Acid-sensitive TASK3 was also detected in western blots of human sperm proteins. Incubation of sperm membrane proteins with TASK3 antibodies yielded 2 specific bands of 49 and 75 kDa. The value of 49 kDa is identical to the value reported by the supplier for rat cerebellum lysates, but the band at 75 kDa has not been reported. Immunolocalization was not possible owing to lack of appropriate antibodies. TASK3 is sensitive to Ba^{2+} in somatic cells (Kim and others 2000), and Ba^{2+} did have a slight effect on motility.

MinK is a 130 amino acid protein that, when associated with KvLQT1 (Kv7.1), forms the potassium channel complex responsible for the underlying slow cardiac current (Barhanin and others 1996, Sanguinetti and others 1996). This protein has been implicated in RVD of some populations of renal proximal tubule cells (Millar and others 2004); furthermore, murine

tracheal epithelial cells from IsK (minK) knockout mice are unable to recover normal size when exposed to hypotonic solutions (Lock and Valverde 2000). Vestibular dark cells of the inner ear (Wangemann and others 1995) and Xenopus oocytes (Busch and others 1992) also have minK-mediated currents that are activated by cell swelling. Specific bands for the minK protein were detected on western blots. Exposure to anti-minK antibodies revealed a specific band at 14 kDa which is similar to one of two bands found at 17 and 23 kDa in rat heart membranes (Alomone Labs).

Voltage-gated Kv1.5 was one of the first potassium channels implicated in RVD of somatic cells, and there continues to be evidence from electrically excitable and non-excitable cells that this channel is involved in RVD (Wehner and others 2003). In addition to evidence from the inhibitor experiments, one specific band for Kv1.5 was detected during western blotting and estimated to be of size 76 kDa. Previous reports have indicated two bands corresponding to Kv1.5 at 65 and 90 kDa in murine Schwann cells (Sobko and others 1998) and 60 and 75 kDa in rat atrium (Yamashita and others 2000). Immunolocalization of Kv1.5 resulted in the most intense staining of the proteins tested and was concentrated at the neck of the spermatozoa. This corresponds to the site of the cytoplasmic droplet and where there was an intact droplet fluorescence was especially strong.

Western blots confirmed the presence in spermatozoa of some of the channel proteins considered from the inhibitor experiments to mediate RVD. These proteins had molecular sizes similar to, or larger than, those found in somatic cells. The latter could reflect glycosylation of the channel. Indeed TASK2 (Kcnk5) has N-glycosylation sites and treatment with N-glycanase action reduces its size from 71 to 55 compatible with its 499 amino acid sequence (Yeung and Cooper, unpublished data).

Relationship between Kinematic Parameters and Cell Swelling
Monkey

Correlating changes in cell volume and kinematic parameters has been suggested to be a more sensitive assay for detecting failure of RVD in spermatozoa, particularly for human spermatozoa, the responses of which are smaller than those of murine spermatozoa, presumably because of a smaller volume and thus less (osmotically) active fluid (Yeung and

others 2003). Since monkey spermatozoa more closely resemble human than murine spermatozoa in volume (monkey 37 μm^3, Rutllant and others 2003; human 28-34 μm^3, mouse 53-81 μm^3; see Gao and others 1997), computer-assisted sperm analaysis (CASA) measurements were recorded for monkey spermatozoa in the presence of channel blockers. Indeed, inhibitors affecting monkey sperm volume also affected sperm motility parameters. Quinine and 4AP created a hyperactivation-like motility by reducing BCF, STR, and LIN and increasing ALH. This is similar to motility patterns of human ejaculated spermatozoa exposed to quinine and 4AP where STR, LIN and VSL are depressed.

Mouse

Changes in murine sperm motility, while not measured by CASA here, are clearly altered by exposure to quinine as this causes the cells to thrash without much forward progress, again indicative of a hyperactivation-like motility (Yeung and others 2002).

Human

In addition to larger forward scatter values, spermatozoa swollen by incubation in quinine and 4AP exhibited a hyperactivation-like motility. This corroborates evidence from other studies that also found higher incidences of hyperactivated motility of human and monkey spermatozoa exposed to QUI or 4AP (Yeung and Cooper 2001, Yeung and others 2003, Gu and others 2004). Clofilium caused a less marked change in motility, perhaps indicating an incomplete transition towards hyperactivated motility. Although Cd^{2+} and Ba^{2+} caused changes in sperm motility, the flagellar movements induced did not resemble the hyperactivated-like motility that was associated with cell swelling. This analysis of motility supports the conclusions from measurement of size with the flow cytometer—that the channels sensitive to 4AP and clofilium are candidates for RVD.

Changes in cell volume in all three species were associated with changes in motility parameters as demonstrated previously for spermatozoa from monkeys (Yeung and others 2004b), mice (Yeung and others 2002) and man (Yeung and others 2003). This mechanism remains to be investigated but it could reflect changes in intracellular ionic strength as a result of the swelling or a physical property of a swollen flagellum, altering the fluid dynamics of axonemal sliding and flagellar force generation.

Repeatability of Response to Quinine

Monkey

For the ejaculates from monkeys collected on several occasions there was inconsistency in the size of spermatozoa from a given male. The intra-class correlation coefficient for control spermatozoa suggested that approximately half of the variation in sperm size occurred between ejaculates from the same male while the other half of variation was between males. Furthermore, the response to quinine was extremely variable in ejaculates from the same male. Some males seemed to produce spermatozoa that were consistent in size between ejaculates both in the presence and absence of quinine, whereas others produced spermatozoa that were quite different from ejaculate to ejaculate both in their control size and in the presence of quinine. This difference may reflect the few males available for study but more likely may reflect the method of collecting the ejaculates, which was via rectal probe ejaculation.

