HYPERACTIVATION OF HUMAN SPERM BY 4-AMINOPYRIDINE: KEY ROLE FOR MOBILISATION OF STORED Ca^{2+} IN THE SPERM NECK

by

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ABSTRACT

When first deposited in the female reproductive tract mammalian sperm show progressive (activated) motility, with high flagellar beat frequency but low bend angle, particularly of the proximal flagellum. Hyperactivated motility is triggered by increased flagellar Ca^{2+} resulting in deep flagellar bends and exaggerated head movement. Two sources of Ca^{2+} may contribute to the regulation of hyperactivation. Ca^{2+} influx at the plasma membrane is crucial and CatSpers, sperm-specific, pH regulated, Ca^{2+}-permeable channels expressed in the principal piece of mammalian sperm are of great importance. In addition, studies on sperm from rodents, bulls and humans have provided strong evidence that Ca^{2+} stored in the region of the sperm neck contributes to regulation of flagellar activity. 4-aminopyridine caused robust and persistent hyperactivation of motility in human sperm. Ca^{2+} imaging showed that treatment with 4-aminopyridine induced a parallel elevation of $[Ca^{2+}]_{i,}$ which initiated at the sperm neck/midpiece and was associated with asymmetric flagellar bending in this region. The data presented here demonstrates that 4-aminopyridine induced hyperactivation in human sperm is not solely pH/CatSper channel dependent and that mobilisation of stored Ca^{2+}, possibly causing activation of store-operated Ca^{2+} influx, is essential for hyperactivation in a population of human sperm. Furthermore, 4-aminopyridine induced IP_3R and RyR store release in sacroplasmic reticulum and brain microsomal vesicles.

Although the physiological factor(s) that activate hyperactivation *in vivo* remains elusive we can conclude that release of the neck/midpiece intracellular Ca^{2+} store was sufficient to initiate hyperactivation in a population of human sperm.

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LIST OF ABBREVIATIONS

AA – Arachidonic acid

ACE – Angiotension-converting enzyme

ADA – N-(2-acetamido)iminodiacetic acid

ADP – Adenosine 5'-diphosphate

AKAP - A-Kinase anchor protein

ALH - Amplitude of lateral head displacement

ALHmax - The maximum ALH found along the trajectory

2-APB - 2-aminoethoxydipenylborane

AR – Acrosome reaction

ART - Assisted reproductive techniques

ATP – Adenosine 5'-triphosphate

BCECF- 2'-7'-bis-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein acetoxymethyl ester

8-bromo cGMP - 8-bromoguanosine-3'-5'-cyclophosphate sodium salt

BSA - Bovine serum albumin

Ca^{2+} - Calcium ion

$[Ca^{2+}]_i$ - Intracellular calcium concentration

CaM – Calmodulin

CaMK – calmodulin kinase

cAMP - Cyclic adenosine 3',5'- monophosphate

CASA - Computer-assisted semen analysis

Ca_v – Voltage activated calcium channel

CCE – Capacitative calcium entry

CD – Cluster of differentiation, cell membrane molecule identified by monoclonal antibodies and used to classify leukocytes into subsets.

CD9 – A type III transmembrane protein crosses the plasma membrane more than once

Cd^{2+} - Cadmium ion

CFsEBSS – Nominal Ca^{2+}-free supplemented Earle's balanced salt solution

CFTR - Cystic fibrosis transmembrane conductance regulator

cGMP - Cyclic guanosine 3',5'-monophosphate

CICR - Ca^{2+}-induced Ca^{2+} release

Cl^- - Chloride ions

4CmC - Chloro-m-cresol

CNG - Cyclic nucleotide-gated

CRAC – Ca^{2+} release activated Ca^{2+}

DNA – deoxyribonucleic acid

DAG - Diacylglycerol **DMSO** - Dimethyl sulfoxide

EGTA - Ethylene glycol-bis (β-amino-ethylether)-N,N,N'N'-tetraacetic acid

E_m – Membrane potential

ENaC - Epithelial Na^+ channels

ER- Endoplasmic reticulum

Fluo3 - 1-[2-Amino-5-(2,7-dichlooro-6-hydroxy-3-oxy-9-xanthenyl)phenoxyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid ammonium potassium salt

FS - Fibrous sheath

FSH - Follicle stimulating hormone

Fura2/AM – 5-Oxazolecarboxylic acid, 2-6-(bis(2-((acetloxy)methoxy)-2-oxoethyl) amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-, (acetyloxy)methyl ester

$GABA_A$ - Gamma-aminobutyric acid type A

GalTase – β 1,4-galactosyltransferase

GAPDH – glyceraldehyde phosphate dehydrogenase

GC – Guanylyl cyclase

GLR - Glycine receptor/Cl^- channel

H^+ - Hydrogen ions

HCO_3^- - Bicarbonate ion

Hepes - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HOS – Hypo-osmotic swelling solution

HNG - hyperpolarisation-activated nucleotide-gated

HPG – Hypothalmic-pituitary-gonadal

Hv1- Voltage gated proton pump 1

IBMX- 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor)

ICS – Intra-calcium store

IP_3 - Inositol-1,4,5-triphosphate

IP_3R - Inositol-1,4,5-triphosphate receptor

IVF - *In vitro* fertilization

K^+ - Potassium ion

Kir – Inwardly rectifying K^+ channel

KO – Knock out

La^{3+} - Lanthanum ion

LH - Luteinizing hormone

LIN - % linearity (straight line velocity/ curvilinear velocity x 100)

mAC - Membrane-associated adenylyl cyclase

MCU – Mitochondrial Ca^{2+} uniporter

mPR - Membrane progesterone receptor

MS - Mitochondrial sheath

Na^+ - Sodium ions

NaK-ATPase – Sodium potassium ATPase

NCX – Sodium calcium exchanger

Ni^{2+} - Nickel ion

NKCC – $Na^+/K^+/Cl^-$ co-transporter

1NM-PP1 – pyrazolo[3,4-*d*]pyrimidine (novel inhibitor)

NO – Nitric oxide

ODFs - Outer dense fibers

OGB-1AM - Oregon green 488 BAPTA 1-
acetoxymethyl

OR - Olfactory receptor

Orai - A protein that forms a calcium channel in the plasma membrane

PBS - Phosphate buffered saline

PDL - Poly-D-lysine

PGRMC1 - Progesterone membrane receptor component 1

PGRMC2 - Progesterone membrane receptor component 2

pH_i - intracellular pH

PIP_2 - Phosphatidylinositol 4,5-biphosphate

PKA - Protein kinase A

PKC - Protein kinase C

PKG - Protein kinase G

PLA – Phospholipase A

PLC - Phospholipase C

PMCA - Plasma membrane Ca^{2+}-ATPase

PP – protein phosphatase

PTP – Phosphotyrosine phosphatase

PTP - Permeability transition pore

RNE - Redundant nuclear envelope

Rh123 - Rhodamine 123 **(**2-(6-Amino-3-imino-3H-xanthen-9-l)benzoic acid methyl ester)

RNE – Redundant nuclear envelope

ROC – Receptor operated channels

RR – Ruthenium red dye

RyRs - Ryanodine receptors

sAC - Soluble adenylyl cyclase

sEBSS - Supplemented Earle's balanced salt solution

SED1- secondary zona-binding factor1

SERCA - Sarcoplasmic-Endoplasmic Ca^{2+}-ATPase

sGC - Soluble guanylyl cyclase

SKF96365 - 1-[β-[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCL

SNARE - Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor

sNHE – Sperm specific sodium hydrogen exchanger

SOAF - Sperm oocyte activating factors

SOC - Store-operated Ca^{2+} channel

SP56- Sperm protein 56

SPAM1 – Sperm adhesion molecule 1

SPCA - Secretory pathway Ca^{2+}-ATPase

SR – Sacroplasmic reticulum

STIM1 – Stromal interacting protein 1

tmACs - Transmembrane adenylyl cyclases

TAPS - [(2-hydroxy-1,1-bis(hydromethyl)ethyl)amino]-1-propanesulfonic acid

2TM – 2 transmembrane

6TM – 6 transmembrane

tmAC – Transmembrane adenylyl cyclase

TRP - Transient receptor potential

TRPC - Transient receptor potential canonical

Tsp-2 – Tyrosylprotein sulfotransferase-2

VAP - Average path velocity

VCL - Curvilinear velocity

VSL – Straight line velocity

VGCC - Voltage-gated Ca^{2+} channels

VOC – Voltage-operated channels

VOCCs - Voltage-operated Ca^{2+} channels

VSL - Straight-line velocity

WHO - World Health Organization

ZP – Zona pellucida

ZPBP1 – Zona pellucida binding protein 1

ZPBP2 – Zona pellucida binding protein 2

CHAPTER ONE

SIGNAL TRANSDUCTION IN HUMAN SPERMATOZOA

Foreword to Chapter One

This chapter summarises the recent progress that has been made in understanding the complex series of activities that spermatozoa must perform to fertilise the oocyte. This chapter describes the physical structure of the human gametes, in particular the spermatozoa. Emphasis is placed on regulation of motility and hyperactivation and the Ca^{2+} signalling events that occur within the sperm cell.

1.1 Mammalian spermatogenesis

Spermatogenesis is a complex process of proliferation and differentiation, occurring in the seminiferious tubules of the testis, resulting in the continuous production of highly differentiated spermatozoa (figure 1.1) (Sutovsky and Manandhar, 2006). During embryogenesis, the primordial germ cells migrate to the testis and are incorporated within the tubules to form spermatogonia. The reproductive system and germ line remain relatively quiescent until the peri-pubertal period (Sutovsky and Manandhar, 2006). At puberty, the diploid spermatogonia are re-activated and undergo mitosis in the basal compartment of the seminiferious tubules to form a reservoir of self renewing stem cells or differentiate to A1 type spermatogonia (figure 1.1). A1 spermatogonia undergo a number of mitotic divisions to form a clone of cells, some of which differentiate to primary spermatocytes before migrating through the blood-testis barrier. The blood-testis barrier is formed by intricate cell-cell junctions (Setchell, 2008) between adjacent cytoplasmic projections of Sertoli cells (nurse cells). Primary spermatocytes penetrate through this barrier to enter the immunoprivileged adluminal compartment and undergo meiotic divisions to form round haploid spermatids (Sutovsky and Manandhar, 2006).

Spermiogenesis involves the differentiation of the spermatid into elongated spermatid and finally spermatozoa. This process involves extensive cellular remodeling and the reduction of many spermatid organelles synchronised with the formation of specialised sperm structures such as the acrosome, flagellum and nucleus. Spermatogenesis is terminated at spermiation, when fully differentiated sperm detach from each other and from the apical surface of the seminiferous epithelium. Spermatozoa within the testis are quiescent and passage through the male reproductive tract is facilitated by the the flow of testicular secretions and ciliated epithelial cells. Following release from the seminiferious tubules, spermatozoa are transported through the epididymis and vas deferens where further biochemical and functional modifications (maturation) occurs (Marengo, 2008). Sperm are subsequently stored in the

cauda epididymis prior to ejaculation (Wassarman and Florman, 1997). Spermatogenesis is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus releases gonadotrophin-releasing hormone which stimulates the anterior pituitary to release the hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The human spermatogenic cycle takes approximately 70 days to complete (Sutovsky and Manandhar, 2006).

Figure 1.1

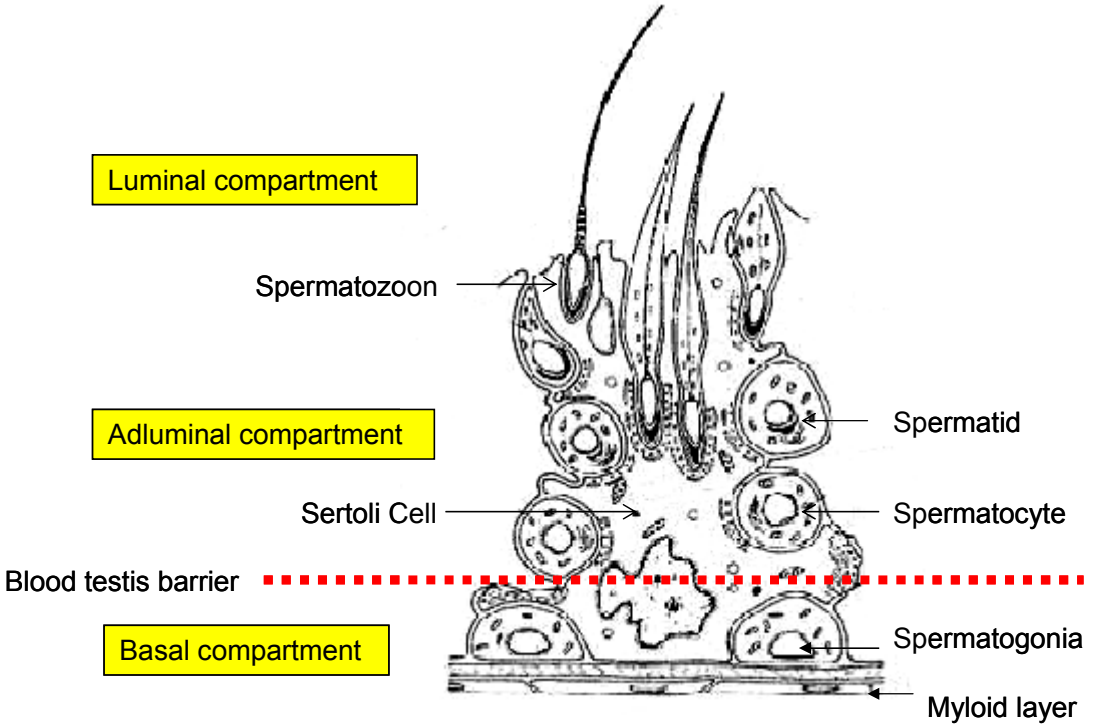

Figure 1.1: Epithelium of the seminiferous tubule, showing the relationship between Sertoli cells and developing sperm cells. As sperm cells mature, they progress towards the lumen of the seminiferous tubule (adapted from Glover *et al*., 1990).

1.1.1 The Sperm cell

Mature spermatozoa are highly polarised, compartmentalised, terminally differentiated cells (Barratt, 1995; Flesch and Gadella, 2000; Naz and Rajesh, 2004). The ungulate, carnivore and primate sperm head is pleomorphic, oval in shape and slightly bilaterally flattened, whereas a rodent sperm head is hook shaped. Taking into consideration any shrinkage that fixation and staining induce, the World Health Organisation (WHO, 1999) morphology guidelines state that the human spermatozoon head should be 4.0-5.0μm in length and 2.5-3.5μm in width. All eutherian mammalian sperm share the general features of the head and tail as described below.

The head contains the nucleus, acrosome and cytoplasmic droplet(s) (figure 1.2). The highly condensed nucleus contains tightly packed chromatin consisting of haploid supercoiled DNA (transcriptionally and translationally inactive) complexed with highly basic protamines. The nuclear envelope pore complexes are reduced during spermiogenesis with some nuclear pores remaining in the redundant nuclear envelope (RNE) located at the base of the nucleus (Ho, 2010). The perinuclear theca forms a unique cytoskeletal shell that encapsulates and protects the mammalian sperm nucleus. This extranuclear structure can be subdivided into three structurally continuous regions: the subacrosomal layer, equatorial segment and the postacrosomal sheath. The subacrosomal layer underlies the acrosomal segment of the sperm head, and functions to anchor the Golgi derived acrosome cap. The inner and outer acrosomal membranes encase the dense acrosomal matrix. The equatorial segment is a folded-over complex of periniuclear theca, inner and outer acrosomal membranes which remains intact following the acrosome reaction. The equatorial segment carries receptors (including the Izumo proteins- Inoue *et al*., 2005; Isotani and Okabe, 2005; Ellerman *et al*., 2009; Ikawa *et al*., 2008, 2010) involved in secondary sperm-oolemma binding. The postacrosomal sheath of the sperm perinuclear theca is believed to contain a complex of sperm borne, oocyte activating factors (including phospholipase C ζ (PLCζ)) (Ciapa and Chiri, 2000; Swann and Lai, 2002;

Swann *et al*., 2004, 2006; Swann and Yu, 2008; Dale *et al.,* 2010).

During spermatogenesis approximately 50% of the mitochondria are lost and the remainder migrate to the base of the nucleus and are arranged in a helix around the proximal piece of the flagella surrounded by the mitochondrial sheath (Cataldo *et al.,* 1996). The cytoplasmic droplet(s) contains redundant cytoplasm and organelles such as mitochondria and ribosomes. The head is separated from the midpiece by the posterior ring.

Spermatozoa are immotile in the testis and are activated upon release into physiological fluid (containing HCO_3^- and Ca^{2+}). The function of the flagellum (figure 1.2) is to produce energy and propel the spermatozoon from the site of semen deposition in the vagina, through the female reproductive tract and finally aid penetration of the outer vestments of the oocyte. The innermost structure of the flagellum consists of the axoneme paralleled by the outer dense fibres (ODF). Sperm motility is provided by the 9+2 arrangement of microtubules that form the axoneme. The nine outer microtubule doubles (figure 1.2a) are paralleled by nine outer dense fibers that provide flexibility and support. The external structure of the sperm flagellum can be divided into four subdivisions – the connecting piece, the mid-piece, the principal piece and the end-piece (Fawcett, 1975). The connecting piece is a short linking segment and is most proximal portion of the flagellum that attaches to the sperm head (0.5μm). The connecting piece contains the sperm proximal centriole (the distal centriole disintegrates during spermiogenesis). The proximal centriole marks the origin of the pair of central microtubule of the axoneme. The midpiece is covered by the mitochondrial sheath containing the helical arrangement of mitochondria (3.5μm). The principal piece (~55μm) is covered by the fibrous sheath, composed of two longitudinal columns running parallel to the outer dense fibres three and eight, and stabilised by circumferential or transverse ribs (figure 1.2b). Finally, the end-piece is a terminal portion of the flagellum containing the axoneme only surrounded by plasma membrane (~3μm) (Turner, 2006).