There are reports that electro-ejaculation has a negative influence on semen quality (Morrell and Hodges 1998). With the exception of a few trained Great Apes, collection of uncontaminated naturally-ejaculated semen is rare; Orangutans (*Pongo pygmaeus*) and chimpanzees (*Pan troglodytes*) have been conditioned to ejaculate into an artificial vagina (VandeVoort and others 1993, Young and others 1995, respectively), and a lowland gorilla (*Gorilla gorilla gorilla*) into a hand-held plastic bag (Pope and others 1997). The problem of decreased semen quality with electro-ejaculation is difficult to solve as many studies have used different stimulation regimes and probes. Nevertheless, there is at least one report that describes no difference in post-thaw motility of spermatozoa collected naturally or by electro-ejaculation from baboons (Kraemer and Cruz 1969).

Electro-ejaculation is believed to alter the composition of the semen, which could lead to a change in osmolality or pH. Unfortunately, there are no previous reports of the osmolality of male reproductive tract fluids in monkeys. The mean osmolality of cynomolgus semen used in these studies was 333 ± 6 mmol/kg and the medium used for osmotic challenge was 290 mmol/kg. Therefore despite variation in semen composition, all spermatozoa would have faced an osmotic challenge, allowing significant volume changes to be detected were RVD to be inhibited. It is possible, however, that the differences seen between ejaculates from the same individual could be attributed to the method of collection if, for example, the probe had a

145

slightly different position in the rectum from one collection to another, stimulating the accessory organs differently, and resulting in altered composition of the seminal plasma.

Mouse

Comparisons of the size of spermatozoa from separate epididymides of the same mouse produced intra-class correlation coefficients in the absence and presence of quinine similar to those calculated for ejaculated spermatozoa from the monkey. These imply low reproducibility between samples but closer agreement between individuals. If volume regulation depends on the epididymal provision of osmolytes to maturing spermatozoa, then these results may reflect differences in epididymal secretion of osmolytes or their uptake, which may depend on the androgenic status or the sexual activity of the particular male.

Human

For the ejaculated human spermatozoa there was consistency in the size of spermatozoa from a given male on different occasions. The intra-class correlation coefficient for control and quinine-treated spermatozoa suggested that only 5% of the variation in sperm size occurred between ejaculates from the same male while approximately 95% of variation was between males. These results are very different from those for monkey and murine spermatozoa which demonstrated far greater within-individual responses. This consistency in swelling response to quinine is in agreement with post-thaw motility responses of human spermatozoa that varies greatly between, but less within, men (Leibo and others 2002).

Until more is known of osmolyte fluxes in spermatozoa, the reasons for the differences in extent of response must remain the speculations given above. As only the mean responses were taken and an even wider response would be expected from individual spermatozoa in an ejaculate, this may have a physiological function. If osmolyte depletion were responsible for swelling and swelling alters the pattern of motility, hyperactivation could be linked to osmolyte depletion and only those depleted of osmolytes would undergo hyperactivation at the best time. As the time of ovulation is variable with respect to the time of insemination, a wide range of responses within the ejaculate may enhance the chance of one spermatozoon achieving fertilization.

pH Sensitivity of Osmotic Responses to Quinine

Mouse

Clofilium-sensitive TASK2 (an acid-sensitive channel) seemed to be involved in RVD of murine spermatozoa as indicated by the channel inhibitor and western blot experiments. This prompted a closer look at the effect of pH on murine sperm RVD. Results from these experiments indicated that regulation of volume may be influenced by the extracellular pH of the cell, although not in the manner expected. The conductance of K^+ through this acid-sensitive channel (when expressed in Ehrlich ascites tumour cells) is strongly inhibited at an acidic pH (6.3) and enhanced at an alkaline pH (8.4) (Niemeyer and others 2001). The voltage-gated K^+ channel Kv1.5, which was also implicated in RVD of mouse sperm cells in these experiments, is similarly sensitive to changes in pH. Kv1.5 channels expressed in *Xenopus* oocytes are blocked at pH 6.3 and are insensitive to alkaline pH (Steidl and Yool 1999). If these channels were involved one would expect that the return to normal volume would be faster in alkaline hypotonic medium than in acidic hypotonic medium, as reported for Ehrlich cells (Hougaard and others 2001). However, in the current experiments only spermatozoa in alkaline Tris-buffer were significantly larger than the control (BWW_{330} pH 7.4) and not spermatozoa incubated at acidic pH.

Because spermatozoa exposed to an alkaline medium buffered with Hepes (although not an optimal buffer at this pH range) were not significantly different from the control, it can not be excluded that this increase in volume was an effect of the Tris buffer rather than the pH. On the other hand, this variation in response to pH and the differences found for the size of TASK2 proteins in these experiments compared to those previously reported for somatic cells may indicate that an unknown acid-insensitive channel exists in the murine sperm membrane that is inhibited by Hepes.

Human

The possible involvement of an acid-sensitive channel (TASK2) in RVD prompted the investigation of the effect of pH on human spermatozoa, as described for the mouse. The results did not indicate acid-sensitivity of human sperm volume regulation. In fact, there was a tendency for the cells incubated at pH 8.4 with Tris to be larger than those at other pH after 30

min incubation. However, because the cells incubated at pH 8.4 with Hepes were not larger than those at 7.4 and 6.3, this may indicate a pH-independent effect of Tris that hinders RVD.

Immunocytochemical Localization of Putative K⁺ Channels

Mouse

Of those channels that could be detected by western blotting, Kv1.5, TASK2, and minK could be localized on the murine sperm plasma membrane by immunocytochemistry. The strongest fluorescence of all three proteins was observed at the connecting piece suggesting a concentration of volume regulatory channels there. The connecting piece anchors the head to the neck which is the site of the cytoplasmic droplet while the spermatozoa are in the epididymis and before the droplet's migration along the midpiece to a more distal position. Isovolumetric regulation (IVR) is the slow accumulation of osmolytes without a measurable change in volume of the spermatozoa in response to a gradual increase in osmolality of the epididymal fluid. If IVR is an active process during epididymal migration, the location of these channels at the cytoplasmic droplet, the sperm's largest reservoir of cytoplasm and potentially of fluid exchange (Cooper and Yeung 2003), would make sense. When murine spermatozoa are exposed to hypotonic conditions, there is an obvious swelling at the cytoplasmic droplet, and the cell accommodates to the change in volume and avoids excessive increase in surface area by angulating at the site of the droplet or assuming a hairpin-like morphology. Indeed, when a cytoplasmic droplet was present the fluorescence on the droplet was intense.

Human

As for murine spermatozoa, the strongest fluorescence of human spermatozoa was observed at the connecting piece, the site of the cytoplasmic droplet. Despite confusion in the literature over use of the terms cytoplasmic droplet and excess residual cytoplasm when describing human spermatozoa (Cooper 2005), the cytoplasmic droplet has recently been demonstrated to be a normal component of functioning human ejaculated spermatozoa (Cooper and others 2004, Fetic and others 2006). The localization of all three potential human RVD channel proteins (TASK2, mink, Kv1.5) to the neck region, combined with the evidence from murine spermatozoa, supports the hypothesis that most RVD is mediated through the membrane of the cytoplasmic droplet.

Summary

The research presented in this dissertation provides evidence that several potassium channels may be involved in RVD of spermatozoa, and while these channels vary among monkey, mice, and men, there are commonalities among the species. Many of the candidate channels are suggested for at least two of the species examined here, and it is not surprising that these channels have been found to be active in RVD of somatic cells. That there is an effect on motility is obvious, visually for murine spermatozoa and measurably with CASA for human and monkey spermatozoa. The Western blots and immunolocalizations have lent support to the presence of the channels in murine and human spermatozoa and validation for future research to elucidate the true function of these channels in volume regulation of spermatozoa. The broad applications of knowledge on RVD of spermatozoa are discussed below.

Channel	Gene	Inhibitors								
		4AP	CLO	Ba	TEA	MTX	CTX	AP	GLY	DTX
Kv1.1	Kcna1	✓			✓					✓
Kv1.2	Kcna2	✓	✓		✓		✓			✓
Kv1.3	Kcna3	✓			✓	✓	✓			✓
Kv1.4	Kcna4	✓	✓							✓
Kv1.5	Kcna5	✓	✓							✓
Kv1.6	Kcna6	✓			✓	✓				✓
Kv1.7	Kcna7	✓				✓				✓
Kv2.1	Kcnb1	✓			✓					
Kv2.2	Kcnb2	✓			✓					
Kv3.1	Kcnc1	✓			✓					
Kv3.2	Kcnc2	✓			✓					
Kv3.3	Kcnc3	✓			✓					
Kv3.4	Kcnc4	✓			✓					
Kv4.1	Kcnd1	✓								
Kv4.2	Kcnd2	✓								
Kv4.3	Kcnd3	✓								
Kv7.1	Kcnq1	✓	✓	✓	✓					
Kv7.2	Kcnq2	✓	✓		✓					
Kv10.1	Kcnh1	✓	✓		✓					
Kv10.2	Kcnh5	✓			✓					
Kv11.1	Kcnh2		✓		✓					
Kv11.2	Kcnh6				✓					
Kv11.3	Kcnh7				✓					
Kv12.1	Kcnh8				✓					
Slo1	Kcnma1				✓		✓			
Slo3	Kcnma3				✓					
BK1	Kcna1	✓			✓		✓			
Mink (Isk)	Kcne1		✓	X						
SK2	Kcnn2							✓		
SK3	Kcnn3							✓		
TWIK1	Kcnk1			✓						
TWIK2	Kcnk6			✓						
TASK1	Kcnk3	X		✓/X	X					
TASK2	Kcnk5	X	✓	X	X					
TASK3	Kcnk9			✓						
TREK1	Kcnk2			✓						
TREK2	Kcnk10			✓						
Kir6.1	Kcnj8								✓	
Kir6.2	Kcnj11								✓	

Table 6.1. Pharmacological sensitivities of potassium channels potentially inhibited by channel blockers used on monkey spermatozoa (see Chapter 4 for abbreviations; quinine not shown). A ✓ indicates that the channel is blocked by the corresponding inhibitor and an X indicates that the given channel is insensitive to the inhibitor. Conflicting data are represented by ✓/X. The shaded column highlights the inhibitor that blocked RVD in monkey ejaculated spermatozoa, and the bold, underlined channels have profiles that are consistent with results obtained in the channel blocker experiments making them potential candidates for involvement in monkey sperm RVD.

Channel	Gene	Inhibitors											
		4AP	CLO	PTX	FLE	Ba	Cd	TEA	MTX	CTX	AP	GLY	Gad
Kv1.1	Kcna1	✓						✓					
Kv1.2	Kcna2	✓	✓					✓		✓			
Kv1.3	Kcna3	✓						✓	✓	✓			
Kv1.4	Kcna4	✓	✓										
Kv1.5	Kcna5	✓	✓										
Kv1.6	Kcna6	✓						✓	✓	✓			
Kv1.7	Kcna7	✓							✓				
Kv2.1	Kcnb1	✓						✓					
Kv2.2	Kcnb2	✓						✓					
Kv3.1	Kcnc1	✓						✓					
Kv3.2	Kcnc2	✓						✓					
Kv3.3	Kcnc3	✓						✓					
Kv3.4	Kcnc4	✓						✓					
Kv4.1	Kcnd1	✓					✓						
Kv4.2	Kcnd2	✓		✓	✓		✓						
Kv4.3	Kcnd3	✓		✓	✓		✓						
Kv7.1	Kcnq1	✓	✓			✓		✓					
Kv7.2	Kcnq2	✓	✓					✓					
Kv10.1	Kcnh1	✓	✓					✓					
Kv10.2	Kcnh5	✓						✓					
Kv11.1	Kcnh2		✓					✓					
Kv11.2	Kcnh6							✓					
Kv11.3	Kcnh7							✓					
Kv12.1	Kcnh8							✓					
Slo1	Kcnma1							✓		✓			
Slo3	Kcnma3							✓					✓
BK1	Kcna1	✓						✓		✓			