Figure 1.2

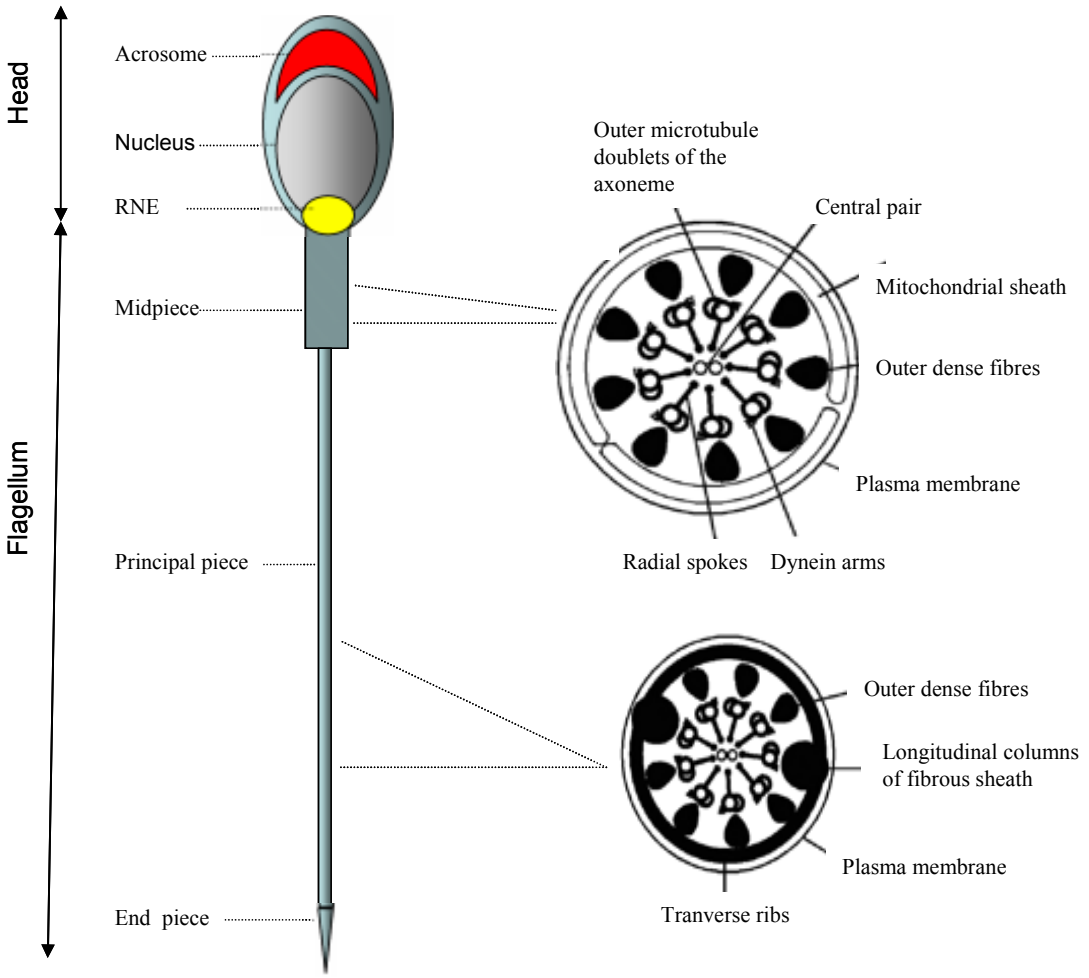

Figure 1.2: Schematic representation of a mammalian sperm cell and the ultrastructure of the flagellum. (a) Schematic cross-section through the midpiece showing the plasma membrane, mitochondrial sheath surrounding the 9 outer dense fibres. Within the outer dense fibres (ODF) are the components of the axoneme: the 9 outer microtubule doublets with associated dynein arms, radial spokes and the central pair of microtubule doublets. (b) Schematic cross-section through the principal piece showing the plasma membrane surrounding 7 ODFs (3 and 8 have been replaced with longitudinal columns of fibrous sheath. The two columns are connected by transverse ribs) (adapted from Turner, 2006 and Lefièvre *et al*., 2009)

1.1.2 Semen

Seminal fluid is a heterogeneous mixture consisting of ejaculated spermatozoa (1-5%) and accessory gland secretions mainly from the prostate gland (30%) and seminal vesicles (60%) (Polakoski *et al.,* 1976; Mann and Lutwak-Mann, 1981; Coffey, 1995). The ejaculate of normozoospermic males contains ≥20 million spermatozoa per ml, ≥50% of these sperm are progressively motile and ≥30% of the sperm are morphologically normal (WHO, 1990). The function of seminal fluid is to provide nutrients, a transport medium and to facilitate sperm movement. Prostate gland secretions include citrate (a nutrient source), inositol, calcium, zinc, magnesium, fibrinolysin and acid phosphatase (involved in activating sperm). Seminal vesicle secretions include fructose, ascorbic acid, vesiculase (a coagulating enzyme) and prostaglandins. Sperm stored in the cauda epididymis are immotile but are immediately activated when sperm are released from the epididymis into semen (high bicarbonate (HCO_3^-) and calcium (Ca^{2+}) conc.) (Visconti and Kopf, 1998; Visconti *et al*., 1998; 2002; Visconti, 2009). Sperm maintained in seminal plasma are unable to capacitate due to the presence of de-capacitating factors (de Lamirande *et al*., 1997; Luconi *et al*., 2000; Fraser, 2010). The first fraction of the ejaculate contains the majority of the spermatozoa which quickly swim out and penetrate the cervical mucus (Katz *et al*., 1978a). Human semen coagulates immediately following ejaculation, although the exact physiological role remains unclear (Burkman, 1995; Mortimer, 1995).

1.2 Oogenesis

During embryogenesis, oogonia develop from the primordial germ cells and undergo a limited number of mitotic divisions. Many of these primary oocytes undergo degeneration or follicular atresia. The primary oocytes (2N) that continue to develop are enclosed in a primary follicle and arrest at meiotic prophase1.

At birth the eutherian primary oocyte is surrounded by the zona pellucida (ZP) glycoproteins and enveloped by a follicle consisting of a single layer of epithelial cells and a less organised layer of mesenchymal thecal cells. With the onset of puberty and stimulated by hormones a number of oocytes periodically mature and complete meiosis 1. The cytoplasm of the primary oocyte divides asymmetrically so that the one of the daughter cells or secondary oocyte (2N) retains most of the cytoplasm and the other cell is a nonviable small polar body (2N). Secondary oocyte maturation proceeds to metaphase 11 and arrests until fertilisation. The follicle undergoes folliculogenesis until it forms a mature pre-ovulatory Graafian follicle. The Graafian follicle consists of several layers of concentric cumulus cells, a fluid filled antrum and the secondary oocyte surrounded by the corona radiata granulosa cells. At ovulation the arrested secondary oocyte is released from the ovary surrounded by ZP and the cumulus oophorus. Upon fertilisation the oocyte is stimulated to complete meiosis 11 whereby the secondary oocyte divides asymmetrically to produce an ovum (1N) and another polar body (1N).

The zona pellucida (ZP) is a thick, translucent extra-cellular sulphated glycoprotein matrix (Wassarman, 1999; Wassarman *et al.*, 2001; 2004). This oocyte specific structure is secreted during folliculogenesis and comprises 3 or 4 ZP glycoproteins which interact via non-covalent bonds (Greve and Wassarman, 1985; Wassarman *et al*., 2004). Due to a paucity of human zona pellucida most ZP research involves murine models. Mouse zona pellucida is composed of three glycoproteins (ZP1, ZP2 and ZP3). The ZP1 glycoprotein acts as a linker molecule between ZP filaments and provides the structural integrity of the ZP matrix (Green, 1997). The

human oocyte expresses four ZP genes (ZP1, ZP2, ZP3 and ZP4 or ZPB, ZP4 is a pseudogene in mice) (Lefièvre *et al*., 2004; Conner *et al*., 2005; Gupta *et al*., 2009). The role of mammalian ZP is to ensure species-specific sperm-oocyte binding, prevent polyspermy and protection of the embryo until blastocyst implantation in the uterus (Flesch and Gadella, 2000; Wassarman *et al*., 2001; 2004).

The cumulus oophorus surrounds the ovum and consists of an inner layer (corona radiata) and outer layers of follicular granulosa cells dispersed in a hyaluronic acid matrix. Mammalian spermatozoa must initially penetrate the cumulus matrix in order to bind to ZP proteins. Before ovulation the cumulus oophorus supports oocyte maturation and during ovulation it guides the oocyte into the oviduct (Tanghe *et al.*, 2002). Cumulus cells facilitate sperm capacitation and the acrosome reaction in human, bovine, porcine and hamster spermatozoa (Laufer *et al.*, 1984; Tanghe *et al*., 2002). The cells of the cumulus secrete steroids including progesterone and synthesise nitric oxide (Tesarik *et al*., 1988, 1990; Osman *et al*., 1989; Patrat *et al*., 2000; Kirkman-Brown *et al*., 2000, 2002; Sun *et al.*, 2005; Machado-Oliveira *et al*., 2008).

1.3 Sperm transport in the female reproductive tract and fertilisation

If coitus occurs mid-cycle, a limited number of sperm quickly leave the seminal plasma, and begin the physiological maturation process of capacitation as they penetrate the cervical mucus and travel through the female reproductive tract (Katz *et al*., 1978a; Burkman, 1995). Passage of spermatozoa through the female tract is regulated to maximise the chance of fertilisation and ensure that sperm with normal morphology and vigorous motility will be the ones that arrive in the Fallopian tube. The cervical mucus serves as a filter to trap damaged (by physical stress during ejaculation) or abnormal spermatozoa (with poor motility and morphology) (Suarez and Pacey, 2006). Since, spermatozoa are allogenic and targeted by the female immune system they have a limited time within the female reproductive tract (Austin, 1957; Menge and

Edwards, 1993). In humans, contractions of the uterine muscle may draw spermatozoa from the cervix into the uterus (Kunz *et al*., 1996). Spermatozoa may be inseminated days (humans, cattle, pigs and horses) or months (some bat species) prior to ooctye arrival and sperm may be stored or trapped at the uterotubal junction (Suarez, 2002a). Although there is little evidence for a human sperm storage reservoir in the Fallopian tubes, oviduct cells prolong sperm survival *in vitro* (De Mott et al., 1995; Pacey et al., 1995a, 1995b; De Jonge, 2005; Suarez and Pacey, 2006).

1.4 Capacitation

Mammalian spermatozoa are unable to fertilize the oocyte immediately after ejaculation but must undergo a maturation process termed capacitation (Austin 1951; Chang, 1951). Physiologically, capacitation occurs as spermatozoa leave the seminal plasma and swim through the female genital tract (Yanagimachi, 1970). Capacitation is a complex series of molecular events that occurs in spermatozoa after epididymal maturation and confers on spermatozoa the ability to fertilise an oocyte (Austin 1951; Chang, 1951; Burkman, 1995; Ho and Suarez, 2001a; Visconti *et al*., 2002). Capacitation can therefore be described as a time dependent activation process where sperm must reside in the female genital tract or a defined culture medium for a period of time (Visconti *et al.*, 1998; Visconti and Kopf, 1998; Flesch and Gadella, 2000; Baldi *et al*., 2000, 2002; Gadella and Visconti, 2006). Capacitated spermatozoa have the ability to exhibit hyperactivated motility (section 1.13) and undergo the ZP-induced acrosome reaction (section 1.5.1) (Yanagimachi and Usui, 1974; Suarez and Osman, 1987; Cross *et al*., 1988; Suarez, 2002b; 2008a; Schuffner *et al*., 2002).

The molecular mechanisms involved in capacitation are still under investigation. Capacitation includes reorganisation of the plasma membrane, increase in protein tyrosine phosphorylation (section 1.9), hyperpolarisation of the plasma membrane potential (E_m) (section 1.7) and

increase in intracellular pH (pH_i) (section 1.8) and Ca^{2+} ($[Ca^{2+}]_i$) (section 1.6) (Parrish *et al*., 1989; Burks *et al*., 1995; Zeng *et al*., 1995, 1996; Darszon *et al*., 2001; Visconti *et al*., 2002; Hernandez-Gonzalez *et al*., 2006, 2007). Capacitation may be induced *in vitro* by removal of sperm from the seminal plasma followed by incubation in defined culture media containing calcium, serum albumin (to serve as a cholesterol acceptor) and bicarbonate (activation of bicarbonate sensitive soluble adenylyl cyclase (sAC)(section 1.9)) (Wuttke *et al*., 2001; Chen *et al*., 2000; Gadella and Visconti, 2006).

Sperm hyperactivation is critical for *in vivo* fertilisation, enabling sperm to detach from the oviduct epithelium and facilitating penetration of the outer vestments of the oocyte (section 1.3) (Morales *et al*., 1988; Suarez, 2008b; Ho *et al*., 2009). The acrosome reaction induced by a physiological stimulus (ZP or progesterone) is used as a marker for the completion of capacitation as only capacitated sperm can acrosome react (Cummins and Yanagimachi, 1986; Morales and Llanos, 1996; Jaiswal *et al*., 1998) .

1.5 The acrosome reaction

The acrosome, is a Golgi derived membrane bound cap like secretory vesicle (Curry and Watson, 1995; Moreno *et al.,* 2000), which comprises 40-70% of the anterior head volume (WHO, 1999) containing various hydrolytic enzymes including hyaluronidase, protease, phosphatase, neuraminidase and inactive zymogens. The function of the acrosome is to facilitate passage of the sperm through the outer vestments of the oocyte. The acrosome reaction (AR) is a Ca^{2+} dependent secretory event characterised by multiple fusions of the plasma membrane. The plasma membrane of the anterior sperm head fuses with the outer acrosomal membrane (Baibakov *et al.*, 2007) to produce numerous hybrid membrane vesicles and expose new membrane domains, both of which are essential for fertilisation to proceed. Dispersion of the acrosomal contents dissolves the ZP matrix and the inner acrosomal membrane forms a continuous membrane hairpin like structure with the plasma membrane

(figure 1.3) (Flesch and Gadella, 2000). The acrosome reacted spermatozoa enter the perivitelline space to initiate secondary binding, including Izumo sperm protein binding with CD9 on the oolemma (figure 1.3) (Miyado *et al*., 2000; 2008). Following membrane fusion the sperm borne oocyte activating factors (including PLCζ) are released to trigger calcium oscillations and the resumption of meiosis in the oocyte (Swann and Lai, 2002; Swann *et al*., 2006; Swann and Yu, 2008). Izumo null mice sperm maintain the ability to penetrate the ZP but accumulate within the perivitelline space and fail to bind with the oocyte (Ikawa *et al*., 2008, 2010; Ellerman *et al*., 2009). Oocyte re-activation releases the cortical granules (specialised secretory vesicles just under the oolemma) to alter the structure of the ZP and prevent polyspermy (Bleil *et al*., 1981). In mouse the physiological inducer of the acrosome reaction *in vivo* is the ZP3 glycoprotein (Bleil and Wassarman, 1980a, 1980b, 1983; Bailey and Storey, 1994; Arnoult *et al*., 1996a, 1996b). Both ZP3 and ZP4 are required to induce the acrosome reaction in capacitated human spermatozoa (Chakravarty *et al*., 2008; Gupta *et al.,* 2009). Progesterone synthesised by the cumulus cells surrounding the oocyte may have a priming effect on sperm AR (Morales and Llanos, 1996; Schuffner *et al*., 2002).

1.5.1 Zona Pellucida3 induced acrosome reaction

A plethora of molecules have been investigated in sperm-oocyte interactions but the identity of the sperm ZP3 receptor(s) remains unclear (table 1.1). Given the number of candidates it is possible that a number of structurally/functionally distinct receptors exist (Shur *et al.,* 2006; Correia *et al.,* 2007; Shur, 2008; José *et al*., 2010). It has been demonstrated that the acrosome reaction occurs in stages whereby components of the acrosomal matrix peripherally re-locate to the surface and interact with the ZP (Buffone *et al*., 2009). There is evidence of a degree of redundancy in mouse as mutations to many of the putative sperm ZP3 receptor (targeted deletion of secondary zona-binding factor (SED-1), β-1,4-galactosyltransferase (GalTase), zona pellucida binding protein (ZPB1 or ZPB2) or proacrosin do not necessarily interfere with

sperm-ZP binding and AR (Shur, 2008). However, the ability of knockout (KO) animals to remain fertile may reflect spontaneous AR during IVF. The AR is a pre-requisite for fertilisation in mammalian sperm (Nagae *et al*., 1986; Yanagimachi, 1994a, 1994b) as only reacted sperm can fuse with the egg plasma membrane (Yanagimachi 1994a; Florman *et al*., 1999).

Table 1.1- Putative mouse sperm ZP3 receptor

Beta 1,4-galactosyltransferase (GalTase)	KO mice are fertile, ZP3-induced AR defective (Lu and Shur, 1997; Shur *et al.*, 2006).
Secondary zona-binding factor (SED1)	KO mice fertile (Ensslin and Shur, 2003). SED1 undergoes sulfation (mediated in Golgi by Tyrosylprotein sulfotransferase-2 (TSP-2). *Tpst-2* KO mice infertile, severe defects in motility (Hoffhines *et al*., 2009).
Zonadhesion	KO mice are fertile, normal ZP3 binding. Confers species specificity to ZP adhesion (component of acrosomal matrix) (Shur *et al*., 2006).
Angiotensin-Converting Enzyme (ACE)	*ACE* KO mice are fertile, mild defect in ZP binding, impaired migration into the oviduct (Hagaman *et al*., 1998; Inoune *et al*., 2010).
Fertilin β (ADAM2)	*Ferilin β* KO fertile, decreased sperm-oocyte fusion, defective ZP binding, impaired migration into oviduct (Primakoff and Myles, 2000; 2002; Kim *et al*., 2006). Calmegin (testis-specific chaperone protein) KO mice reduced fertility due to loss of Fertilinαβ and ZP binding (Ikawa *et al*., 1997)
Cyritestin (ADAM3)	KO mice were infertile, defective ZP binding, impaired migration into the oviduct (Yamaguchi *et al*., 2009). ADAM3 is a pseudogene in humans (Frayne *et al*., 1999).
sp56	Potential ZP3 receptor, however sp56 is located within acrosomal matrix. Sp56 relocated to capacitated plasma membrane where may interact with ZP3 (Wassarman, 2009).
Proacrosin/acrosin	KO mice fertile but penetration of ZP delayed compared to wild type, located acrosomal matrix (Buffone *et al.*, 2009).
PH-20 or SPAM1 (sperm adhesion molecule 1)	KO mice fertile, slow to disperse cumulus cells (Baba *et al*., 2002).
Zona Pellucida Binding Protein 1(sp38) Zona Pellucida Binding Protein2	Components of acrosomal matrix. ZPBP1 KO mice are sterile, ZPBP2 KO mice are subfertile (small defects in sperm) (Lin *et al*., 2007)
Glycine receptor/ Cl^- channel (GLR)	Mice with mutations in GLRα or GLRβ defective ZP3 or glycine induced AR (Sato *et al*., 2000).

Table 1.1 Summary of putative sperm ZP3 receptors identified in mouse (including the effects of mutations on male fertility and sperm motility).

Sperm binding with ZP3 triggers various responses required to produce a sustained calcium influx which drive the acrosome reaction. ZP3 induced signal transduction includes activation of a pertussis toxin-sensitive G_i-coupled receptor resulting in cytoplasmic alkalinisation (Florman *et al.,* 1989; Ward *et al*., 1994; Arnoult *et al*., 1996b, 1998). Sperm-ZP3 binding activates phospholipase C (PLC). The resulting inositol triphosphate (IP_3) dependent mobilisation of stored acrosomal calcium is essential (De Blas *et al*., 2002; Herrick *et al*., 2005) and likely to induce store operated Ca^{2+} influx possibly via canonical Transient Receptor Potential (TRP) channels (Clapham *et al.*, 2005). Jungnickel *et al.,* (2001) reported that TRPC2 co-localised with ZP3 binding sites and application of a TRPC2 antibody prevented the sustained calcium response to ZP3. However, TRPC2 is a pseudogene in humans (Wes *et al*., 1995; Vannier *et al*., 1999) and bovine sperm (Wissenbach *et al*., 1998) and TRPC2 mutant mice remain fertile (Leypold *et al*., 2002). These results suggest that other channels in the anterior head (including other TRPC channels) are involved in the sustained calcium influx and substitute for TPPC2. In addition ZP3 binding triggers a transient Ca^{2+} influx, the activation time course and effects of pharmacological antagonists suggest this transient calcium entry is carried by a low voltage calcium channel (possibly Cav3) (Arnoult *et al*., 1996a; Jimenez-Gonzalez *et al.*, 2006; Florman *et al*., 2008). However, a recent study provided evidence for an alternative model in which the initial transient $[Ca^{2+}]_i$ influx in mouse sperm was via CatSper channels (section 1.13.1) located in the flagellum, the signal propagating rapidly (~3 sec at 37^oC) from the principal piece to the head (Xia and Ren, 2009b).