151

		4AP	CLO	PTX	FLE	Ba	Cd	TEA	MTX	CTX	AP	GLY	Gad
Mink (Isk)	Kcne1		✓			X							
SK2	Kcnn2										✓		
SK3	Kcnn3										✓		
SK4	Kcnn4									✓			
TWIK1	Kcnk1					✓							
TWIK2	Kcnk6					✓							
TASK1	Kcnk3	X				✓/X		X					
TRAAK	Kcnk4												✓
TASK2	Kcnk5	X	✓			X		X					
TASK3	Kcnk9					✓							
TREK1	Kcnk2					✓							✓
TREK2	Kcnk10					✓							
Kir6.1	Kcnj8											✓	
Kir6.2	Kcnj11											✓	

Table 6.2. Pharmacological sensitivities of potassium channels potentially inhibited by channel blockers used on murine spermatozoa (see Chapter 4 for abbreviations; quinine not shown). A ✓ indicates that the channel is blocked by the corresponding inhibitor and an X indicates that the given channel is insensitive to the inhibitor. Conflicting data are represented by ✓/X. The shaded columns highlight those of inhibitors that blocked RVD in murine epididymal spermatozoa, and the bold, underlined channels have profiles that are consistent with results obtained in the channel blocker experiments making them potential candidates for involvement in murine sperm RVD.

Channel	Gene	Inhibitors						
		4AP	CLO	TEA	PTX	FLC	Ba	Cd
Kv1.1	Kcna1	✓		✓				
Kv1.2	Kcna2	✓	✓	✓				
Kv1.3	Kcna3	✓		✓				
Kv1.4	Kcna4	✓	✓					
Kv1.5	Kcna5	✓	✓					
Kv1.6	Kcna6	✓		✓				
Kv1.7	Kcna7	✓						
Kv2.1	Kcnb1	✓		✓				
Kv2.2	Kcnb2	✓		✓				
Kv3.1	Kcnc1	✓		✓				
Kv3.2	Kcnc2	✓		✓				
Kv3.3	Kcnc3	✓		✓				
Kv3.4	Kcnc4	✓		✓				
Kv4.1	Kcnd1	✓						✓
Kv4.2	Kcnd2	✓			✓	✓		✓
Kv4.3	Kcnd3	✓			✓	✓		✓
Kv7.1	Kcnq1	✓	✓	✓			✓	
Kv7.2	Kcnq2	✓	✓	✓				
Kv10.1 (EAG1)	Kcnh1	✓	✓	✓				
Kv10.2 (EAG2)	Kcnh5	✓		✓				
Kv11.1 (ERG1)	Kcnh2		✓	✓				
Kv11.2 (ERG2)	Kcnh6			✓				
Kv11.3 (ERG3)	Kcnh7			✓				
Kv12.1 (ELK1)	Kcnh8			✓				
Slo1	Kcnma1			✓				
Slo3	Kcnma3			✓				
BK1	Kcna1	✓		✓				
Mink (Isk)	Kcne1		✓				X	
TWIK1	Kcnk1						✓	
TWIK2	Kcnk6						✓	
TASK1	Kcnk3	X		X			✓/X	
TASK2	Kcnk5	X	✓	X			X	
TASK3	Kcnk9						✓	
TREK1	Kcnk2						✓	
TREK2	Kcnk10						✓	

Table 6.3. Pharmacological sensitivities of potassium channels potentially inhibited by channel blockers used on human spermatozoa (see Chapter 4 for abbreviations; quinine not shown). A ✓ indicates that the channel is blocked by the corresponding inhibitor and an X indicates that the given channel is insensitive to the inhibitor. Conflicting data are represented by ✓/X. The shaded columns highlight those of inhibitors that blocked RVD in human ejaculated spermatozoa, and the bold, underlined channels have profiles that are consistent with results obtained in the channel blocker experiments making them potential candidates for involvement in human sperm RVD.

Organic Osmolytes and Sperm RVD

   Osmolyte influx and efflux through volume-sensitive channels are driven by extracellular osmolality. During volume regulation in hypotonic media, osmolytes are lost from the cell and while this is a natural event for spermatozoa that experience hypotonic conditions upon ejaculation, any premature loss of osmolytes (such as during handling for cryopreservation) may leave the cell ill-prepared for the critical osmotic challenges that occur after manipulation (i.e. after insemination). Premature loss of osmolytes may be prevented, and potentially compensated for, if more were known about the permeability to, and uptake of, osmolytes into spermatozoa.

Establishing isotonicity of murine spermatozoa

   Many studies on murine epididymal spermatozoa have described them as being ideal osmometers with isotonicity being assumed to be around 286, 290, or 300 mmol/kg (Du and others 1994, Noiles and others 1995,1997, Willoughby and others 1996, Agca and others 2002, Walters and others 2005). These values are nowhere near the reported osmolality of epididymal fluid, some 100 mmol/kg higher (Cooper and Barfield 2005). High cauda epididymidal osmolality explains why transfer of caudal spermatozoa to media of approximately 300 mmol/kg induces swelling that requires volume regulation (Yeung and others 2002). Such volume-regulating cells cannot be described as ideal (passive) osmometers. In fact, the anticipated deviation from linearity of the relationship between cell volume and extra-cellular osmolality around physiological values has been recorded, although not adequately addressed (Du and others 1994).

   Cytoplasmic osmolality can be determined when cells bathed in a medium have no need to respond osmotically. In the present experiments, this was measured for spermatozoa from the cauda epididymidis by exposing them to media of various osmolalities. The osmolality of the medium in which the cells did not change volume regardless of the presence of quinine was taken to be the intracellular osmolality. The degree of cell swelling in the presence of quinine reflected the extent of osmotic water flux that would have occurred had normal volume regulation not been inhibited. As anticipated, spermatozoa swelled less as extracellular osmolality was increased but the extent of volume regulation depended on the nature of the medium. Different values for isotonicity were determined in high and low ionic strength media,

with values in the former (~530) close to the upper limits of the osmolality of caudal fluid (~500 mmol/kg) and values in the latter (~430) closer to the measured mean values (Cooper and Barfield 2005). As the ionic strength of cauda epididymidal fluid is low (Cooper 1998), the lower values, established in the low ionic strength media, may reflect the true tonicity of epididymal spermatozoa *in situ*. Whichever value is taken to be the tonicity of murine epididymal spermatozoa, it is far higher than the often given "isotonic" values of 290-310 mmol/kg.

In low ionic strength media, swelling was less extensive than in high ionic strength media containing sodium or chloride although dependent on the nature of the non-reducing sugar present. This could be indicating a better volume regulatory performance of spermatozoa in low ionic strength, or low Cl$^-$ media. However, it could also reflect the entry of NaCl into the cell at the higher concentrations which would draw in water and exaggerate cell swelling. Irrespective of the mechanism, the ionic strength and the nature of the major extra-cellular compound responsible for raising the osmolality of the medium can influence intracellular tonicity. Alteration in the ionic strength of the environment would affect the charge of the membrane and could alter the activity of pumps and channels directly or indirectly related to ion fluxes across the membrane. This, in turn, could alter the channels activated during RVD, preventing the same osmotic response as would occur in low ionic strength media and creating the difference in isotonicity values estimated here.

Permeability of osmolytes and cryoprotectants

In the study of permeability towards water-soluble compounds, large molecules (C$_{18}$ tri- and C$_{12}$ di-saccharides) were impermeant as expected. Other studies have suggested the impermeability of the murine sperm membrane to di- and tri-saccharides. In fact, non-permeating solutes, such as sugars, are used to augment the effectiveness of permeating CPAs in cryopreservation schemes for certain cell types, allowing the use of lower concentrations of permeating CPAs (Gao and others 1997). Agca and others (2002) found that the induction of non-linear flagellar forms (angulated swollen spermatozoa, Yeung and others 2002) by penetrating cryoprotectants was prevented upon co-incubation with sucrose and raffinose, suggesting that these sugars remain outside the cell and prevent excess water influx to the cell.

Some, but not all, compounds with 6 or fewer carbon atoms penetrated spermatozoa enough to induce swelling. The permeability of murine spermatozoa to the polyols (mannitol >

D-arabitol > dulcitol > sorbitol > adonitol > *m*-erythritol > L-arabitol) contrasts with that of bovine epididymal spermatozoa (*m*-erythritol > adonitol > D-arabitol > sorbitol > L-arabitol = mannitol = dulcitol) measured by Drevius (1971) from microscopical observations. Arabitol, fructose, and mannitol were more penetrating than compounds of the same molecular size, which may indicate the presence of transporters in spermatozoa. Of the cryoprotectants tested, penetrating order was found to be DMS > EG > PD (2 and 3) = GLY > THM = EHM. Other volume measurements of murine spermatozoa have demonstrated a greater permeability at room temperature for EG than GLY for B6C3F1 and ICR mice (Agca and others 2002) whereas Phelps and others (1999) found the relative permeability EG > GLY > PD2 for ICR mice but GLY > EG > PD2 for sperm from the B6C3F1 strain.

The finding that the propanediols THM and EHM were less penetrating agents than glycerol implies that as a cryoprotectant these compounds may result in less swelling during initial exposure to the cells than glycerol, which causes a large swelling event owing to the time required for this molecule to move into the cell. Limiting the stress on the plasma membrane during the addition and removal of cryoprotectant by preventing large changes in volume (via dilution addition and removal) has been shown to result in higher numbers of motile spermatozoa (Gao and others 1995). The use of cryoprotectants that cause less swelling, such as THM and EHM, may help avoid damage to the plasma membrane during the cryopreservation process. Although the addition of these polyols to CPA including glycerol was deleterious to murine spermatozoa resulting in fewer intact cells post thaw, the effects of these agents alone (without the presence of glycerol) were not studied (Storey and others 1998).

The major epididymal secretions (GPC, glutamate, L-carnitine, *myo*-inositol) have been postulated to provide spermatozoa with the osmolytes needed for volume regulation in the female tract (Cooper and Yeung 2003). This may require these osmolytes to be membrane-permeant, but they did not induce sperm swelling at isotonicity. Estimates of intracellular concentrations based on murine sperm volume (Willoughby and others 1996) and osmolyte content (Yeung and others 2004a) are 33 mM for L-carnitine and 38 mM for *myo*-inositol, confirming that a substantially higher concentration (250 mM) was applied in these experiments. The inability of the epididymal osmolytes to enter the cells could reflect the fact that these osmolytes are membrane-impermeant, which may reflect their polarity: *myo*-inositol is uncharged but highly polar whereas L-carnitine and GPC are zwitterions with little charge at

156

neutral pH; GPC is impermeant to renal cells (Zablocki and others 1991). Whatever the explanation, the present results suggest that, at epididymal osmolality, spermatozoa are impermeable to these secretions. Uptake of these osmolytes may only occur when volume-sensitive channels are activated during hypertonic stress experienced during epididymal transit (Cooper and Yeung 2003), a speculation that requires to be proven.