Combined calcium channel opening and depletion of Ca^{2+} from acrosomal stores results in elevation of $[Ca^{2+}]_i$ to drive exocytosis of the acrosomal contents (Florman *et al,* 1989; Florman, 1994).

The fusion proteins SNARE (soluble N-ethylmaleimide-sensitive attachment protein receptors), which are localised to the outer acrosomal membrane of mammalian and sea urchin sperm (Schulz *et al*., 1997; Michaut *et al*., 2000; Hutt *et al*., 2002; Tomes *et al*., 2002) couple calcium

influx with exocytosis (Evans and Florman, 2002). Relatively high $[Ca^{2+}]_i$ activates actin severing proteins such as gelsolin, inducing actin filament depolymerisation between the outer acrosomal membrane and the overlaying plasma membrane (Cabello-Agueros *et al*., 2003). The apical plasma membrane of the sperm head fuses with the underlying outer acrosomal membrane at multiple sites, forming mixed vesicles that disperse and the acrosomal contents are released (figure 1.3) (Breitbart, 2003).

Other molecular components of the acrosome reaction include calcium dependent Phospholipase A_2 (PLA_2). Hydrolysis of phospholipids in the acrosome reaction generates free fatty acids (arachidonic acid (AA)) and formation of lysophosphatidylcholine, metabolites involved in membrane fusion and activation of Protein Kinase C (PKC) (Yuan *et al*., 2003; Abou-haila and Tulsiani, 2009). PKC activators and inhibitors modulate the AR in sperm cells (De Jonge *et al*., 1991; Roldan and Harrison, 1993).

Figure 1.3

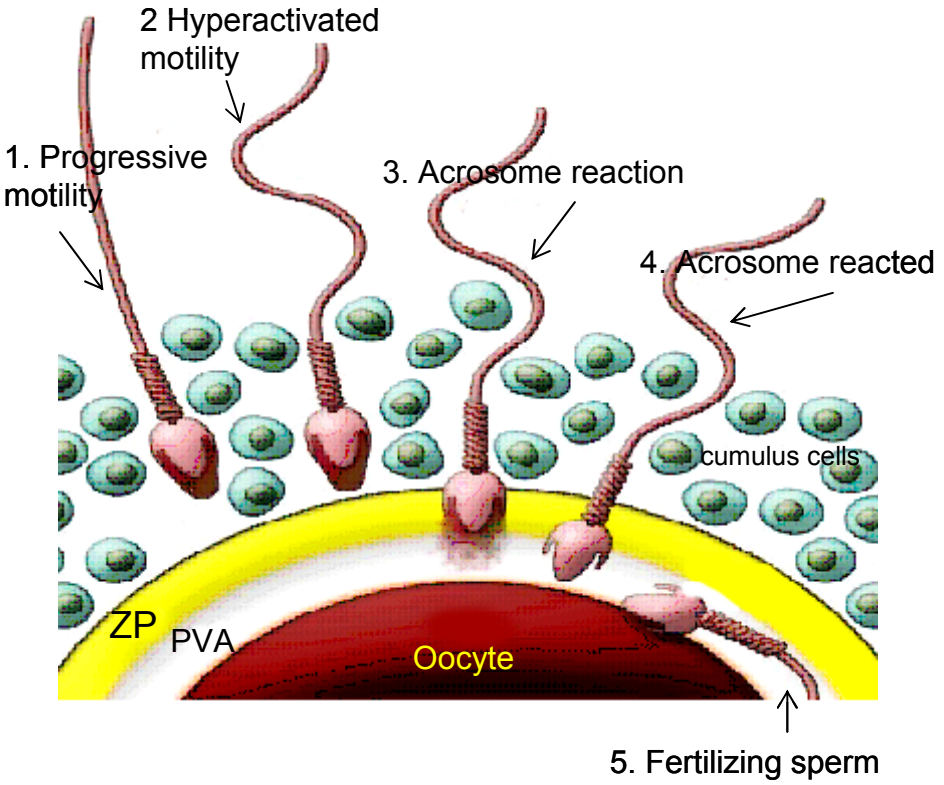

Figure 1.3 Mechanism of sperm-egg interaction. As spermatozoa move through the female reproductive tract they undergo a maturation process termed capacitation. As capacitation progresses, spermatozoa alter motility from progressive motility (1) to the relatively non-progressive hyperactivated motility (2) enabling cells to push through the cumulus cells surrounding the oocyte. (3) Sperm undergo the zona pellucida induced acrosome reaction and (4) release of the acrosomal contents enables the sperm cell to enter the perivitelline space. (5) The equatorial segment carries receptors involved in secondary sperm-oolemma binding and fusion with the oolemma (adapted from Ikawa *et al.*, 2010)

1.5.2 Progesterone induced acrosome reaction

Progesterone is synthesised by the cumulus cells and present in micromolar concentrations surrounding the oocyte (Osman *et al*., 1989; Thomas and Meizel, 1989). Due to the paucity of human ZP, the dose-dependent progesterone induced acrosome reaction has been extensively studied in human sperm (Blackmore *et al*, 1990; Baldi *et al*, 1991; Foresta *et al.*, 1993; Plant *et al.*, 1995; Aitken *et al*., 1996; Yanagimachi, 1994b; Kirkman-Brown *et al.,* 2000; Harper *et al*, 2003, 2006, 2008). Progesterone binds to a cell surface receptor (Meizel and Turner, 1991), although the identity of the receptor remains unclear (table 1.2) and may consist of more than one candidate receptor forming a complex progesterone binding molecule (Correia *et al*., 2007). It has been shown that progesterone and follicular fluid act in synergy with ZP3 to induce the acrosome reaction in mouse sperm (Roldan *et al*., 1994; Schuffner *et al.,* 2002).

In human spermatozoa, progesterone effects are not associated with G_i-protein activation (Tesarik *et al*., 1993; Abou-haila and Tulsiani, 2009). Capacitated human spermatozoa respond to progesterone *in vitro* with a rapid influx of calcium due to receptor operated mechanism and activation of a non-specific calcium ion channel (Meizel *et al.*, 1997; Blackmore and Eisoldt, 1999). A role for voltage operated calcium channels (VOCC, section 1.6.1) in this effect is unclear (Blackmore and Eisoldt, 1999; Garcia and Meizel, 1999). As with mouse ZP3 induced AR, the initial transient influx is followed by a slower, more sustained increase in intracellular calcium possibly triggered by store mobilisation and capacitative calcium entry (CCE, section 1.6.4) (Thomas and Meizel, 1989; Bedu-Addo *et al*., 2007). Progesterone also stimulates PLA_2 in murine acrosomal reaction (Pietrobon *et al*., 2005). Fukami *et al*., (2003) demonstrated that the initial $[Ca^{2+}]_i$ response initiated in the acrosome in mice treated with ZP3 whereas the progesterone induced calcium signal intiated in the neck/midpiece region.

Furthermore, human spermatozoa undergo the acrosome reaction in response to low dose progesterone *in vitro* (Harper *et al.,* 2004). Should this happen *in vivo* as the sperm cell

approaches the cumulus-oocyte complex these prematurely reacted cells would lose their ability to fertilise (Harper and Publicover, 2005). Premature acrosome reaction may serve as a quality control function potentially removing poorer quality cells (Harper and Publicover, 2005).

Table 1.2- Putative sperm progesterone receptor

Truncated classical progesterone receptor	hmPR1/PGRMC1 and hmPR2/PGRMC2 immunolocalised PGRMC1 to the inner plasma membrane of permeabilised porcine spermatozoa (Losel *et al*., 2004, 2005). Progesterone may diffuse through the membrane to bind on the inner plasma membrane.
G-coupled membrane progesterone receptors	Novel G-coupled PR identified, cloned and characterised in humans and other vertebrates (Zhu *et al*., 2003). However, Krietsch *et al.* (2006) found that no progesterone binding activity or calcium mobilisation when receptors expressed in mammalian cell systems.
γ-aminobutyric acid ($GABA_A$)-receptor /Cl^- channel	Inhibition of Cl^- efflux by channels blockers or incubation in Cl^- free media reduces the progesterone induced AR rates in murine, porcine and human spermatozoa (Roldan *et al*., 1994; Shi and Roldan, 1995; Wistrom and Meizel, 1993; Calogero *et al*., 1999). However, others report that inhibition with $GABA_A$ receptor blocker (bicuculline) did not affect the progesterone induced calcium influx or AR (Baldi *et al*., 1991).

Table 1.2 Summary of putative sperm progesterone receptors identified in mammalian sperm.

1.6 The sperm calcium signalling 'toolkit'

Spermatozoa rely on post translational modifications of existing proteins regulated by second messengers including cyclic adenosine 3',5'-monophosphate (cAMP), nitric oxide (NO) and Ca^{2+} (San Agustin and Witman, 1994; Jimenez-Gonzalez *et al*., 2006). Calcium functions as a ubiquitous second messenger and fluctuations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) mediate rapid transmission of information from extracellular signals to internal response systems (Berridge, 1997; Breitbart, 2003; Bedu-Addo *et al*., 2008). Mature spermatozoa are 'stripped down' cells lacking an endoplasmic reticulum, have minimal cytoplasm with a highly condensed nucleus and consequently appears to have a much simplified calcium signalling toolkit (figure 1.4) in comparison to somatic cells (Publicover *et al.,* 2007; Bedu-Addo *et al*., 2008; Barratt *et al*., 2009). Numerous studies have established that changes in Ca^{2+}-signalling regulate or contribute to the regulation of many different aspects of mammalian sperm function (Kirkman-Brown *et al*., 2002; Ho and Suarez, 2003; Jimenez-Gonzalez *et al*, 2006; Suarez *et al*., 2007; Publicover *et al*, 2007). Spermatozoa generate discrete, spatiotemporal Ca^{2+} signals to selectively regulate specific activities in response to extracellular cues (e.g. ZP induced acrosome reaction, chemotaxis in response to low dose progesterone) (Publicover *et al*., 2007; Machado-Oliveira *et al*., 2008; Teves *et al*., 2006, 2009). $[Ca^{2+}]_i$ has been shown to increase during capacitation, hyperactivation and the acrosome reaction in several mammalian species including human (Baldi *et al*., 1991; Yanagimachi, 1994a; Garcia and Meizel, 1999). Impairment of calcium signalling in sperm is associated with male sub-fertility (Baldi *et al*., 1999, Espino *et al*., 2009). The application of standard cell electrophysiological techniques is technically demanding in spermatozoa therefore identification and characterisation of the sperm calcium 'toolkit' is complex (figure 1.4).

Figure 1.4

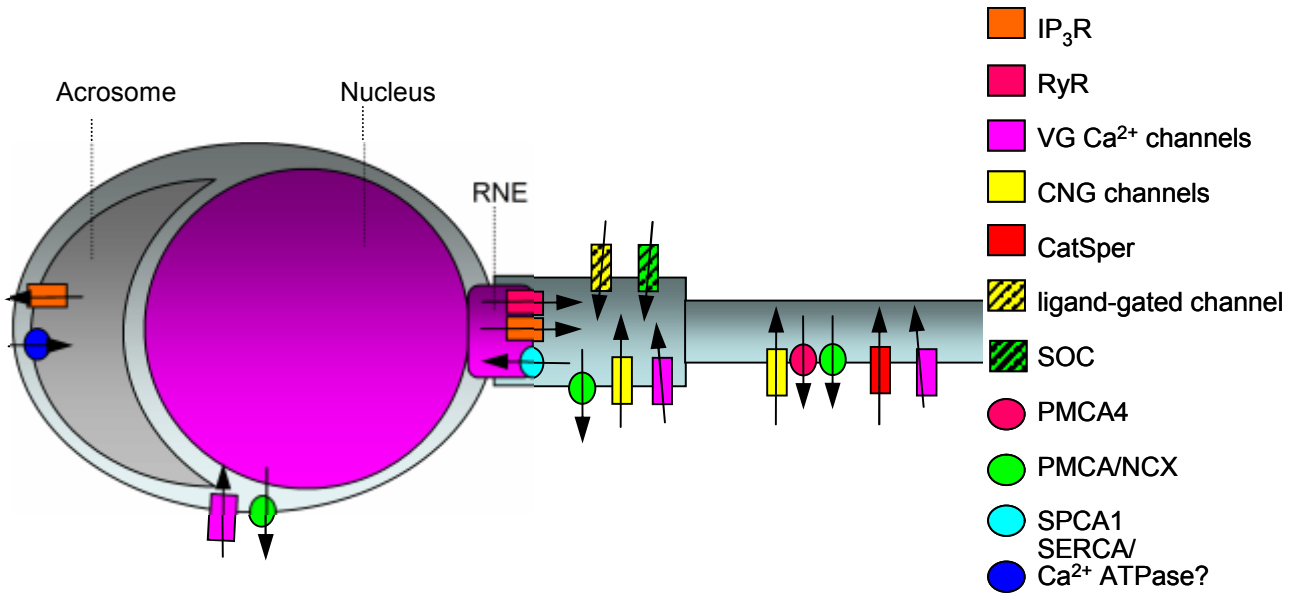

Figure 1.4 Summary of sperm cell $[Ca^{2+}]_i$ signalling toolkit (refer to text for details) (adapted from Publicover *et al*., 2008; Bedu-Addo *et al*., 2008)

1.6.1 Voltage Gated Calcium Channels and CatSper Channels

Voltage gated calcium channels ($Ca_{v,}$ refer to appendix I and table 7.0 for further details) are multi-subunit transmembrane channels which open in response to membrane depolarisation (figure 8.0) (translate membrane potential (E_m)) (Darszon *et al*., 2006). Molecular and immunohistochemistry studies show that several types of Ca_v are present in sperm (Wennemuth *et al*., 2000; Jagannathan *et al*, 2002; Felix, 2005). Park *et al* (2003) detected transcripts for Ca_v1 and Ca_v2 (high voltage activated channels) and Ca_v3 (low voltage activated channels) in human spermatozoa. $Ca_v3.1$ and $Ca_v3.2$ have been immunolocalised to the sperm

head and $Ca_v3.3$ is limited to the mid-piece region of human sperm (Trevino *et al*., 2004; Jimenez-Gonzales *et al*., 2006). High voltage activated (Ca_v1 and Ca_v2) channels has shown similar localisation in mouse and human. However, electrophysiological studies on mouse and human spermatogenic cells generate only Ca_v3 (transient) currents (Trevino *et al*., 2004). Faire-Zamora and Gonzalez-Martinez (2004) demonstrated that intracellular alkalinisation stimulated a depolarisation-evoked calcium influx in human sperm and this effect was enhanced in capacitated cells (Neri-Vidaurri Pdel *et al*., 2006). However, disruption of the genes that encode $Ca_v3.1$ and $Ca_v3.2$ channels did not affect fertility in mice (Escoffier *et al.,* 2007; Florman *et al*., 2008). Similar results were obtained with $Ca_v2.2$ and $Ca_v2.3$ deficient mice (Sakata *et al*., 2002). Recently, Xia and Ren (2009b) detected Ca_v3 currents in mouse spermatocyes and spermatids but the current gradually decreased as sperm became more mature. No current was detectable in mouse eipdidymal sperm.

Catspers (refer to Appendix I, table 8.0) are a novel family of Ca^{2+} permeable cation channels, resembling voltage-gated Ca^{2+} channels, which are expressed exclusively in sperm. Recent studies have shown that CatSper channels are important in hyperactivation and the acrosome reaction in mouse models (Xia and Ren, 2009b). The CatSper channels (formed from putative 6TM subunits), are voltage gated, Ca^{2+} permeant channels that mediate $I_{CatSper}$ (Quill *et al*., 2001; Clapham and Garbers, 2005). CatSper channel subunits are structurally most closely related to individual domains of Ca_v channels (Ren *et al.,* 2001). The CatSper proteins are located on the plasma membrane of the principal piece the largest segment of the flagellum (Ren *et al*., 2001; Quill *et al*., 2001, 2003; Xia *et al*., 2007). Four different proteins have been identified, CatSper1-4 (Ren *et al*., 2001; Quill *et al*., 2001, 2003; Lobley *et al*., 2003; Jin *et al*., 2007). $I_{CatSper}$ is constitutively active, weakly facilitated by membrane depolarisation but strongly augmented by cytoplasmic alkalinisation (Navarro *et al.,* 2007). Alkalinisation shifts the voltage dependence of activation towards negative potentials ($V_{1/2}$ @ pH6.0 = +87mV; $V_{1/2}$ @ pH7.5 = +11mV). CatSper channels may regulate basal Ca^{2+} levels and motility since

elimination of any of the CatSper proteins gradually decreases sperm motility (Quill *et al*., 2003; Qi *et al*., 2007). Calcium entry through CatSper channels is induced by alkalinisation leading to increased flagellar bending. Targeted disruption of *CatSper*1, 2, 3 or 4 genes results in an identical phenotype in which spermatozoa fail to exhibit hyperactivation. Such disruptions are associated with a deficit in alkalinisation and depolarisation-evoked Ca^{2+} entry into sperm (Carlson *et al*., 2003, 2005; Navarro *et al.,* 2007; Qi *et al.,* 2007). The driving force for Ca^{2+} entry is principally determined by the weak outwardly rectifying K^+ channel (I_{KSper}) that, like CatSpers, is actived by intracellular alkalinisation (Navarro *et al*., 2007). The HCO_3^- anion accelerates flagellar beat in CatSper1 and 2 null sperm indicating that downstream cAMP mediated responses are not affected by the CatSper proteins loss of function phenotype. In several experiments it has been shown that the application of extracellular cell permeant cyclic nucleotide analogues induces a rapid increase in $[Ca^{2+}]_i$ (Kobori *et al*., 2000; Ren *et al*., 2001; Xia *et al*., 2007). The direct mechanism by which cyclic nucleotides activates Ca^{2+} influx is not known but this response is absent in CatSper1 null mice (Carlson *et al*., 2003). Patch clamp experiments suggests that cyclic nucleotides do not directly open CatSper channels (Ren *et al*., 2001; Kirichok *et al*., 2006) it is probable that HCO_3^-/sAC/cAMP/PKA (section 1.9) pathway facilitates phosphorylation of CatSper channel on one or more subunits (Carlson *et al*., 2003, 2005, 2007; Xie *et al*., 2006; Babcock, 2007; Qi *et al*., 2007). Recently Ho *et al*., (2009) demonstrated that CatSper null spermatozoa are unable to detach from oviductal epithelium. Mutations in CatSper protein result in human infertility (Avenarius *et al*., 2009)

1.6.2 Cyclic nucleotide-gated channels

Cyclic nucleotide signalling (cyclic adenosine 3',5'-monophosphate (cAMP) or cyclic guanosine 3',5'-monophosphate (cGMP)) is essential in mammalian sperm function (Leclerc *et al*., 1996; Lefièvre *et al*., 2000) and a cyclic-nucleotide gated (CNG), Ca^{2+} permeable channel

similar to those found in olfactory neurons has been described in human sperm (refer to Appendix I) (Parmentier *et al*., 1992; Kaupp, 1995; Wiesner *et al.*, 1998). It has been suggested that the human olfactory receptor (hOR17-4) expressed in sperm are involved in chemotaxis by regulation of cyclic-nucleotide mediated Ca^{2+} influx and motility (Spehr *et al*., 2003, 2004).

1.6.3 Store Operated Channels

In both excitable and non-excitable cells, mobilisation of stored intracellular calcium (leading to a fall in the $[Ca^{2+}]_i$ in the storage organelle), triggers a Ca^{2+} influx across the plasma membrane via store-operated channels (SOC) or calcium release activated Ca^{2+} channels (CRAC) by a process termed capacitative Ca^{2+} entry (CCE) (Putney, 1990; Parekh and Putney, 2005). Pharmacological mobilisation of stored calcium activates calcium influx in non-capacitated human spermatozoa (Blackmore, 1993). This influx of calcium results in the sustained increase of $[Ca^{2+}]_i$ necessary to drive the acrosome reaction (O'Toole *et al*., 2000; Gonzalez-Martinez *et al.,* 2001; Hirohashi and Vacquier, 2003; Jimenez-Gonzalez *et al*., 2006; Conner *et al*., 2007). The molecular components of the highly selective I_{CRAC} current have recently been identified including proteins of the STIM (stromal interaction molecule 1) (Roos *et al.,* 2005; Zhang *et al*., 2005; Lewis, 2007; Luik *et al*., 2008) and Orai (Feske *et al*., 2006; Prakriya *et al.,* 2006; Yuan *et al.*, 2007; Várnai *et al*., 2009) families. STIM1 is the putative sensor for detection of store filling status and Orai1 forms the membrane Ca^{2+}-permeable channel (Strange *et al*, 2007). Upon depletion of the endoplasmic reticulum (ER) Ca^{2+} store, STIM1 and Orai protein aggregate into a macromolecular complex. Both STIM1 and Orai1 are expressed in human spermatozoa and immunolocalise mainly to the redundant nuclear envelope (RNE, section 1.6.4) in the neck/midpiece region (Lefièvre, in prep; Costello *et al*., 2009). These proteins may also combine/interact with members of the canonical transient receptor potential (TRPCs) (refer to Appendix I and table 9.0) to form a SOC macromolecular

complex to faciliate Ca^{2+} influx (Abramowitz and Birmbaumer, 2009; Vaca, 2010). Various TRP(s) (refer to appendix 1, table 9.0) have been identified on the head and flagellum of human sperm (Trevino *et al*., 2001; Castellano *et al*., 2003). TRPC1, 3, 4 and 6 have been localised to the flagellum in human sperm and SKF96365, a blocker of TRPC channels significantly reduced motility (Castellano *et al*., 2003). Recently TRPM8, a thermo sensitive channel has been identified and immunolocalised to the tail and head in human sperm (De Blas *et al*., 2009). TRPM8 agonist (methanol and temperature) induce an increase in $[Ca^{2+}]_i$ and AR in a dose dependent manner but did not modify motility.

1.6.4 Calcium stores in sperm

In many cell types at rest the endoplasmic reticulum (ER) Ca^{2+} store is in a dynamic steady-state with cytoplasmic Ca^{2+} (Wennemuth *et al*., 2003b). Although mature spermatozoa lack an ER, there is evidence for the presence of at least three discrete intracellular Ca^{2+} stores in mammalian sperm. The acrosome contains IP_3-gated calcium channels, a Ca^{2+}-ATPase (sacroplasmic-endoplasmic Ca^{2+}-ATPases (SERCAs) and calreticulin (a low affinity, high capacity calcium buffering protein) and is thought to serve as an intracellular Ca^{2+} store (Walensky and Snyder, 1995; Kuroda *et al*., 1999; Minelli *et al*., 2000; Rossato *et al*., 2001; De Blas *et al*, 2002; Publicover *et al*., 2007; Lawson *et al*., 2007) .

A second Ca^{2+} store (or stores) is present in the sperm neck/midpiece region. The identity of the membranous compartment in which Ca^{2+} is stored is not yet clear, but it appears to involve the redundant nuclear envelope (RNE) and/or calreticulin-containing vesicles in the cytoplasmic droplet (Ho and Suarez, 2001a, 2001b, 2003; Naaby-Hansen *et al*, 2001; Harper *et al*., 2004, 2005). Both IP_3R and ryanodine receptors (RyRs) have been localised to this region and are sensitive to local Ca^{2+} concentration, increased cytosolic Ca^{2+} induces further release from the store, a process known as Ca^{2+}-induced Ca^{2+} release (CICR). The calcium sensitivity of these channels decreases as cytosolic Ca^{2+} increases (i.e. the channels begin to close again).

RyR receptors are large proteins organised into a complicated tetrameric arrangement with only ~20% of the protein associated with channel activity. The remainder form a prominent structure that extends into the cytoplasm and possesses binding sites for a number of physiological modulators including calcium. IP_3R are also tetramers, each of the component subunits possess 6 transmembrane segments. Both the N- and C-termini lie within the cytosol and possess a number of modulatory sites. IP_3 binds to the IP_3R (located on the N-terminal) and increases sensitivity of the channel to the local Ca^{2+} concentration. IP_3 is generated as a result of PLC induced hydrolysis of inositol 4', 5' bisphosphate (Fukami *et al.*, 2001; 2003). The IP_3-sensitive calcium channel has been well studied in numerous cell types, including sperm (Jimenez-Gonzalez *et al*., 2006). Isoform-specific IP_3R antibody labelling has been used on various mammalian spermatozoa including human where IP_3R1 antibody predominantly labelled the acrosomal area and IP_3R3 antibody labelled the posterior head region, mid-piece and part of the flagellum (Kuroda *et al*., 1999; Minelli *et al.,* 2000; Naaby-Hansen *et al.,* 2001; Ho and Suarez, 2003). It is possible that the Ca^{2+} sensitivity of the IP_3R could be responsible for Ca^{2+} induced Ca^{2+} release (Taylor *et al*., 2009) with CatSper dependent Ca^{2+} influx increasing IP_3 production. Treatment with the Ca^{2+} ionophore (A23187) activates PLC generating diacylglycerol (DAG) and IP_3 in a variety of mammalian sperm (Roldan and Fraser, 1998; Felix, 2005). Furthermore, cAMP can stimulate PLC to generate IP_3 (Schmidt *et al*., 2001), with various subtypes identified in the head and midpiece of sperm-PLCζ, PLCδ4 and PLCβ (Walensky and Snyder, 1995; Choi *et al.,* 2001; Swann *et al*., 2004; Irino *et al.*, 2005).

In addition to IP_3-sensitive Ca^{2+} channels, there is evidence for the presence of a functional ryanodine (RyRs; so-called because of their sensitivity to the plant alkaloid ryanodine) receptor in sperm cells (Giannini *et al*., 1995; Minelli *et al.,* 2000; Harper *et al*., 2004). RyR1 and RyR3 are expressed in mouse spermatocytes and spermatids (Giannini *et al*., 1995; Trevino *et al*., 1998; Chiarella *et al*., 2004), RyR3 immunolocalised to the acrosomal area in mature

spermatozoa (Trevino *et al.,* 1998). Immunolocalisation of RyR in human spermatozoa was focussed at the rear of the head with a lower signal from the acrosomal region (Harper *et al.,* 2004). Pharmacological modulators of RyR have been shown to significantly modify progesterone induced $[Ca^{2+}]_i$ oscillations (Harper *et al*., 2004; Machado-Oliveira *et al.,* 2008).

In human spermatozoa, the mitochondria are restricted to the midpiece. Mitochondria can act as Ca^{2+} buffers, accumulating Ca^{2+} from the cytosol (Gunter *et al*., 2000; Nicholls and Chalmers, 2004). The mitochondrial Ca^{2+} uniporter (MCU) brings Ca^{2+} into the energised mitochondria only when Ca^{2+} is elevated to 0.5μM (Gunter *et al*., 2000). Treatment with uncouplers has little effect on $[Ca^{2+}]_i$ signals in human spermatozoa (Wennemuth *et al*., 2003b; Ho and Suarez, 2003; Harper *et al.,* 2004; Machado-Oliveira *et al.,* 2008).

1.6.5 Calcium clearance mechanisms in sperm

Ca^{2+} is a highly effective signalling agent, however, excessive intracellular Ca^{2+} is cytotoxic and Ca^{2+} clearance mechanisms are essential to return the cell to resting levels after Ca^{2+} signalling events have occurred. Sperm Ca^{2+} clearance mechanisms include the plasma membrane Ca^{2+}-ATPases (PMCA) and the Na^{2+}-Ca^{2+} exchangers (NCXs), which extrude calcium from the cell (Breitbart *et al*., 1983; Michelangeli *et al*., 2005). Plasma membrane Ca^{2+} ATPase4 is expressed at high levels in the flagellum and is essential to sperm motility and hyperactivation (Okunade *et al*, 2004; Schuh *et al,* 2004). A plasma membrane Ca^{2+} ATPase is also expressed in sea urchin sperm localised to the sperm head. Pharmacological inhibition of this pump disrupts control of $[Ca^{2+}]_i$ in the sperm and resulting in loss of flagellar activity (Gunaratne and Vacquier, 2006). Na^{+}-Ca^{2+} exchangers are also present in sperm and contribute to $[Ca^{2+}]_i$ regulation (Wennemuth *et al*, 2003b).

There is evidence that the intracellular Ca^{2+} store pumps (sacroplasmic-endoplasmic Ca^{2+}-ATPases (SERCA) and the secretory pathway Ca^{2+}-ATPase (SPCA)) are involved in calcium

clearance as they sequester calcium to the intracellular calcium stores (Harper *et al*., 2005; Jimenez-Gonzalez *et al*., 2006). Analysis of calcium clearance in mouse spermatozoa suggests that both calcium exchangers and calcium pumps are important contributors to intracellular calcium buffering but the relative importance of each is still to be identified (reviewed in Jimenez-Gonzalez *et al*., 2006; Conner *et al*., 2007). In sperm, high doses of thapsigargin (inhibits SERCA) does not induce a rapid elevation of $[Ca^{2+}]_i$ but a gradual release, the effect is irreversible resulting in a loss of motility (Williams and Ford, 2003).

1.7 Sperm E_m and K^+ channels

Capacitation in mammalian sperm is accompanied by hyperpolarisation of membrane potential (E_m) (Espinosa and Darszon, 1995; Zeng *et al*., 1995, 1996; Arnoult *et al.,* 1999). Hyperpolarisation is observed as an increase in the intracellular negative charge (-30-45mV to -65mV) when compared to the extracellular environment (Arnoult *et al*., 1996a; 1998, 1999; Espinosa and Darszon, 1995; Muñoz-Garay *et al*., 2001; Acevedo *et al*., 2006). Ion channels contribute significantly to E_m, but electrophysiological characterisation of ion channels is technically demanding in mature sperm cells therefore many studies utilise spermatogenic cells. Capacitation associated elevation of pH_i may activate K^+ channels permitting K^+ efflux, driving E_m (-45mV) towards the K^+ equilibrium potential (-90-110mV) and hyperpolarising sperm (-80mV) (Arnoult *et al*., 1996a; 1999; Espinosa and Darszon, 1995; Muñoz -Garay *et al*., 2001; Acevedo *et al.*, 2006). In whole cell patch clamp, a constitutively active, weakly outwardly rectifying K^+ current was measured (I_{KSper}), localised to the principal piece of mouse sperm flagellum (Navarro *et al*., 2007). Intracellular alkalinisation strongly potentiates I_{KSper} and the sperm specific pH sensitive Slo3 K^+ channel (identified in mice spermatocytes) (Schreiber *et al*., 1998) has been proposed as a likely candidate to carry this current (Navarro *et al*., 2008). I_{KSper} is not affected by cell permeant cyclic nucleotides (Navarro *et al*., 2007). Other studies report that Slo3 does not mediate KSper current in mouse since the voltage

dependency of Slo3 current is significantly stronger that KSper (Yang *et al*., 2009). K^+ currents recorded from *Xenopus* oocytes (expressing Slo3) and mouse sperm were stimulated by increased pH_i and cAMP (Marinez-Lopez *et al*., 2009). Slo3 knockout mice are infertile; Slo3 mutant sperm depolarise during capacitation (wild type hyperpolarise during capacitation), lack a pH-sensitive K^+ current and have impaired motility and AR (Santi *et al*., 2010). In addition, studies indicate the presence of an inwardly rectifying pH_i sensitive K^+ channel (K_{ir}) in mice sperm (Muñoz -Garay *et al*., 2001). K_{ir} channels selectively carry larger inward currents at membrane potential (E_m) negative to E_K than outward currents at voltages positive to E_K even with equal [K^+] on both sides of the membrane (Nichols and Lopatin, 1997). Uncapacitated sperm cells have a relatively acidic pH_i which may act as a negative regulator of K_{ir}, maintaining a depolarised E_m. Capacitation induced cytoplasmic alkalinisation would enhance the open probability of a small but significant proportion of K_{ir} channels at potentials more positive than E_K, leading to K^+ efflux and hyperpolarisation (Muñoz-Garay *et al*., 2001). The presence of a pH-sensitive K_{ATP} channels has been shown, blockers of adenosine 5'-triphosphate (ATP) synthesis and channel activators, enhanced whole cell currents in mouse spermatogenic cells. During capacitation ATP content of the cell decreases (Baker and Aitken, 2004) increasing K_{ATP} channel open time resulting in E_m hyperpolarisation. Hernandez-Gonzalez *et al*., 2006 have shown that mouse sperm resting E_m is Na^+- dependent and epithelial Na^+ channels participate in the regulation of capacitation in mouse spermatogenic cells. Capacitation associated hyperpolarisation may result from decreased Na^+ permeability. Hernandez-Gonzalez *et al*., 2006 demonstrated the presence of epithelial Na^+ channels (ENaCs) type currents in mouse sperm. Experiments designed to inhibit ENaC activity (treatment with amiloride) and reduce Na^+ permeability (replacing Na^+ with non-permeant cations) result in E_m hyperpolarisation.

Furthermore, this group report the involvement of the cystic fibrosis transmembrane conductance regulator (CFTR, a Cl^- channel) in controlling hyperpolarisation and regulating

the activity of ENaC in human sperm (Hernandez-Gonzalez *et al*., 2007). Inhibition of CFTR blocks capacitation-associated hyperpolarisation. The CFTR Cl^- channel is cAMP/PKA regulated, treatment of noncapacitated sperm with cAMP or IBMX increased $[Cl^-]_i$ and significantly diminished ENaC activity (decreased Na^+ permeability) resulting in E_m hyperpolarisation in mice sperm. Wertheimer *et al.,* (2008) confirm that in addition to CFTR a $Na^+/K^+/Cl^-$ co-transporter (NKCC) mediates Cl^- transport into the cell. Inward translocation of Cl^- is coupled to the activity of the Cl^-/HCO_3^- antiporter (efflux of Cl^- and influx of HCO_3^-, enhanced cAMP/PKA activity and tyrosine phosphorylation). Demarco *et al*., (2003) have shown that increased extracellular HCO_3^- (that occurs during ejaculation) activates a Na^+/HCO_3^- cotransporter involved in mouse sperm E_m hyperpolarisation.

In summary, membrane hyperpolarisation associated with capacitation is likely to involve numerous channels and ion fluxes.

1.8 Intracellular pH and sperm

Intracellular pH is a critical regulator of sperm motility (Parrish *et al*., 1989; Zeng *et al*., 1996; Galantino-Homer *et al*., 2004). The male reproductive tract renders sperm cytoplasm pH_i acidic (pH~6.5), apparently maintaining spermatozoa quiescent prior to ejaculation (Acott and Carr, 1984; Carr and Acott, 1989; Jones and Bavister, 2000; Lishko *et al*., 2010). Capacitation associated cytoplasmic alkalinisation is essential for normal and hyperactivated motility (Hamamah and Gatti, 1998; Marquez and Suarez, 2007) and the acrosome reaction (Florman *et al*., 1989; Parrish *et al*., 1989; Darszon *et al*., 2006). Proton extrusion from the cytoplasm would increase pH_i. Proton transfer may involve a Na^+/H^+ exchanger (Garcia and Meizel, 1999; Woo *et al*., 2002) or a voltage gated proton channel (Hv1) (Lishko *et al*., 2010)

Lishko *et al*., (2010) identified a voltage-gated proton channel (Hv1) localised to the principal piece of human sperm flagellum. Hv1 currents were activated by membrane depolarisation, removal of extracellular zinc (a potent Hv1 inhibitor) and an extracellular alkaline environment.

Hv1 allows only the outward transport of protons, resulting in extracellular alkalinisation. Since physiological membrane potential in capacitated sperm cells hyperpolarises to values which should prevent Hv1 channel activity, activity may be regulated by another mechanism (Florman *et al*., 2010). Lishko *et al.,* (2010) suggest a degree of species specificity in sperm pH regulation; a robust proton current in human sperm but a barely detectable current in mice sperm. The authors conclude that mouse sperm do not have a functional Hv1 channel and that proton extrusion from these cells is mediated by a different mechanism.

Mammalian sperm, including humans have a sperm-specific Na^+/H^+ exchanger (sNHE) which plays a role in capacitation (Wang *et al*., 2003, 2007). The driving force for sNHE activation (proton efflux leading to pH_i alkalinisation), would occur as sperm enter the slightly alkaline female tract environment (Wang *et al*., 2003; Florman *et al*., 2010). The sNHE plays an important role in maintaining pH_i, motility and infertility. The sNHE is present in the principal piece of sperm flagellum. Mutations of the sNHE gene causes complete infertility, sperm immotility was partly rescued by addition of NH_4Cl and completely restored by cell permeant cAMP analogues (Wang *et al*., 2003; Quill *et al*., 2006). The soluble adenylyl cyclase (sAC) is mainly responsible for cAMP synthesis in mammalian sperm (Sinclair *et al*., 2000; Esposito *et al*., 2004; Fraser *et al*., 2005; Hess *et al.*, 2005; Xie *et al*., 2006). sNHE protein contains a voltage sensitive domain and a cyclic nucleotide binding domain and may therefore be sensitive to cAMP and/or membrane potential. cAMP may activate (directly or indirectly) sNHE, which could account for intracellular alkalinisation.