The inability of quinine to restrict the entry of osmolytes into murine spermatozoa at isotonicity contrasts with the results of Kulkarni and others (1997) who demonstrated that quinine was able to block the swelling of bovine spermatozoa caused by high extracellular concentrations of many of the same compounds. The explanation could lie in the different species but is more likely to reflect the use of ejaculated rather than epididymal spermatozoa and the osmotic changes involved in preparing these cells for study. Firstly, bovine seminal plasma has an osmolality (mmol/kg) from 341 (White and MacLeod 1963) to 355 (Salisbury and Cragle 1956) and in the study by Kulkarni and others (1997) spermatozoa were prepared by swim up in 0.15 M NaCl + 5 mM Na phosphate (approx. 310 mmol/kg). This so-called isotonic medium (without test compounds) was actually hypotonic since spermatozoa swelled in it in the presence of quinine. This swelling would have resulted in an efflux of osmolytes causing the volume regulatory capabilities of the spermatozoa to be compromised even before the spermatozoa were exposed to quinine or the compounds being tested. Secondly, during the uptake experiments spermatozoa were subjected to a hypertonic medium (0.31 M osmolytes + 35 mM Na Cl + 5 mM Na phosphate or approx. 390 mmol/kg). Therefore, the inability of the spermatozoa to swell in the presence of quinine may actually reflect a blockage of RVI (which occurs in response to cell shrinkage) rather than RVD.

The present results demonstrating that quinine increased the volume of murine spermatozoa under isotonic conditions in the presence of penetrating solutes indicates that volume regulation occurs even when water entry is associated with that of a permeating solute. This increase in volume also implies that cells will regulate volume when penetrating solutes enter from hypertonic solutions, which has important implications for cryobiology. The use of penetrating cryoprotectants is widespread because in many species they can prevent intracellular ice crystal formation. However, the loss of osmolytes from the spermatozoa treated in this way may impact post-thaw fertility of the sample. This is discussed further below.

157

Osmotic Response of Spermatozoa from Two Strains of Mice

An indirect measure of the volume regulation capacity of spermatozoa can be gauged from the volume response of spermatozoa to a range of osmolalities over time. It may be that the more the osmolyte reserve (those available within the cell for RVD events), the greater the ability of the spermatozoon to maintain volume in face of an osmotic challenge. As cytoplasmic isotonicity depends on the ionic strength, the actual osmotic challenge presented by the same osmolality differs whether NaCl or sucrose is the major osmotic species. NaCl-containing medium of 330 mmol/kg was a sufficient stimulus to raise sperm volume initially to a size that initiated a time-dependent decline in size (RVD), as reported before (Yeung and others 2002). However, in sucrose at 330 mmol/kg swelling was less marked and sperm volume was maintained throughout incubation, which can be explained by the more efficient volume regulation by spermatozoa in this low Cl⁻ medium. A larger osmotic challenge (245 mmol/kg) induced a time-dependent increase in sperm volume in both NaCl and sucrose media. This failure to maintain volume at low osmolality is reminiscent of the response of immature (caput) spermatozoa that have not acquired their ability to regulate volume (Yeung and others 2002) and could be explained by the depletion of the osmolytes responsible for RVD during a non-physiological osmotic challenge.

If loss of intracellular osmolytes prevents effective RVD in media of 245 mmol/kg, then the indication of some extent of volume regulation (the maintenance of volume over 15-30 min before unopposed water entry) in media of made to 160 mmol/kg with NaCl requires explanation. This could be the operation of different channels employing other osmolytes at the lower osmolality. The extent of osmotic challenge can dictate the sequential operation of cation and anion channels (MacLeod and Hamilton 1999, Junankar and others 2004) or sequential release of different organic osmolytes (Kinne 1998) in somatic cells, depending on cell type. For murine spermatozoa, a physiological challenge (330 mmol/kg in NaCl medium) stimulates the release of anions or organic osmolytes initially followed by potassium efflux (Yeung and others 2005b). At 160 mmol/kg, other osmolytes may be preferentially liberated to maintain volume initially but, when supplies are exhausted, a rapid increase in cell volume occurs.

Finally, although the trends in volume regulation with an anisotonic media were parallel in both mouse strains, spermatozoa from the B6D2F1 strain were consistently better at regulating volume than those from the C57BL6 strain. Whether the basis for this is a greater

initial osmolyte reserve to call upon during hypotonic stress (due to greater epididymal reserves of osmolytes) or a greater ability to take up osmolytes during epididymal transit, requires detailed study. Nevertheless, the superior volume-regulating ability of B6D2F1 spermatozoa could, in part, contribute to the higher rates of in vitro fertilization initiated by frozen-thawed B6D2F1 spermatozoa than frozen-thawed C57BL6 spermatozoa (Songsassen and Leibo 1997). Screening of the amount of osmolytes in the epididymal fluid and in the spermatozoa of the different strains may be helpful in predicting post-thaw survival of spermatozoa from different species or in detecting individual differences in potential cryodamage. In this regard, several recent publications have indicated that the volume regulatory ability of spermatozoa is a useful tool in determining cryoprotective efficiency (Petrunkina and others 2005) and predicting freezability of canine spermatozoa (Petrunkina and others 2004).

Applications and Perspectives

Cellular volume regulation is vital for all cells and many physiological functions depend on the maintenance of a homeostatic volume. For spermatozoa, the inability to sustain a critical volume results in failure during unassisted fertilization. The research presented here, while valuable for its contribution to knowledge on basic sperm physiology, has many applications to the field of conservation biology. These applications have been presented in the introductory chapters and will be discussed below with regard to the results of this dissertation and future directions of this research topic.

Contraceptive potential

In previous chapters, the contraceptive potential of mimicking failed volume regulation has been mentioned but the approaches not discussed. As the ability for spermatozoa to perform RVD is developed during transport through the epididymis (Yeung and others 2002, 2004a, Cooper and Yeung 2003), any contraceptive method directed at RVD in spermatozoa would have its primary action in this organ. There are three possible approaches to disrupting volume regulatory mechanisms of spermatozoa while they are in the epididymis (Cooper and Yeung 1999).