1.9 sAC/cAMP/ PKA and motility

An increase in intracellular pH triggers the activation of sperm flagellum through cAMP-dependent protein kinase A (PKA) (Brokaw, 1987; Lindemann and Goltz, 1988; Scott *et al*., 2000; Lefièvre *et al*., 2002; Harayama *et al.*, 2003; Hayashi and Shingyoji, 2009; Goto and Harayama, 2009). Bicarbonate and Ca^{2+} ions activate the soluble adenylyl cyclase

(sAC) increasing cAMP synthesis and activation of PKA (Tash and Means, 1982; Visconti *et al*., 1995; Visconti and Kopf, 1998; Boatman and Robbins, 1991; Buck *et al*., 1999; Wennemuth *et al.,* 2003a; Marquez and Suarez, 2008) Sperm AC differs molecularly and biochemically from the transmembrane adenylyl cyclase (tmAC), partly because sAC is uniquely sensitive to bicarbonate (Wuttke *et al.*, 2001; Jaiswal and Conti, 2003; Litvin *et al*., 2003). Motility is defective in sAC null mice sperm and males are sterile but can be rescued with the addition of cell permeant cAMP (Esposito *et al*., 2004). Targeted mutation of ATP binding pocket of mouse sperm PKACα2 catalytic subunit, results in infertility. Mutated sperm lack cAMP, depolarisation induced calcium influx and protein tyrosine phosphorylation (Skalhegg *et al.,* 2002; Nolan *et al*., 2004). Morgan *et al.,* (2008) engineered a "knockin" mouse that can be switched from expressing wild-type PKACα to mutated PKACα. Furthermore, this mutation confers sensitivity to inhibition by 1NM-PP1 (pyrazolo[3,4-*d*]pyrimide, a novel PKA catalytic subunit inhibitor) (Morgan *et al*., 2008). This group was able to examine the HCO_3^-/sAC/cAMP/ PKA pathway regulated fast and slow capacitation events in mouse sperm (Morgan *et al.*, 2008; Visconti, 2009). When murine sperm are exposed to Ca^{2+} and HCO_3^-, cAMP rises to a maximum within 60 seconds and the increase in PKA-dependent phosphorylation begins after 90 seconds resulting in vigorous flagellar movement (Harrison, 2004; Morgan *et al.,* 2008; Kaneto *et al*., 2008). Slower sAC/cAMP/ PKA dependent events occur as sperm are capacitated *in vivo* or *in vitro*, resulting in increased tyrosine phosphorylation (Visconti, 2009). Application of HCO_3^- resulted in vigorous flagellar activity in mutated sperm however flagellar beat frequency was quickly lowed in the presence of the inhibitor. Furthermore, continuous incubation with the inhibitor prevented protein tyrosine phosphorylation of late-stage capacitation (Morgan *et al*., 2008) and loss of motility in the majority of cells. However, acute treatment with inhibitor did not affect the established waveform asymmetry of hyperactivated mutant cells. This agrees with studies that bending of flagellum is Ca^{2+} dependent (Lindemann and Goltz, 1988; Lindemann *et al*.,

1991; Ishijima *et al.,* 2006).

Once activated, PKA phosphorylates various target proteins which initiate several signalling pathways. PKA cannot directly phosphorylate proteins on tyrosine residues which may occur due to the involvement of an intermediate unidentified tyrosine kinase, or inhibition of a phosphotyrosine phosphatase (PTP) or PKA phosphorylation of proteins on serine or threonine residues that prime these proteins for subsequent phosphorylation on tyrosine residues (Breitbart and Spungin, 1997). The majority of tyrosine phosphorylation occurs in the flagellum (Visconti *et al.,* 1995; Carrera *et al*., 1996; Vijayaraghavan *et al.,* 1997; Si and Okuno, 1999; Naz and Rajesh, 2004; Tomes *et al.,* 2004). Capacitation is associated with increased tyrosine phosphorylation of sperm flagellar proteins and is related to the acquisition of hyperactivated motility. The regulatory subunits of PKA also are important for intracellular localisation of the kinase. Multiple A kinase anchoring proteins (AKAPs) have been localised throughout the sperm and AKAP4 is a major protein of the sperm fibrous sheath. AKAP4 recruits PKA to the fibrous sheath to facilitate phosphorylation of neighbouring proteins and regulate flagellar function (Carrera *et al,* 1996; Miki *et al*., 2002; Brown *et al*., 2003). Targeted disruption of the *Akap4* gene causes defects in sperm flagellum and motility (Miki *et al*., 2002; Huang *et al*., 2005). Protein phophatases (PP) have also been identified in mouse sperm and co-immunoprecipate with the regulatory subunit of PKA and may be involved in maintaining progressive motility by suppression of PKA activity (Goto and Harayama, 2009) by dephosphorylation of axonemal proteins. Protein phosphatase 1γ2 is a unique isoform of PP1 which has been detected in the head and flagellar principal piece in mice sperm (Huang *et al.*, 2005; Goto and Harayama, 2009).

1.10 Calmodulin -dependent protein kinase and motility

In addition to PKA-dependent phosphorylation, several studies have suggested that Ca^{2+}/Calmodulin (CaM)-dependent protein kinase (CaMK) is involved in flagellar motility initiation and regulation (Jones *et al.,* 1980; Tash *et al.*, 1988; Tash, 1989; Tash and Bracho, 1998; Si and Olds-Clarke, 2000; Nomura *et al*., 2004; Ignotz and Suarez, 2005; Marín-Briggiler *et al.,* 2005; Schlingmann *et al.*, 2007; Suarez, 2008a). CaM has been identified as a component of ciliary and flagellar axonemes and is thought to act as a calcium sensor to mediate motility (Lindemann *et al.,* 1991). CaM kinase (CaMK) has been immunolocalised to the flagellum in human sperm and incubation with CaMK inhibitors reduced motility and sperm ATP content but does not alter patterns of protein tyrosine phosphorylation or induce the acrosome reaction (Marín-Briggiler *et al*., 2005). A recent study in *Chlamydomonas* demonstrated that calcium controls dynein-driven microtubule sliding through the activation of axonemal CaM and CaMK phosphorylation of the radical spoke proteins (Dymek and Smith, 2007). These changes increase flagellar bending and enhance ATP production.

1.11 Motility – the mechanics of movement

The axoneme or axial filament stretches the full length of the flagellum and is the motor apparatus of the sperm tail consisting of a classical 9+2 pattern (2 central microtubules (figure 1.5 (yellow boxed region) surrounded by 9 evenly spaced microtubule doublets (figure 1.5 (red boxed region)) (Curry and Watson, 1995; Porter and Johnson, 1989). Nine radial spokes, each derived from one of the outer microtubular doublet pairs, extend inwards towards the central pair in a helical fashion. Permanently attached to the A tubule of each doublet microtubule are the inner and outer dynein arm. Activation of the (Ca^{2+} Mg^{2+} dependent) dyenin ATPase (ATP hydrolysis and phosphorylation of dynein arms (Gibbons and Rowe, 1965) causes the dynein arms to interact with their B tubule of the neighbouring microtubular doublet and produce a

power stroke, causing the microtubules to slide past each other (Satir 1968; Brokaw, 1972, 1987, 1989; Shingyoji *et al.,* 1977, Summers and Gibbons, 1971; Tash and Means, 1983, 1987; Tash, 1989; Ishijima and Hamaguchi, 1993; Ishijima *et al.,* 2002a, 2002b; Turner, 2006). The axoneme is connected to the base of the sperm so that sliding force generates bending of the flagellum and involves mechanically induced switching of dynein activity between the two sides of the central pair (Nakano *et al*., 2003; Hayashi and Shingyoji, 2008, 2009). The peripheral microtubles are connected to the adjacent microtubule by nexin, acting as an elastic element, regulating the extent of sheer force while retaining the symmetry of the axoneme. The key regulatory proteins required for modulating motility are located in the flagellum and involves key calcium sensors calmodulin and calmodulin dependent kinases (Yang *et al*., 2001; Smith, 2002; Nomura *et al.,* 2004; Morita *et al.,* 2009). Regulation of dynein activity by Ca^{2+} involves the central pair or apparatus and the radial spoke complex (Ishijima *et al*., 1996; Smith, 2002; Nakano *et al*., 2003). Axonemes are highly conserved in ciliated and flagellated eukaryotic cells although the outer dense fibres, mitochondrial sheath and fibrous sheath are exclusive to mammalian spermatozoa flagellum (Turner, 2006).

Figure 1.5

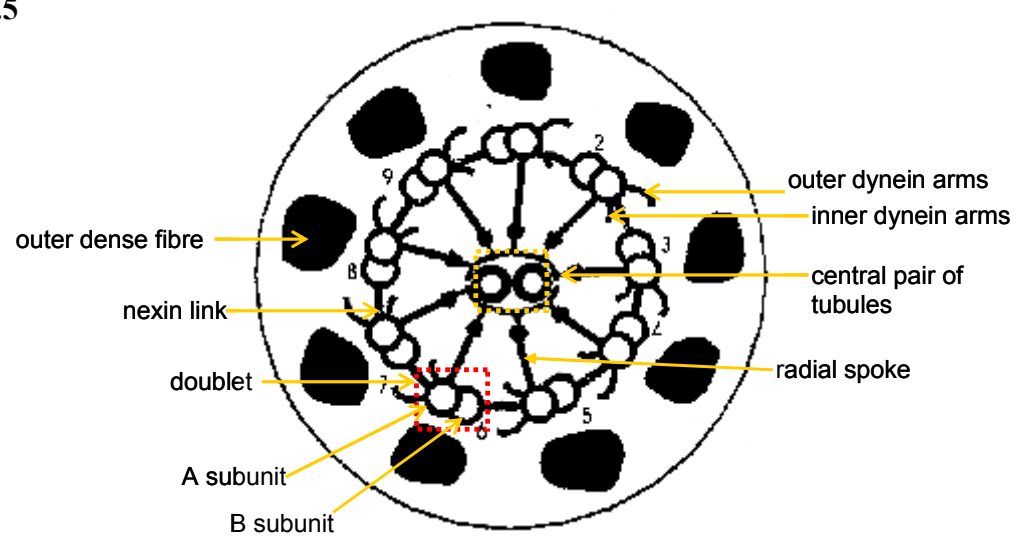

Figure 1.5: Diagrammatic cross-section of a mammalian sperm tail in the proximal region of the principal piece (refer to text for details, adapted from Mortimer, 2000).

In spermatozoa, mitochondria are restricted to the mid-piece and generate ATP through aerobic respiration. Sperm specific mitochondrial proteins include isoforms of lactate dehydrogenase and hexokinase but due to their location production of sufficiently high levels ATP and effective diffusion to supply dynein ATPases is unlikely (Williams and Ford, 2003; Turner *et al.,* 2003; Miki *et al*., 2004; Ford, 2006). Mice spermatozoa defective in mitochondrial oxidative phosphorylation remain fertile but motility is reduced with lower levels of ATP generated (Narisawa *et al*., 2002). This data suggests oxidative phosphorylation is not the sole source of ATP and motility. Dynein ATPases generate ATP locally by glycolysis along the length of the principal piece for flagellar movement (Eddy *et al*., 2007). The glycolytic enzymes hexokinase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been identified in the fibrous sheath/principal piece of mammalian species (Westhoff and Kamp, 1997; Bunch *et al*., 1998; Miki *et al.*, 2004). All the glycolytic enzymes downstream of GAPDH are tightly bound to the cytoskeleton, even after membrane removal suggesting they are components of either the fibrous sheath or ODFs. Furthermore mammalian sperm produce lactate from glucose under aerobic conditions (Storey and Kayne, 1975). Ejaculated sperm can produce 90% of their ATP by anaerobic metabolism and blocking aerobic ATP production does not significantly reduce ATP levels or motility (Mukai and Okuno, 2004). Null mutant mice for the sperm's fibrous sheath-specific GAPDH are immotile (Miki *et al.,* 2004). Both ATP and adenosine 5'-diphosphate (ADP) binding to dynein regulates motile activity of the flagellum (Inoue and Shingyoji, 2007; Yoshimura *et al*., 2007).

1.11.1 Quantitation of Sperm Movement

Axonemal bending and the flagellar wave occur as a result of attachment-detachment cycles between the dynein arms and the adjacent doublet although the exact mechanism has not been fully elucidated (figure 1.5) (Ishijima, 1995; Mortimer, 1997; Ishijima *et al.*, 2002a). The flagellar wave is seen as a flattened helical beat of the tail, the wave travels the length of the

tail to propel the sperm cell forward, while the sperm head is forced to rotate along the axis of the direction of travel. The initial phase of the flagellar wave occurs near the head and if small in amplitude the sperm head has little lateral movement (figure 1.6a) and a pronounced forward movement. When the flagellar waves are of higher amplitude the head movement has a more pronounced, wider lateral movement (figure 1.6b). Movement patterns of sperm are influenced by their external environment. Recently, Smith *et al.,* (2009) utilised high frame rate digital imaging to examine in detail bend propagation in the flagella of free swimming human sperm in low viscosity and high viscosity medium (to mimic tract mucus). They report that sperm swimming in high viscosity medium had a lower wavespeed (frequency X wavelength) than sperm swimming in low viscosity medium (200µm/s and 890µm/s respectively) but that progressive velocity remained similar (62µm/s and 65µm/s respectively). In summary, cells in high viscosity media flagella beat less frequently (11Hz versus 23Hz) and have shorter wavelength (18µm versus 39µm) but progression per beat was >50% higher (5.8µm versus 2.7µm). Sperm swimming in low viscosity medium have a high head rolling rate because of the non-planarity of the flagellar bend. The flagellar envelope is described as an elliptical cone with a head rolling rate between 9-11 Hz. Ishijima *et al*., (1992) reported the rotational movement of sperm attached vertically to a coverslip but swimming freely in media. Human spermatozoa rolled counter clockwise (viewed from the anterior end) and occasionally changed rotational direction (head rolling rate~9Hz). Smith *et al*., (2009) report that sperm migrating through high viscosity media have a reduced head rolling rate and an almost planar flagellar beat to maintain effective cell progression despite increased resistance. Analysis of motility in low viscosity media is essential to understanding how cells swim in IVF media or follicular fluid around the time of ovulation (Smith *et al*., 2009).

Since flagellar beat pattern determines the pattern of head movement computer assisted semen analysis (CASA) instruments exploits this relationship to track the sperm head movement, which does not move as fast as the flagellum. CASA analysis is routinely used in clinics to

assess motility parameters in human spermatozoa and has two main advantages over manual method – high precision and provision of quantitative data on sperm kinematics.

Motility kinematic values determined include the velocity of movement, the width of the sperm head trajectory and the frequency of change of direction of the sperm head (Mortimer, 2000). Velocity values (μm/sec) include curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP). VCL refers to the total distance the sperm head travels and is the highest value (figure 1.6, red line linking black dots (dots represent individual head position points). VSL is determined from the straight line distance between the first head position point and the last point (figure 1.6a and b; blue lines). VAP is the distance the sperm has travelled in the average direction of movement during the observation period. VAP is calculated by taking the (x,y) coordinates for each head position point as well as those of two track point on either side, to give a smoothed (x,y) coordinate for that track point. When all the track points are smoothed the average path is calculated from the total distance travelled in the observation period. In cases, where the sperm position points are regular and linear, VAP (broken line, figure 1.6a) is similar to VSL (blue line, figure 1.6a). Linearity (%) can be used to describe the trajectory of the cells (VSL/VCL x 100). The amplitude of lateral head displacement (ALH, μm) is used to approximate the flagellar beat envelope. To determine ALH manually, parallel lines are drawn between adjacent peaks and troughs and the perpendicular distance is measured, for regular trajectories the true head position point is perpendicular to the smoothed point on the average path (figure 1.6a). CASA calculates ALHmax (the maximum ALH found along the trajectory) using the Risers method, the distance from the true track coordinate (figure 1.6c, black dots linked with red lines) to the corresponding smoothed track coordinate (green dots) is calculated (Mortimer, 2000).

It is unusual for all spermatozoa (especially human) in a preparation to have the same motility pattern therefore population averages are unlikely to give an indication of the proportion of hyperactivated cells. However, since the motility pattern of hyperactivated sperm is very

different to non-hyperactivated sperm (compare sperm tracks in figure 1.7a with 1.7c and d), a defined range of kinematic criteria (Boolean argument) has been published (Mortimer, 2000). For a human sperm to be considered hyperactivated it must meet all the following criteria: curvilinear velocity ≥150µm/s $_{AND}$ linearity <50% $_{AND}$ lateral head displacement ≥7µm (Mortimer, 2000).

Figure 1.6

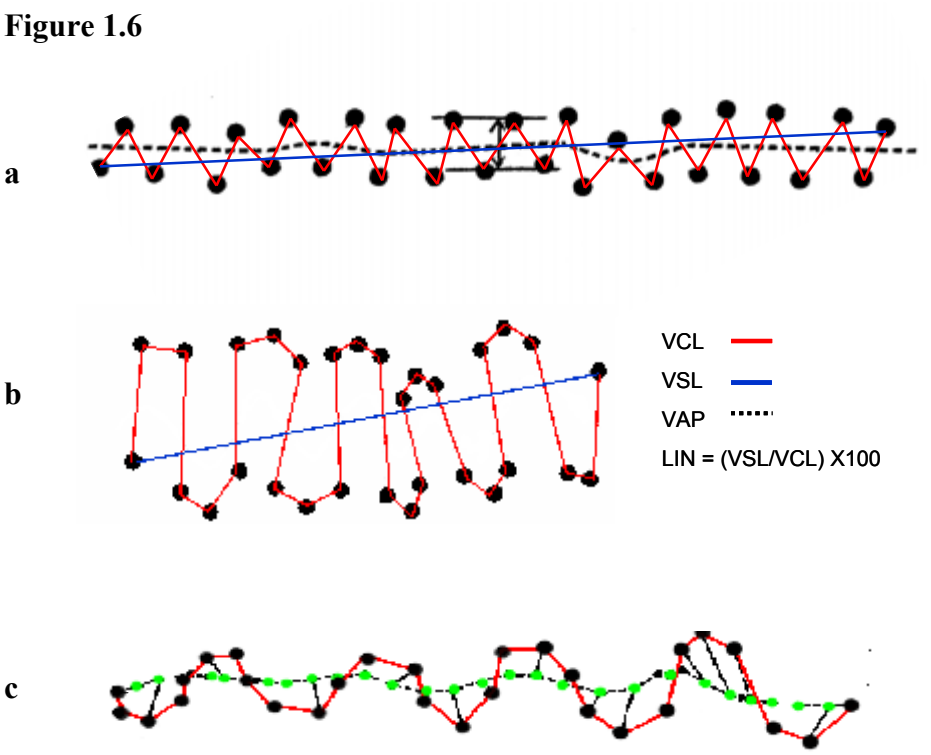

Figure 1.6: Motility patterns for capacitated human sperm. Individual head position points (black dots with red links) are shown. Refer to text for VSL (blue line) and VCL (red line) details (a) head position points of sperm (black dots) with regular motility pattern and (b) irregular motility pattern. (c) Risers method for determining ALH using smoothed head position points (green dots) and true head position points (black dots linked with red lines). Refer to text for ALH calculation details (adapted from Mortimer, 2000).