Blocking sperm RVD channels

The first option is to target spermatozoa with an anti-fertility agent that actively blocks the channels in the sperm membrane participating in RVD. For this approach to be effective, the osmolyte channels would have to be specific to spermatozoa or the delivery of the drug specific to the epididymal lumen. Since the completion of this dissertation research, the nucleotide sequences of the potassium channels identified here (minK, Kv1.5, and TASK2) have been determined for human spermatozoa and are identical to those found in other tissues and published in genomic databases (Yeung and Cooper, unpublished data). Indeed, the ubiquitous nature of ion channels in particular makes this approach problematical, especially as the concentrations of drug effective on spermatozoa are in the same range as those effective on somatic cells.

Targeting organic osmolyte channels on spermatozoa may also produce an effective contraceptive. For this, the mechanism of osmolyte uptake into spermatozoa needs to be known. The fact that the compounds found in high concentration in the epididymis (i.e. GPC, carnitine, and *myo*-inositol) were not permeant in these experiments conducted at isotonicity, when no volume regulatory channels are open, suggests that these osmolytes need transporters or channels to enter the cells. Indeed, an organic cation/carnitine transporter has been identified in human spermatozoa (Xuan and others 2003). Further tests conducted using media of a higher osmolality, such as experienced by spermatozoa during epididymal transport, may reveal that these molecules can permeate the membrane during RVI. Before this approach becomes a reality, the osmolytes released during RVD must be determined. Preliminary studies on compounds preventing RVD in hypotonic media have suggested that several small organic compounds may be lost by spermatozoa (Yeung and others 2004b, 2006). The experiments described here under isotonic conditions serve as a preliminary screening of molecules that have the capability to traverse the plasma membrane, making them potential osmolytes.

Because volume regulation appears to involve a cascade of different channels that are activated under different degrees and duration of osmotic challenge (Kinne 1998), any inhibitory compound would need to target many different channels; otherwise, the cell may circumvent the drug and activate alternative channels. The finding that spermatozoa from C57BL6 and B6D2F1 mice maintain volume over 30 min incubation in media of 160 mmol/kg, but not 245 mmol/kg, may indeed indicate that the pathways of activation at these varying

osmolalities are different. The extent of blockade would be most effective if not limited to potassium channels, as Cl⁻ channels are also important in RVD (Yeung and others 2005a,b). The diversity of channels involved and the large numbers of spermatozoa that must be affected for the contraceptive method to be acceptable make this approach impracticable.

Preventing or reducing the epididymal provision of osmolytes

A second option would be to target the epididymal epithelium. An action at this level could aim to prevent or reduce the synthesis or transport of ions and organic solutes, which would alter the composition of the epididymal fluid. As the changing components and characteristics of this fluid during epididymal transport are considered to be important for maturation of spermatozoa, alterations in the milieu could incapacitate sperm function (Cooper 1992). Targeting the transport of specific osmolytes known to be major players in the RVD process, so that the spermatozoa are unable to acquire them during isovolumetric regulation, may leave the sperm ill-prepared for the osmotic challenges that await them at ejaculation. Organic cation transporters CT2 and OCTN have been identified in the rat and human epididymis (Rodriguez and others 2002, Enomoto and others 2002), but targeting them to reduce epididymal carnitine has not yet been attempted. Lowering epididymal carnitine has been achieved in another fashion by increasing its urinary excretion (Cooper and others 1997, Lewin and others 1997), but infertility was not induced in rats or hamsters and rat spermatozoa retained their normal carnitine content. Thus, depleting spermatozoa of epididymal osmolytes may be difficult.

Affecting sperm transport

Finally, a modification of the peritubular muscle action intended to speed the rate at which spermatozoa are transported through the epididymis may result in the ejaculation of immature spermatozoa. Normally spermatozoa remain in the epididymis for long periods of time, from 25 days in the guinea pig (Leibfried-Rutledge and others 1997), one week in the rabbit (Orgebin-Crist 1965) to 5 days in the mouse (Dadoune and Alfonsi 1994). If spermatozoa require a minimum amount of time in the epididymis to acquire the necessary amounts of osmolytes for RVD, then a reduced time in the epididymis may produce osmotically defective spermatozoa.

Targeting the volume regulatory mechanism of spermatozoa may have many advantages over other methods of contraception currently available to men, which include not interfering with hormone secretion. There is also the possibility of targeting spermatozoa once they reach the female tract with the application of a gel or spermicide. Here, the chances of depleting sperm osmolytes may be higher as the sperm concentration is lower. However, because there is a variety of acceptable contraceptive methods available to females, this approach is not likely to be investigated by pharmaceutical companies.

Handling of spermatozoa

Of particular importance for the human reproduction field is the finding that the osmolality of the ejaculate increases during the liquefaction process. Thus, the immediate environment of ejaculated spermatozoa obtained in the laboratory and assessed after liquefaction differs markedly from that at sexual intercourse, where the spermatozoa would quickly leave the seminal fluid and enter cervical mucus (Sobrero and Macleod 1962). The implications of these observations are profound, for spermatozoa ejaculated into the vagina are already at an osmolality close to that of cervical mucus (287 mmol/kg, Rossato and others 1996) and would not undergo the osmotic changes that occur to spermatozoa obtained in the laboratory after liquefaction and transferred to medium of serum-like tonicity for processing. New methods of processing semen in the laboratory that avoid extended exposure to the seminal plasma may improve the quality of spermatozoa obtained for ART. Whether or not the osmolality of semen from other animals also rises with time after ejaculation is unknown and would be also important for semen handling considerations.

Short-term storage of spermatozoa

The determination of isotonicity for epididymal spermatozoa and minimizing volume changes to preserve sperm osmolytes may also be important for short-term storage of spermatozoa. Many of the diluents currently used for semen are around 300 mmol/kg. Depending on the species, this may cause a hypotonic challenge that compromises the spermatozoa's ability to adjust to subsequent osmotic challenges. Attempts have been made to mimic epididymal fluid in an effort to preserve spermatozoa in alternative ways to cryopreservation, especially for relatively short storage duration. These are based on evidence

162

that spermatozoa held in the epididymis can remain viable for days after death of the animal (Kikuchi and others 1999, Soler and others 2003, Yu and Leibo 2002) and even be used to produce offspring (Songsasen and others 1998).