1.12 Chemotaxis and Sperm

Sperm are attracted and swim towards chemical factors released by the oocyte- a process called chemotaxis (Kaupp *et al.,* 2003*,* 2008). Much of the chemotatic research has centered on marine sperm where chemotatic factors from the egg jelly causes the sperm to re-orientate and swim towards the oocyte following a concentration gradient (Cohen-Dayag *et al.,* 1994; Eisenbach, 1999; Eisenbach and Giojalas, 2006; Neill and Vacquier, 2004). Binding of the egg derived factor to the receptor guanylyl cyclase (GC) activates cGMP synthesis. cGMP opens a K^+ selective cyclic nucleotide-gated (KCNG) channel, causing E_m hyperpolarisation (Strünker *et al*., 2006; Bönigk *et al.,* 2009). Hyperpolarisation activates a cyclic-nucleotide gated channel (HCN) and low voltage activated Ca^{2+} channels (LVA Ca^{2+}) allowing the influx of Na^+ and Ca^{2+} respectively (Strünker *et al.,* 2006). The opening of high voltage activated Ca^{2+} channels may be involved in the sustained elevation of $[Ca^{2+}]_i$. As previously described, Ca^{2+} interacts with the motor proteins of the axoneme to cause a change in flagellar beat and finally a change in the swimming trajectory (Kaupp *et al.,* 2008).

Spehr *et al.,* (2003; 2004) identified the presence of an ordorant receptor (h-OR17-4) which responds to bourgeonal (floral scent) stimulating chemotatic behaviour in human sperm. Similarly lyral (floral scent) binds to a mouse ordorant receptor (mOR23) and stimulates chemotaxis (Fukuda *et al.,* 2004). Binding of the ordorant to the OR activates a G protein (G_{olf}) and activation of the transmembrane adenylyl cyclase (tmAC) to induce a cAMP $[Ca^{2+}]_i$ response. The specific components of this cAMP signalling pathway have not been unequivocally identified (Kaupp *et al*., 2008).

Progesterone is secreted by the cumulus cells surrounding the oocyte and may form a chemotatic gradient (Jaiswal *et al*., 1999). Recently Teves *et al* (2006) demonstrated a chemotatic response in (5-10%) of sperm (rabbit or human) to picomolar concentrations of

progesterone. The progesterone-signalling pathway mediating chemotaxis is unknown but $[Ca^{2+}]_i$ signals generated could lead to chemotatic turns and orientation of cells up the chemotatic gradient (Kaupp *et al.,* 2008). Human sperm stimulated with a progesterone gradient (nanomolar to micromolar) to represent more closely the physiological stimulus that a sperm will encounter upon approaching the oocyte, generate a slow tonic rise in $[Ca^{2+}]_i$ superimposed with Ca^{2+} oscillations in the neck region of sperm (Harper *et al*., 2004). In loosely tethered human sperm, increased flagellar excursion and flagellar asymmetry occur during the initial progesterone-induced $[Ca^{2+}]_i$ response and Ca^{2+} oscillations synchronise with flagellar asymmetry even in calcium free saline (Harper *et al.,* 2004; Bedu-Addo *et al*., 2007). Evidence also indicates that cumulus oophorus express nitric oxide synthase and as spermatozoa approach or enter the cumulus they experience increased concentrations of NO (similar to progesterone gradient). Treatment of spermatozoa with nitric oxide (NO) donors affects sperm motility (Herrero *et al,* 1994; Zhang and Zheng, 1996; Yeoman *et al*, 1998; Herrero and Gagnon, 2001) and causes a sustained rise in $[Ca^{2+}]_i$ due to mobilisation of a calcium store (Machado-Oliveira *et al.,* 2008). NO was shown to affect nitrosylation of RyR and act as a functional switch (Lefièvre *et al*., 2007; Machado-Oliveira *et al*., 2008). Machado-Oliveira *et al.*, (2008) demonstrated that progesterone and NO act synergistically. Human spermatozoa pre-treated with low dose progesterone and NO donor induce an immediate large transit $[Ca^{2+}]_i$, signal which was often followed by Ca^{2+} oscillations synchronised with bending of the proximal flagellum, leading to flagellar asymmetry.

1.13 Hyperactivation

Mammalian spermatozoa exhibit two forms of physiological motility – activated and hyperactivated. Within semen or low viscosity media activated spermatozoa move in a relatively straight path with minimal head rolling and the flagellum generates symmetrical,

lower amplitude waveform that propel the cell forward (figure 1.7a) (Ho and Suarez, 2001a; Turner, 2006; Marquez *et al*., 2007).

As spermatozoa reach the oviduct the pattern of flagellar motility alters to an asymmetric, high amplitude beat termed hyperactivated motility. Hyperactivated spermatozoa (*in vitro*) display extreme bending of the flagellum (high amplitude, high curvature) resulting in large side to side movements of the sperm head (greater lateral displacement of the head) (figure 1.7c and d). *In vitro* hyperactivated sperm have limited forward progression and when viewed on a microscope slide are often swimming in circles (starspin) or figures of eight (Ho and Suarez, 2001a). This pattern of motility is biphasic as spermatozoa can switch from one swimming pattern to another (Katz and Yanagimachi, 1980; Pacey *et al*., 1995a; Mortimer and Swan, 1995).

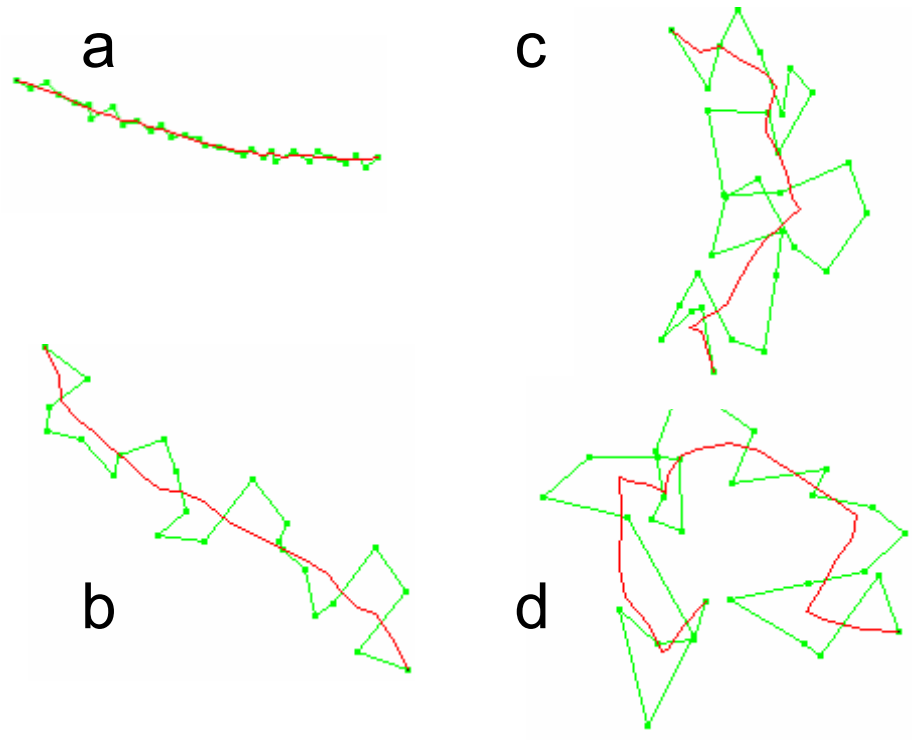

Figure 1.7: Motility pattern for activated and hyperactivated sperm. Sperm tracks show typical motility of cells suspended in (a) control media and (b) stimulated with 3μM progesterone or (c and d) with 2mM 4-AP (adapted from Gu *et al.,* 2004).

Yanagimachi (1970) first observed *in situ* hyperactivated spermatozoa through the walls of the oviductal ampulla of golden hamster indicating a physiological role in fertilisation. Hyperactivation has since been observed *in vitro* for all eutherian spermatozoa studied (Yanagimachi and Mahi, 1976; Battalia and Yanagamachi, 1979; Katz and Yanagamachi, 1980; Overstreet *et al.,* 1980; Mortimer *et al.,* 1984; Burkman *et al.,* 1984). There are a number of similarities in the hyperactivated movement patterns of mammalian spermatozoa, although interspecies differences have been observed. The hyperactivated sperm recovered from the ampulla were observed to swim in circular trajectories, due to the development of asymmetric flagellar beats whereas ejaculated sperm had linear trajectories due to symmetrical flagellar beat patterns. As mentioned previously, motility is influenced by the external environment and in high viscosity media hyperactivated mouse cells are more progressive than activated cells (Suarez and Dai, 1992).

The changes in motility patterns of hyperactivated spermatozoa have been shown to be due to change in flagellar bending rather than to a change in the viscous drag associated with acrosomal loss, as hyperactivated motility has been observed in headless spermatozoa from guinea pig and hamster (Katz *et al.,* 1978b). *In vivo* hyperactivation enables spermatozoa to detach from the oviductal epithelium (Ho *et al.,* 2009) and move progressively through the oviduct and effectively penetrate the outer vestements of the oocyte (Ho and Suarez, 2001a, 2003; Quill *et al*., 2003; Suarez *et al*., 1991; Stauss *et al*., 1995; Marquez *et al*., 2007).

The *in vivo* signals which regulate hyperactivation may include oviductal luminal fluid (containing hormones, ions and secretions), cumulus oophorus and/or follicular fluid but the specific triggers that induce hyperactivation are unknown (Mendoza and Tesarik, 1990; Uhler *et al*., 1992; Ho and Suarez, 2001b). Hyperactivation is regularly associated with capacitation but can occur independently (Marquez and Suarez, 2004).

The signalling mechanisms that govern *in vivo* HA are not known however, evidence for

increased intracellular Ca^{2+}, a role for plasma membrane channels and intra-calcium store (ICS) mobilisation have been demonstrated (Marquez *et al*., 2007). Various calcium channels including the sperm specific CatSper channels have been localised to the flagellum (Marquez *et al*., 2007). Mobilisation of stored calcium has been shown to initiate hyperactivation in mice, bovine and human sperm (Ho and Suarez, 2001b; 2003).

Calcium regulates the axonemal waveform in both activated and hyperactivated motility (Suarez *et al*., 1987, 2007; Ho *et al*., 2002). The axonemal response to changes in calcium concentration has been well reported. Axonemes isolated for sea urchin sperm are reactivated *in vitro* under low calcium concentrations and beat with a symmetrical waveform. Increasing calcium enhanced an asymmetrical axonemal beat (Brokaw *et al*., 1974; Browkaw, 1979) and in high extracellular conditions the axoneme are motionless (Gibbons and Gibbons, 1980; Sale, 1986).

1.13.1 CatSper channels and hyperactivation

Calcium entry through CatSper channels is important to sustain sperm hyperactivated motility in mouse sperm (Carlson *et al*., 2003, 2009) enhanced calcium influx is induced by alkalinisation leading to increased flagellar bending. Kirichok *et al*., (2006) reported that the calcium current ($I_{CatSper}$) is absent in CatSper null mice. The current was weakly voltage sensitive, but this sensitivity showed a pronounced negative shift (to physiological values of membrane potential) when pH_i was raised from its basal vale of <7 to ~7.5 or 8. Recently the same lab detected a weakly outwardly rectifying K^+ current (I_{KSper}) in the principal piece of murine sperm Alkalinisation activates the pH_i sensitive I_{KSper}, setting E_m to negative potentials were Ca^{2+} entry via $I_{CatSper}$ is maximized (Navarro *et al*., 2007). CatSper null sperm are unable to penetrate the outer vestments of the intact oocyte but can fertilise denuded oocytes *in vitro* (Ren *et al*., 2001; Quill *et al*., 2003; Carlson *et al*., 2005). CatSper null sperm have normal patterns of tyrosine phosphorylation and are able to undergo ZP3-induced acrosome reaction

(Xia *et al.*, 2007).

Recently a flagellar voltage-gated proton channel (Hv1) has been identified in human sperm and proton extrusion from the cytoplasm would lead to cytoplasmic alkalinisation and CatSper channel activation (Lishko *et al*., 2010).

Xia *et al*., (2007) recorded relative $[Ca^{2+}]_i$ along the length of the mouse tail using a Ca^{2+} sensitive fluorescent dye after the application of cell permeable cyclic analogues (8-Br-cAMP or 8-Br-cGMP). Increased intracellular Ca^{2+} started in the principal piece and propagated rapidly to the midpiece and head (3.2 sec) (Xia *et al*., 2007). However, with ionomycin, a Ca^{2+} ionophore that facilitates Ca^{2+} transport across the plasma membrane, was applied extracellularly the fluorescence signal was seen immediately in all regions of the cell. Furthermore no significant response to 8-Br-cAMP was seen in CatSper null mutants. Xia *et al.,* (2007) concluded that 8-Br-cAMP indirectly opens CatSper channels, Ca^{2+} influx through the principal piece which propagates rapidly to the midpiece and head. CatSper channels have been implicated in regulating basal motility (Quill *et al*., 2003; Qi *et al*., 2007). Marquez *et al.,* (2007) observed that in CatSper null mice the flagellar bend amplitude is lowered but could be raised to normal pre-hyperactivation levels with the addition of thimerosal (ethyl (2-mercaptobenzoato-(2-)-O,S)mercurate(1-)sodium (thimerosal) is a thiol reactive agent that stimulates Ca^{2+} flux in IP_3R by an as yet unidentified mechanism (Bultynck *et al*., 2004). CatSper channels are also thought to be involved in ZP3 induced $[Ca^{2+}]_i$ increase and therefore CatSpers could play a role in the acrosome reaction (Xia and Ren, 2009b).

1.13.2 Intracellular Ca^{2+} store release/capacitative Ca^{2+} entry and hyperactivation

Marquez *et al*., (2007) provided evidence that release of calcium from intracellular stores contributes to increased $[Ca^{2+}]_i$ in sperm that can induce hyperactivation in sperm lacking CatSper proteins. Ho and Suarez (2001b, 2003) reported that the redundant nuclear envelope

(RNE) may act as a calcium store having immunolocalised both IP_3R and calreticulin to this region. In Ca^{2+} free conditions, the release of stored calcium by thimerosal was sufficient to initiate hyperactivation in mice sperm although extra-cellular calcium influx was required to maximise and sustain hyperactivation for longer periods (Marquez *et al.*, 2007). CatSper1 and CatSper2 null sperm stimulated by thimerosal responded in this way, confirming mobilisation of stored calcium in the neck region was capable of supporting hyperactivation independently of influx through CatSper channels (Marquez *et al*., 2007). Recently Xia *et al*. (2007) demonstrated that the CatSper dependent transient calcium influx signal initiated in the principal piece propagates rapidly to the midpiece and sperm head. Both the initial calcium elevation and the forward progression were lost in CatSper null mice. Suarez *et al.,* (1993) demonstrated that relative intracellular Ca^{2+} was observed to be highest in the head region of intact hyperactivated hamster sperm. But can diffusion account for the sustained increase in head $[Ca^{2+}]_i$ to maintain hyperactivation or is the CatSper dependent Ca^{2+} influx stimulating CICR and mobilising stored Ca^{2+}? A recent paper from Olson *et al.,* (2010) used mathematical modelling of sperm Ca^{2+} dynamics to investigate a pump (PMCA)/ leak system and diffusion. They demonstrated that Ca^{2+} influx through CatSper channels located in the principal piece could increase Ca^{2+} in the principal piece and head but the response in the head could not be sustained (as Ca^{2+} would be pumped out). By extending the model to include IP_3, IP_3R and RNE Ca^{2+} store mobilisation, increased $[Ca^{2+}]_i$ in the head region was sustained for as long as IP_3 was synthesised and Ca^{2+} released from RNE store. In summary, CatSper derived Ca^{2+} propagated along tail midpiece and head triggered IP_3 dependent Ca^{2+} release resulting in a sustained increase in head calcium (characteristic of hyperactivated cells). The presence of IP_3R in mammalian sperm has been confirmed in many studies (Costello et al., 2009). Furthermore RyR may play a role in amplifying the signal initiated by Ca^{2+} influx via the CatSper channel. The acrosome is not thought to be involved since experiments using Ca^{2+} release agents to induce hyperactivation did not enhance acrosome reaction rates (Ho and

Suarez, 2001b).

1.13.3 4-aminopyridine and hyperactivation

4-aminopyridine (figure 1.8) is a widely used voltage-sensitive K^+ channel blocker (Aronson *et al.,* 1992). 4-aminopyridine has also been used therapeutically for a number of neurological and neuromuscular junction disorders, such as Alzheimer's disease, multiple sclerosis and myasthenia gravis. Clinical application of 4-aminopyridine is thought to improve symptoms through blockage of voltage-activated K^+ channels and in turn cause neuronal depolarisation and potentiation of neurotransmission (Smith *et al.*, 2000).

In addition to K^+ channels inhibition, 4-aminopyridine disrupts intra-cellular calcium homeostasis in a number of cell types (Guse *et al*., 1994; Ishida and Honda, 1993; Gobet *et al.,* 1995; Grimaldi *et al*., 2001) and also functions as a cell-permeant weak base. Grimaldi and colleagues (2001) demonstrated that the 4-aminopyridine induced mobilisation of intracellular Ca^{2+} stores, potentiation of neurotransmitter-induced calcium transients and CCE in type 1 astrocytes, neurons and skeletal muscle cells. The $[Ca^{2+}]_i$ elevation induced by 4-aminopyridine was concentration-dependent and consisted of two phases: the first was dependent on store mobilisation and the second was dependent on extra-cellular calcium influx. 4-aminopyridine also enhanced the second messenger IP_3 in both neurons and astrocytes. In astrocytes, 4-aminopyridine potentiate the sustained phase of the $[Ca^{2+}]_i$ elevation induced by ATP and bradykinin. The 4-aminopyridine induced effects were fully reversible. Other voltage-sensitive K^+ channel blockers did not affect agonist-induced calcium transients or CCE indicating that 4-aminopyridine effects on $[Ca^{2+}]_i$ was not caused by blockade of voltage gated K^+ channels.

4-aminopyridine has been shown to have a particularly potent effect on human sperm motility. Data from our laboratory and others show that application of 4-aminopyridine induces a

dramatic increase in hyperactivation in both non-capacitated and capacitated human spermatozoa, with the proportion of hyperactivated sperm increased from 3-5% to 40-50% (Gu *et al.*, 2004; Barfield *et al.*, 2005). As previously discussed the initiation and maintenance of hyperactivation requires enhanced $[Ca^{2+}]_i$ in mammalian sperm.

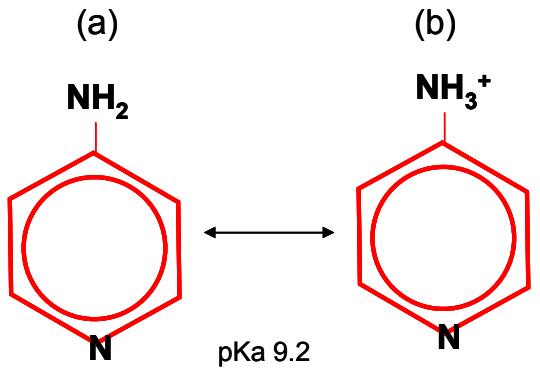

Figure 1.8: Structure of 4-aminopyridine. 4-aminopyridine can exist in an uncharged (a) or protonated form (b) The % of protonated 4-aminopyridine (pKa 9.2 at 25°C, Albert *et al.*, 1948; Babiak and Testa, 1976) is dependent on pH_o (Howe and Ritchie, 1991; Stephens *et al.*, 1994).

In mice sperm, 4-aminopyridine (weak organic base, pKa9.2) induced cytoplasmic alkalinisation and enhanced I_{KSper} induced hyperpolarisation to increase the driving force for Ca^{2+} entry through CatSper channels (Navarro *et al.*, 2007) Similarly, cytoplasmic alkalinisation with 25mM NH_4Cl resulted in hyperactivation in bovine sperm (Marquez and Suarez, 2007). In addition, the weak base procaine had a similar effect and induced hyperactivation in mammalian sperm which further implicates pH as a component in the signalling pathway that control Ca^{2+} entry and hyperactivation (Mújica *et al.,* 1994; Marquez and Suarez, 2004; McPartlin *et al.*, 2009). Despite the importance of pH_i and CatSper activation, studies in rodent and bovine sperm has provided strong evidence that the elevation of $[Ca^{2+}]_i$ that occurs upon mobilisation of Ca^{2+}, from a store in the sperm neck/midpiece region also contribute to regulation of flagellar activity, including (or at least facilitating) aspects of hyperactivation (Ho *et al.*, 2001b; 2003). Hyperactivation can also be induced by thimerosal induced store mobilisation in CatSper null mice. Experiments in human sperm cells have demonstrated that manipulations of the store located at the neck/midpiece result in

oscillations which are synchronised to asymmetrical binding of the proximal piece of the flagellum (Machado-Oliveira *et al.*, 2008). As mentioned previously mathematical modelling demonstrated that CatSper channel Ca^{2+} tail to head propagation is insufficient to maintain a sufficiently high $[Ca^{2+}]_i$ and that store mobilisation and SOC induced Ca^{2+} influx was required (Olson *et al*., 2010).

Since capacitative Ca^{2+} entry (influx of Ca^{2+} through store operated Ca^{2+} channels) has been observed in sperm (Blackmore, 1993; Williams & Ford, 2003; Harper *et al,* 2005; Costello *et al.*, 2009), store mobilisation might even recruit this mechanism to supplement Ca^{2+} influx through CatSper channels. Alternatively this second pathway for induction of hyperactivation may play a separate role from that mediated by CatSpers. Observations of sperm, primarily in humans but also other mammals, suggest that the cells can 'switch' rapidly and probably repeatedly between activated and hyperactivated motility (Pacey *et al*., 1995a, 1995b; Mortimer and Swan, 1995), a behaviour that may mediate interactions with cells of the female tract (Pacey *et al*., 1995b). Mobilisation of stored Ca^{2+}, which is rapidly reversible and localised, may be better suited to such transient regulation of motility whereas pH regulated activation of CatSpers could support the prolonged hyperactivation that permits 'permanent' detachment from the cells of the female tract (Ho *et al.*, 2009) and penetration of the zona. It is however not clear how store mobilisation contributes and interacts with the CatSper channels (Olson *et al*., 2010). The effects of 4-aminopyridine on human sperm hyperactivation and $[Ca^{2+}]_i$ response has been examined in the following chapters. In particular, the relative contribution of cytoplasmic alkalinisation (CatSper activation) and store mobilisation (SOC entry) in 4-aminopyridine induced hyperactivation in human spermatozoa was investigated.

1.14 Summary

The mature sperm cell is a highly specialized cell despite lacking much of the intracellular structure and organisation present in somatic cells. Mammalian sperm are capable of long distance travel through the female reproductive tract to the oviduct, in which fertilisation takes place (Ikawa *et al*., 2010). Transit through the female reproductive is accomplished by the flagellum driving the sperm cell towards its target. Due to ethical constraints very little is known about the human sperm's journey and interaction with the oocyte *in vivo*. This review focused on the Ca^{2+} signalling toolkit essential for sperm motility and hyperactivation (Publicover *et al.*, 2007).

RESEARCH AIMS

Assess the effects of 4-aminopyridine on $[Ca^{2+}]_i$ and hyperactivation in human spermatozoa and the importance of K^+ flux and E_m depolarisation in this process (Q1 and Q2 figure 1.9, results chapter 2).

Investigate the effects of extracellular pH on 4-aminopyridine entry into sperm. Examine the effects of pH_i modulators on 4-aminopyridine induced hyperactivation and $[Ca^{2+}]_i$ response (Q3,Q4 and Q5 figure 1.9, results chapter 3).

Assess the contribution of CatSper induced Ca^{2+} influx in 4-aminopyridine induced hyperactivation and $[Ca^{2+}]_i$ response (Q6 figure 1.9, results chapter 4)

Examine the effects of 4-aminopyridine on intracellular Ca^{2+} store release and the consequent effects on human sperm functions (Q7 figure 1.9, results chapter 5)

Investigate the effects of store operated channels modulation in the 4-aminopyridine induced $[Ca^{2+}]_i$ response (Q8 figure 1.9, results chapter 6).

An experimental matrix/workpath linking the project research aims (and experimental questions Q1-Q8) with results chapters (2-6) is illustrated in figure 1.9 (also included are methods utilised to investigate and results obtained).

Figure 1.9

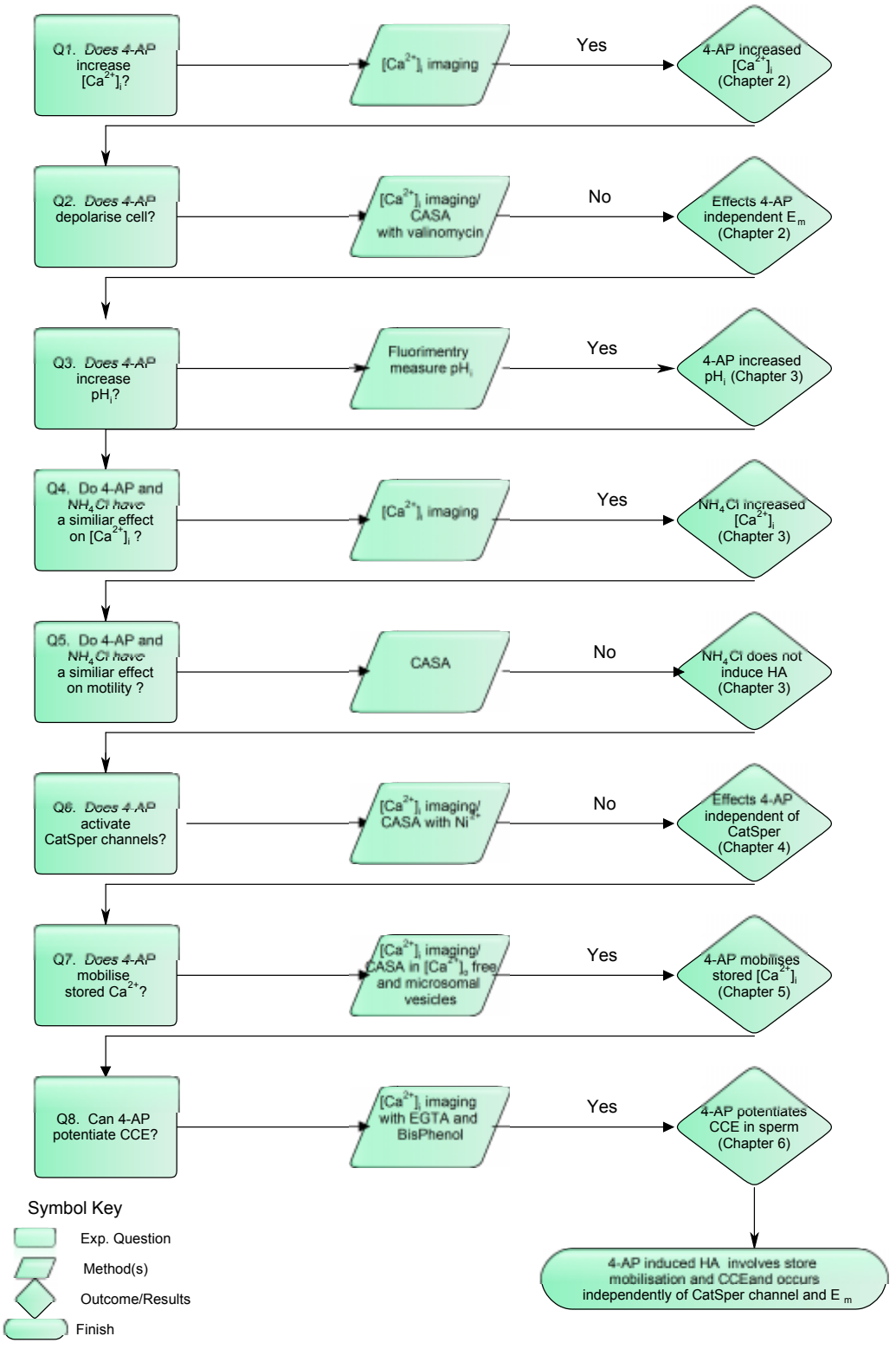

Figure 1.9: Experimental matrix/workpath linking research aims and experimental questions with results chapters

CHAPTER TWO

4-AMINOPYRIDINE INDUCED HYPERACTIVATION AND ELEVATION OF $[Ca^{2+}]_i$ IN HUMAN SPERM IS INDEPENDENT OF K^+ FLUX AND MEMBRANE DEPOLARISATION

2.1 Abstract

4-aminopyridine (4-AP), a blocker of voltage-activated K^+ channels, is one of the most potent inducers of human sperm hyperactivation yet described (Gu *et al*., 2004). Since data from both our studies and those of others laboratories have shown that $[Ca^{2+}]_i$ plays a central role in regulating the activity of the flagellum, we have investigated the effects of 4-aminopyridine on $[Ca^{2+}]_i$ fluorescence in human spermatozoa. We report that 4-aminopyridine (2mM) enhanced $[Ca^{2+}]_i$ fluorescence in OGB labelled sperm and induced hyperactivated motility. These 4-aminopyridine induced effects are independent of K^+ flux and membrane potential.

2.2 Introduction

During fertilisation the highly specialised sperm cell fuses with the oocyte to deliver paternal genetic material and trigger egg activation (Swann *et al*., 2004). Mammalian sperm are unable to fertilise the oocyte immediately after ejaculation but must undergo a maturation process termed capacitation as they journey through the female reproductive tract (Austin 1951; Chang 1951; Yanagimachi and Usui, 1974). Spermatozoa acquire the ability to undergo the agonist induced acrosome reaction and modify motility pattern from activated to hyperactivated (Gadella and Visconti., 2006). Activated or progressive motility is characterised by high flagellar beat frequency with low bend angle, particularly of the proximal flagellum. Transition to hyperactivated swimming involves deep flagellar bends, due to increased flexure of the midpiece/proximal flagellum and a concomitant decreased beat frequency resulting in greater lateral displacement of the head (Ho and Suarez, 2001a; Ishijima, 2007a, 2007b).

In situ hyperactivation was first reported by Yanagimachi (1970) and observed through the oviductal walls of golden hamster. Hyperactivated motility has been described *in vitro* for all eutherian spermatozoa (Suarez and Osman, 1987; Mortimer, 2000) and has been shown to be essential for *in vivo* fertilisation, enabling the sperm to effectively penetrate mucus, detach from the oviductal epithelium and penetrate the outer vestments of the oocyte (Stauss *et al*., 1995; Ren *et al*., 2001; Quill *et al*., 2003; Carlson *et al*., 2003; Ho *et al*., 2009).

The signalling mechanisms that govern *in vivo* hyperactivation in sperm are not fully understood but various studies have shown that pH (Parrish *et al*., 1989) and cAMP are important regulatory components (Marquez and Suarez, 2007). However the increase in intracellular Ca^{2+}, particularily in the mid-piece, is key to the initiation and maintenance of hyperactivation, probably by strongly enhancing flagellar bending (Lindemann and Goltz 1988; Suarez and Dai 1992; Yanagimachi *et al*., 1994b; Visconti *et al*., 1995; Ho *et al*., 2002; Ishijima *et al*., 2006, Ohmuro and Ishijima, 2006; Marquez *et al*., 2007).

Two sources of Ca^{2+} may contribute to the regulation of hyperactivation in sperm. Ca^{2+} influx at the plasma membrane is crucial and/or release of stored intracellular calcium and activation of capacitative calcium entry (CCE). The sperm-specific, pH regulated, Ca^{2+}-permeable CatSper channels expressed in the principal piece of mammalian sperm are of great importance in this process (Kirichok *et al*., 2006; Qi *et al*., 2007). In addition, studies on sperm from rodents, bulls and humans have provided strong evidence that Ca^{2+} stored in the region of the sperm neck contributes to regulation of flagellar activity (Chandler and Battersby, 1976; De Blas *et al*., 2002; Herrick *et al*., 2005).

Due to the ethical constraints, *in vivo* hyperactivation cannot be observed in the human Fallopian tube however failure of human sperm to hyperactivate may be an important contributory factor to infertility (Nikpoor *et al.,* 2004).

A major problem in human sperm samples is the highly asynchronous motility response within a population of cells. However, since the motility pattern of hyperactivated sperm is very different to non-hyperactivated sperm (compare sperm tracks in figure 1.7a with 1.7c and d), it has been possible to define specific kinematic criteria (Mortimer, 2000). As mentioned previously (section 1.11.1), for a human sperm to be considered hyperactivated it must meet all the following criteria: curvilinear velocity $\geq$150μm/s $_{AND}$ linearity <50% $_{AND}$ lateral head displacement $\geq$7μm (Mortimer, 2000). More detailed characterization of flagellar movement involves digital frame by frame analysis which is both tedious and time-consuming and normally depends on some form of cell attachment.

Gu *et al.,* (2004) previously described 4-aminopyridine (2mM) as a potent inducer of hyperactivation in human spermatozoa and potentially a useful tool to assess patient sperm hyperactivation ability. 4-aminopyridine is a widely used and effective blocker of K^+ channels and has been shown to disrupt intra-cellular calcium homeostasis in a number of cell types.

We have examined the effects of 4-aminopyridine on $[Ca^{2+}]_i$ fluorescence and motility (assessed by CASA) of human sperm.

2.3 Materials and Methods

2.3.1 Materials

Supplemented Earle's balanced salt solution (sEBSS) was prepared in the laboratory and contained 1.0167mM NaH_2PO_4, 5.4mM KCl, 0.811mM $MgSO_4.7H_2O$, 5.5mM $C_6H_{12}O_6$, 2.5mM $C_3H_3NaO_3$, 19.0mM $CH_3CH(OH)COONa$, 1.8mM $CaCl_2.2H_2O$, 25.0mM $NaHCO_3$, 118.4mM NaCl and 15mM HEPES (pH 7.35, 285-295 mOsm), supplemented with 0.3% (w/v) fatty acid free BSA. Chemicals used in preparation of media were from Sigma Aldrich (Poole, Dorset, UK). Fatty acid-free BSA was from SAFC Biosciences (Lenexa, KS, USA). Osmotic strength (285-295mOsmol) was maintained by adjusting NaCl. OGB and fura-2/AM were from Invitrogen Molecular Probes (Paisley, UK). PDL was from BD Biosciences (Oxford, UK). All other chemicals referred in the text were from Calbiochem (San Diego, CA, USA), except 4-aminopyridine and Pluronic F-127 were from Sigma-Aldrich (Poole, Dorset, UK). All chemicals were cell-culture-tested grade where available.

2.3.2 Preparation and capacitation of spermatozoa

All donors were recruited in accordance with the Human and Embryology Authority Code of Practice (Version 7) and gave informed consent (LREC 2003/239 and University of Birmingham Life and Health Sciences ERC 07-009). Cells from more than 20 donors were used over the duration of the study and all showed similar responses to stimulation with 4-aminopyridine. Semen was collected by masturbation after 2-3 days of sexual abstinence and allowed to liquefy for approximately 30 minutes, at 37°C and 5.5% CO_2. Highly motile spermatozoa were harvested by direct swim-up (Mortimer, 1994) into supplemented Earle's balanced salt solution (sEBSS) medium (15mM Hepes, pH 7.3-7.4) with 0.3% Bovine Serum Albumin (BSA). Briefly, 1 ml of medium was underlayered with 0.3 ml of semen in Falcon round-bottom tubes. After 1 hour incubation at 37°C and 5% CO_2, the top 0.7ml was removed, pooled and spermatozoa concentration was determined using a Neubauer haemocytometer and

adjusted to 6 x 10^6 cells/ml. Aliquots of 200µl (for single-cell imaging and motility analysis) were left to capacitate for 5-6 hours. .

2.3.3 Single Cell Imaging

200µl aliquots of capacitated spermatozoa at 3-6 x 10^6 cells/ml were loaded with Oregon Green bis-(o-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic (BAPTA 1)-AM (12µM final concentration dispersed with Pluronic F-127). Fluorescence of Oregon Green BAPTA shows negligible pH sensitivity over the range pH 6-9 (Haugland and You, 2008) making it suitable for the experiments in which pH_i is varied. After incubation for 30 min at 37°C and 5.5% CO_2, cells were transferred to an imaging chamber (volume 180 µl) in which the lower surface was a 1% (w/v) poly-$_D$-lysine coated coverslip. A further 30 min incubation was then allowed for the cells to adhere. The chamber was connected to the perfusion apparatus (peristaltic pump and header tank; perfusion rate 0.4ml min^{-1}) and cells were superfused with approximately 10 ml of fresh medium to remove unattached cells and excess dye. All experiments were performed at 25±0.5°C in a continuous flow of medium.

Spermatozoa were imaged on a Nikon TE200 inverted fluorescence microscope. Images were obtained at 10 s intervals using a 40x objective and a Hamamatsu Orca 1 or Q Imaging Rolera-XR cooled CCD camera controlled by a PC running iQ software (Andor Technology, Belfast, UK). For high speed fluorescence imaging (9 Hz) the protocol was as above but cells were viewed using a 60x oil immersion objective and images were captures with an Andor iXon EM^+ camera controlled by a PC running iQ software.

2.3.4 Imaging Data Processing

Data were processed offline using iQ software. A region of interest was drawn around the rear of the head of each sperm in the field of view. The image series was then replayed several

times, enabling close visual inspection of each cell. Cells were excluded from analysis if they moved outside the region of interest or if the fluorescence faded to zero within the duration of the experiment (assumed to reflect loss of dye due to cell death). Raw intensity values were imported into Microsoft Excel and normalized utilizing the equation

$R = [(F - F_{rest}) / F_{rest}] \times 100\%$,

where R is normalised fluorescence intensity, F is Fluorescence intensity at a time t and F_{rest} is the mean of 20-30 determinations of F obtained during the control period. At each time point the average normalised head fluorescence (R_{tot}) was calculated. The total series of R_{tot} was then plotted to give the mean normalized fluorescence intensity for that experiment. For each cell, Microsoft Excel was used to calculate the mean and 95% confidence interval of fluorescence intensity for at least 15 images during the control period ($Con \pm con$) and 8-10 images at a selected time point after stimulation ($X \pm X$). The response was considered significant if

$X - x > Con + con$

where *'X'* and *'x'* are the mean and 95% confidence interval for that sampling point, and *'Con'* and *'con'* are the mean and 95% confidence interval for the control period . Cells were sorted into those showing either an increase, decrease or no change in fluorescence after treatment. Visual examination of fluorescence-time plots for the sorted cells confirmed that this technique resulted in successful sorting of the different response categories. Where more complex $[Ca^{2+}]_i$ responses were observed, quantification of responses was carried out by direct observation of time-fluorescence intensity plots.