For example, bovine spermatozoa can be stored in the epididymis for weeks without significant loss of fertilizing ability (Verberckmoes and others 2004). In an effort to mimic the epididymal environment, some investigators have experimented with electrolyte-free (low ionic strength) media and have been able to prolong human sperm life for long periods at 4°C (Saito and others 1996a,b). Others have recreated the ionic and osmolal environment to create a superior diluent for preserving ejaculated bovine spermatozoa. Preservation in an epididymal-like fluid (CEP-2) was compared against a Tris diluent (Verberckmoes and others 2004), and after 6 days incubation at 5°C, spermatozoa exposed to the Tris diluent had higher percentage motility but also more hyperactivated spermatozoa. Hyperactivation is a feature associated with hypotonic swelling (osmolyte loss) and is to be avoided when spermatozoa are to be stored in a quiescent state. By contrast, the spermatozoa stored in epididymal-like fluid (CEP-2) had more progressively motile spermatozoa, which improved further when sorbitol was added (Verberckmoes and others 2004). Further experiments indicated that spermatozoa stored in CEP-2 for 6 days at 5°C had fertility rates (as indicated by extrusion of 2 polar bodies by oocytes) comparable to those of the commonly used diluent, Caprogen (primarily in New Zealand) and superior to that of Triladyl, another conventional diluent (Verberckmoes and others 2005). In these experiments, the osmolality of the CEP-2 diluent was adjusted to 320-325 mmol/kg to approximate that of bovine cauda epididymidal fluid.

Improvements to short-term sperm preservation have direct application to collection of spermatozoa from animals in the field, especially in countries with limited resources. If a diluent were developed that could maintain spermatozoa for days beyond its natural "shelf life" and avoid the necessity of cryopreservation, then spermatozoa collected from wildlife could be transported nearly anywhere in the world. As spermatozoa that have not gone through the cryopreservation process have higher rates of fertility, this could improve the success of various ART techniques for valuable animals in captivity. For some species cryopreservation is difficult (Holt 2000), and although 52 species of animals have been produced from frozen spermatozoa, for most endangered species cryopreservation methods have not been established.

Improving cryopreservation

During cryopreservation, spermatozoa undergo many drastic volume changes which have been identified as a source of cryodamage. These changes in volume are likely to render spermatozoa surviving the cryopreservation process incapable of responding to the hypotonic challenges they face during insemination or IVF with consequences for fertility (Cooper and Barfield 2006). Indeed, recent evidence from dogs demonstrates that the cryopreservation procedure reduces the ability of frozen-thawed spermatozoa to regulate volume under (non-physiological) hypotonic conditions (Petrunkina and others 2004, 2005).

In this dissertation, comparison of the osmotic responses of spermatozoa from the C57BL6 and B6D2F1 mice to varying osmolalities was stimulated by experiments conducted by Songsasen and Leibo (1997) in which simple exposure to cryoprotective media significantly reduced fertilization rates for the former strain but not the latter. These differences were attributed to differential sensitivity to the osmotic shocks associated with addition and removal of glycerol. This could be related to the volume regulatory capabilities of spermatozoa from the different strains, as the B6D2F1 appear to have superior RVD responses to C57BL6 mice. This suggests that the RVD capability has a genetic component, that if elucidated could be a valuable predictor of "freezability" of spermatozoa from a wide variety of species. From the results of the experiments by Songsasen and Leibo (1997) and others (i.e. Gao and others 1995, Thurston and others 2002), it seems clear that avoiding or minimizing osmotic shock as much as possible is one way to optimize sperm survival during the cryopreservation process (Holt 2000).

It may be that some non-physiological osmotic stress during the cryopreservation process is unavoidable and to this end it may be beneficial to provide the spermatozoa with osmolytes to compensate for those lost during the freezing and thawing process. The osmolytes could be made available to the sperm during the addition of cryoprotectant as the hypertonic media would cause cell shrinking. The activation of regulatory volume increase to counteract the shrinkage may provide an opportunity for the osmolytes to enter the cell. The cell would then have a larger reserve of osmolytes for the RVD that must occur during cryoprotectant removal. Once the major osmolytes used in sperm RVD are identified, future cryobiological experiments could include the addition of these osmolytes to CPA media to determine if this could increase the numbers of live sperm recovered post-thaw and impact fertilization rates.

Conclusion

Conservation biology is a diverse field that combines the expertise of scientists from a variety of backgrounds. The role of reproductive biology is critical for the conservation of endangered species, not only for the development of ART, but for understanding reproductive physiology and how it can be integrated into management of wild and captive animals. This dissertation explores an area of study that is in its early stages of development as it pertains to spermatozoa and for which the full implications are yet to be revealed. Whether the knowledge gained from these experiments contributes to improved sperm handling and preservation techniques, the development of a non-hormonal male contraceptive or perhaps just a better understanding of sperm physiology, is yet to be seen. Basic science is the foundation of scientific progress, and although this dissertation addresses a very narrow aspect of sperm physiology, it may be one of the important areas of study that comes together with others to make an impact in reproductive biology and ultimately conservation biology.

In addition to the scientific value of this doctoral research, this document embodies the results of a collaboration between often separate areas in the field of reproduction - the study of human reproduction and the study of animal reproduction. Exchange of information between clinicians and academic scientists can result in novel applications of old and new techniques, thereby spreading the potential benefits throughout the animal kingdom. An example of this is the application of human contraceptive methods to wildlife (Barfield and others 2006). Multidisciplinary research is a growing trend that must be embraced if we are to protect and preserve the species that compose our diverse ecosystems.

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