2.3.5 Evaluation of 4-aminopyridine fluorescence quenching

Following swim-up approximately 7mls of highly motile spermatozoa (6×10^6 cells/ml) were pooled and loaded with Oregon Green BAPTA 1-AM (6μM final concentration dispersed with Pluronic F-127). To allow maximal de-esterification of the dye, cells were incubated for

approximately 5 hours at 37°C and 5% CO_2 then centrifuged (300g for 5 min). The supernatant was retained (6mls) and diluted with 20mM Hepes buffer deionised H_2O (pH 7.3) so that final Ca^{2+} concentration was 3.6 X 10^{-4}M. Experiments were carried out using a Perkin-Elmer LS50B fluorescence spectrofluorimeter running FLWinlab version 3.0. Fluorimetry was performed with 2ml aliquot of de-esterified dye in a magnetically stirred methylacrylate cuvette maintained at 25^{o}C by a water jacket. An emission spectrum was recorded with 4-aminopyridine (0-5mM final concentration) using the fixed excitation wavelength of 494nm with slit width set at 3nM. Emission was scanned between 495 to 600nm with slit width set at 3nM and scan speed 1000 (nm/min). Fluorescence quenching was measured quantitatively using the Stern-Volmer equation.

$$K_{sv} = ((F_0/F_{[Q]})-1)/[Q]$$

K_{sv} – Stern Volmer Constant

[Q]- concentration of quencher (4-aminopyridine)

F_0 - measured fluorescence intensity in the absence of quencher

$F_{[Q]}$- measured fluorescence intensity in the presence of [Q].

2.3.6 Evaluation of sperm hyperactivation

For motility assessment 200µl aliquots of spermatozoa (≥ 6 x 10^{-6} cells.ml^{-1}) were prepared by direct swim-up into standard media (sEBSS) and incubated for 5-6 hours. 200µl aliquots were treated with either saline (control) or 4-aminopyridine and immediately introduced into a chamber (depth 20µm) (Microcell, Conception Technologies Ltd.) at a concentration of 6-15 million/ml on a Hamilton Thorne HTM IVOS semen analyzer (37^{o}C). Hyperactivation was defined as curvilinear velocity ≥150µm/s; linearity <50%; lateral head displacement ≥7µm. Only cells where tracks of ≥ 13 points were obtained were included in calculation of %

hyperactivated cells. At least 20 fields were scored across each slide. Playback feature also help to ensure the accuracy of the system

2.3.7 High speed imaging of sperm motility

Image series of swimming sperm for assessment of flagellar movement were collected using 200µl aliquots of spermatozoa which were prepared for fluorescence imaging but diluted to approximately 0.5 million cells.ml^{-1} before use. Aliquots were treated with either saline (control) or 4-aminopyridine immediately before recording. For low-speed image series, phase contrast images of free-swimming cells were collected at 10 Hz using the same chamber, camera and software as that employed for fluorescence imaging (see above). For high speed imaging cells were introduced into a Model P-2, series 20 imaging chamber (Warner Instruments Inc) and observed using a positive phase contrast lens (203/0.40 1/0.17 Ph1, depth of field 5.8 um) on an Olympus BX50 microscope. Data capture was via a Hamamatsu Photonics C9300 CCD camera at approximately 150 frames per second, streaming data directly to a Dell Dimension 64 bit Workstation, running Wasabi software (Hamamatsu). The stage was maintained at 37^{o}C using a LINKAM C0102 stage heater.

2.3.8 Statistical analysis

Values in the text giving frequency of each response type are stated as mean ± s.e.m.

Microsoft Excel was used to perform paired or unpaired *t* tests as appropriate. Statistical significance was set at $P<0.05$. Percentage data were arcsine transformed before testing

2.4 Results

2.4.1 4-aminopyridine induced hyperactivation in human spermatozoa

As described previously (Gu *et al*., 2004), 2mM 4-aminopyridine induced an immediate transition to hyperactivated motility in a proportion of human sperm. Since 4-aminopyridine acts as a weak base, CASA experiments were carried out in HEPES buffered (15-25mM sEBSS, pH7.3) saline. In capacitated cells (5-6 h) the proportion of hyperactivated sperm (as assessed by CASA) increased from 3.5± 1.1% to 16.2 ± 2.8% (15-25mM HEPES buffered saline, n= 6. paired *t*-test, $P<0.001$), <2 minutes (minimum delay prior to assessment) (figure 2.1a). Series of phase contrast images of sperm (10 frames/second) showing 'typical' activated sperm under control conditions (left column) and sperm hyperactivated by 2mM 4-aminopyridine (right column) (figure 2.1b).

Figure 2.1

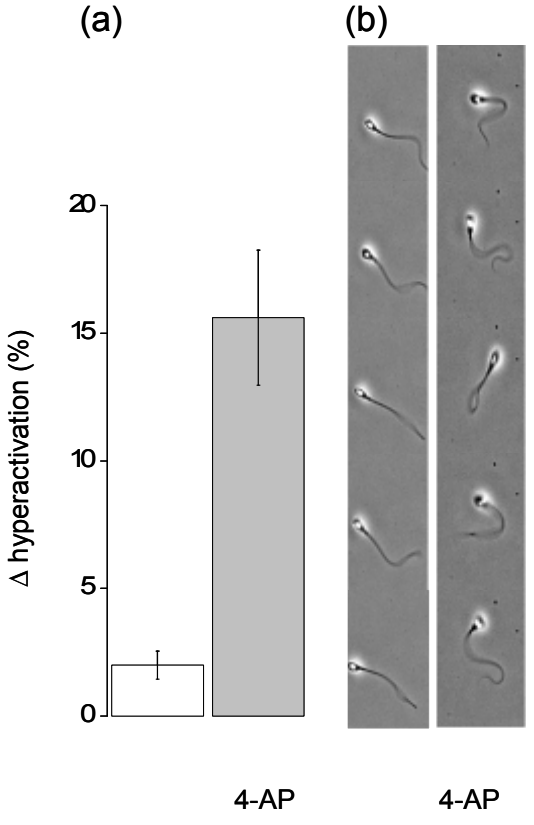

Figure 2.1. 4-aminopyridine (4-AP, 2mM) induced hyperactivation of capacitated human spermatozoa (assessed by CASA) in HEPES buffered sEBSS at pH7.3. **(a)** 4-AP causes an increase in the % of hyperactivated sperm from control conditions (open bar) and after exposure to 4-AP for ≈ 2min (grey bar). Each bar shows mean ± s.e.m. of 6 experiments (P<0.001; paired t). **(b)** Series of phase contrast images of sperm (left column) and sperm hyperactivated by 2mM 4-AP (right column) (inter-frame interval 100ms).

2.4.2 4-aminopyridine elevates $[Ca^{2+}]_i$ fluorescence in OGB labelled spermatozoa

Application of 2mM 4-aminopyridine to cells loaded with Oregon Green BAPTA 1 (OGB) showed that ≈75% of imaged cells responded with a significant increase in fluorescence (see methods, paired *t*-test, $P<0.05$). Fluorescence increased within 10-30 s and stabilized at an elevated level 1-5 min after application. The mean amplitude of this response (Δ fluorescence), 5 minutes after application of 4-aminopyridine was 15.0±1.4% (8 experiments; 515 cells; figure 2.2). Upon washout of 4-aminopyridine $[Ca^{2+}]_i$ fell rapidly to levels at or near those observed before treatment, sometimes with an 'undershoot' of 2-3 minute duration (figure 2.2).

Figure 2.2

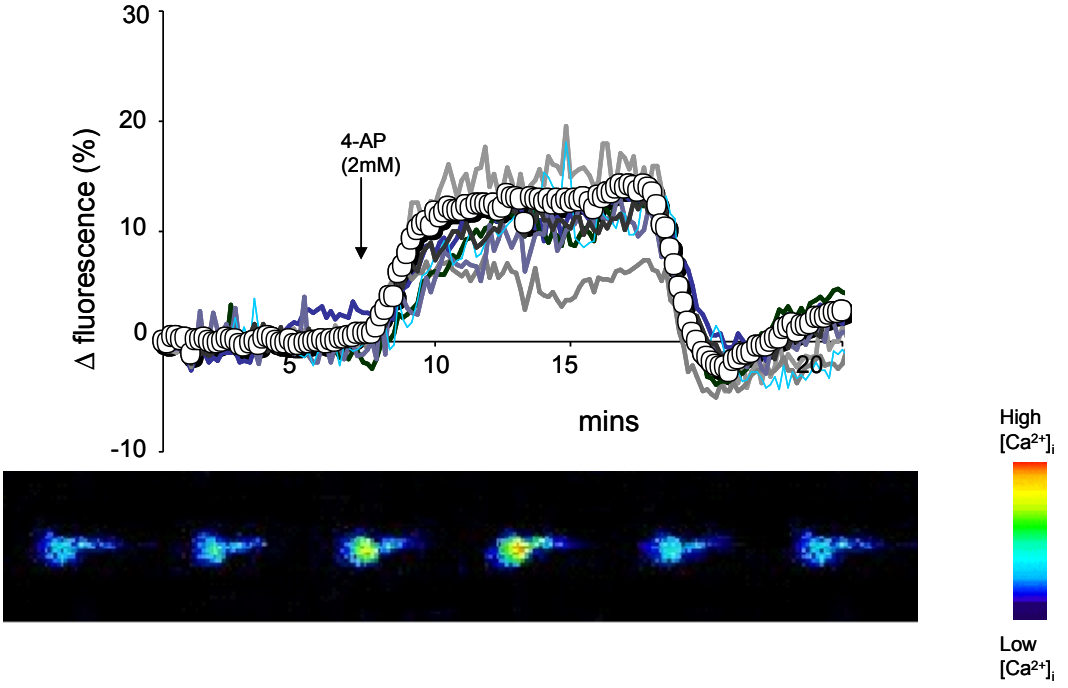

Figure 2.2. The effects of 2mM 4-AP on $[Ca^{2+}]_i$ (Oregon Green BAPTA-1 fluorescence) in capacitated human sperm bathed in standard saline (pH7.3). Traces show 7 responses of representative cells and also mean response (o-o) of all 55 cells in this experiment. Lower panel shows pseudo-colour images (warm colours show high $[Ca^{2+}]_i$) of OGB fluorescence response in a 'typical' sperm cell at various points in the experiment. First two images are prior to treatment, images 3 and 4 are during 4-aminopyridine superfusion and images 5 and 6 are during washout.

2.4.3 4-aminopyridine did not significantly quench OGB emission signal

Aminopyridines are N-heterocyclic tertiary amines and have inherent fluorescent properties. There are three isomeric amines of pyridine depending on the position (2, 3 or 4) of the ring nitrogen. 4-aminopyridine absorbs light at λ 245nm but is essentially non fluorescent in polar and non-polar solvents, with a low quantum yield (0.001) and a molar extinction coefficient of ~11,600 $cm^{-1}M^{-1}$(Steck and Ewing, 1948; Dega-Szafran *et al*., 1994). Direct effects of 4-aminopyridine on the recorded fluorescent signal are therefore unlikely.

Aromatic amines have been shown to quench or decrease the intensity of fluorophore emission. We have therefore examined the effects of 4-aminopyridine on OGB emission intensity. To investigate, de-esterified OGB (as described in the methods) was prepared, OGB fluorescence emission recorded in the presence (F, figure 2.3a) and absence ($F_{0,}$ figure 2.3b) of 4-aminopyridine (0.5-5mM). Fluorescence emission intensity quenching can be quantified by the Stern-Volmer equation (see methods). By plotting 4-aminopyridine concentrations (0.5-5mM) against the ratio of F_0 to F, the value for K_{sv} (Stern-Volmer constant) can be obtained from the slope using linear regression (K_{sv} = 0.023 at 25^{o}C). Addition of 4-aminopyridine did not change the shape of the fluorescence spectra and slightly quenched OGB emission (figure 2.3a). The Stern-Volmer constant serves as a direct measure of the efficiency of the fluorescence quenching process. It is useful to note that $1/K_{sv}$ is the 4-aminopyridine concentration at which $F_0/F_{[Q]}$=2 (or 50% of the fluorescence intensity is quenched), therefore >50mM 4-aminopyridine would be required to quench fluorescence intensity by 50%.

Figure 2.3

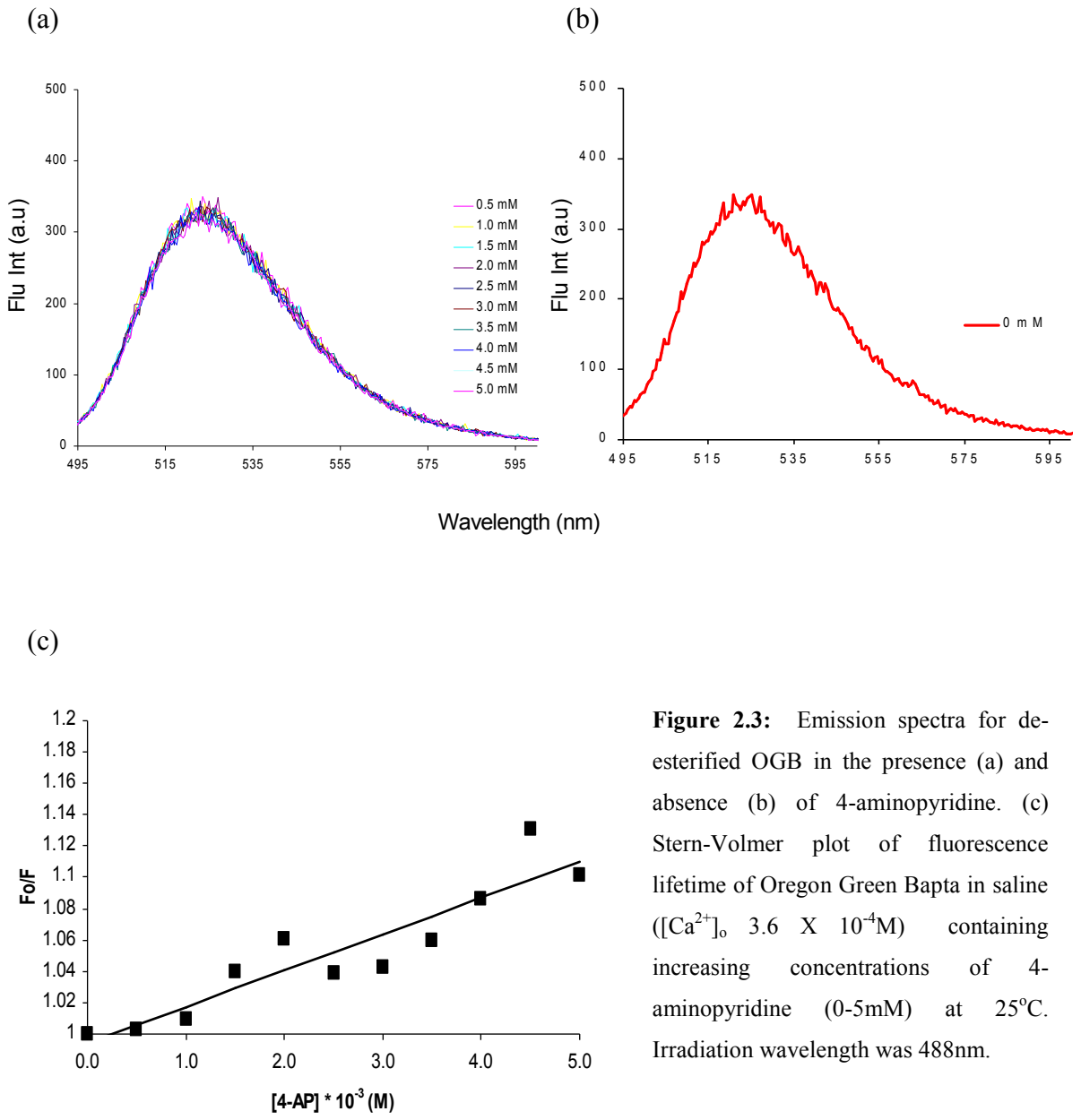

Figure 2.3: Emission spectra for de-esterified OGB in the presence (a) and absence (b) of 4-aminopyridine. (c) Stern-Volmer plot of fluorescence lifetime of Oregon Green Bapta in saline ($[Ca^{2+}]_o$ 3.6 X 10^{-4}M) containing increasing concentrations of 4-aminopyridine (0-5mM) at 25°C. Irradiation wavelength was 488nm.

2.4.4 4-Aminopyridine induced hyperactivation and increased $[Ca^{2+}]_i$ fluorescence does not act through K^+ channel block and modulation of E_m

4-aminopyridine is a widely used and effective blocker of K^+ channels, where it acts at an intracellular site after entering the cell in its uncharged form (Howe and Ritchie, 1991, Stephens *et al.*, 1994). Such an action, for instance leading to depolarisation of the sperm membrane potential (E_m), might underlie the effect of 4-aminopyridine on $[Ca^{2+}]_i$ and hyperactivation. To investigate this possibility, we 'clamped' sperm membrane potential at the potassium equilibrium potential (E_K) by pre-incubating cells with the K^+-ionophore valinomycin (1 μM) (Nernst calculation for E_K= -83mV (125mM intracellular K^+ and 5.4mM extracellular K^+) for 5 min prior to 4-aminopyridine addition. In 7 sets of paired observations, brief pre-treatment with valinomycin (~2mins time taken to load sample and record motility) reduced only slightly the proportion of cells that showed hyperactivated motility in response to 2mM 4-aminopyridine (2mM 4-aminopyridine =26.6±5.3%; valinomycin followed by 2mM 4-aminopyridine =21.1±4.9%; paired *t*-test, $P>0.05$) (figure 2.4b). In single cell imaging experiments, pre-treatment with 1μM valinomycin had a significant effect on resting $[Ca^{2+}]_i$, increasing fluorescence by 12.4% ±0.8%; n=4 experiments; 226 cells, paired *t*-test, $P<0.05$) (figure 2.4a), an effect that may reflect action of valinomycin at the mitochondria (Salvioli *et al.*, 2000; Foreman *et al.*, 2006). However, the response to 4-aminopyridine was essentially unchanged, the mean increase in fluorescence 5 min after exposure to 4-aminopyridine being 24.8 ±2.0% after valinomycin pre-treatment (normalized to fluorescence intensity in the presence of valinomycin; 4 experiments; 220 cells) and 20.8 ±2.0 % in parallel controls (4 paired experiments, 195 cells; paired *t*-test, $P>0.05$).

Figure 2.4

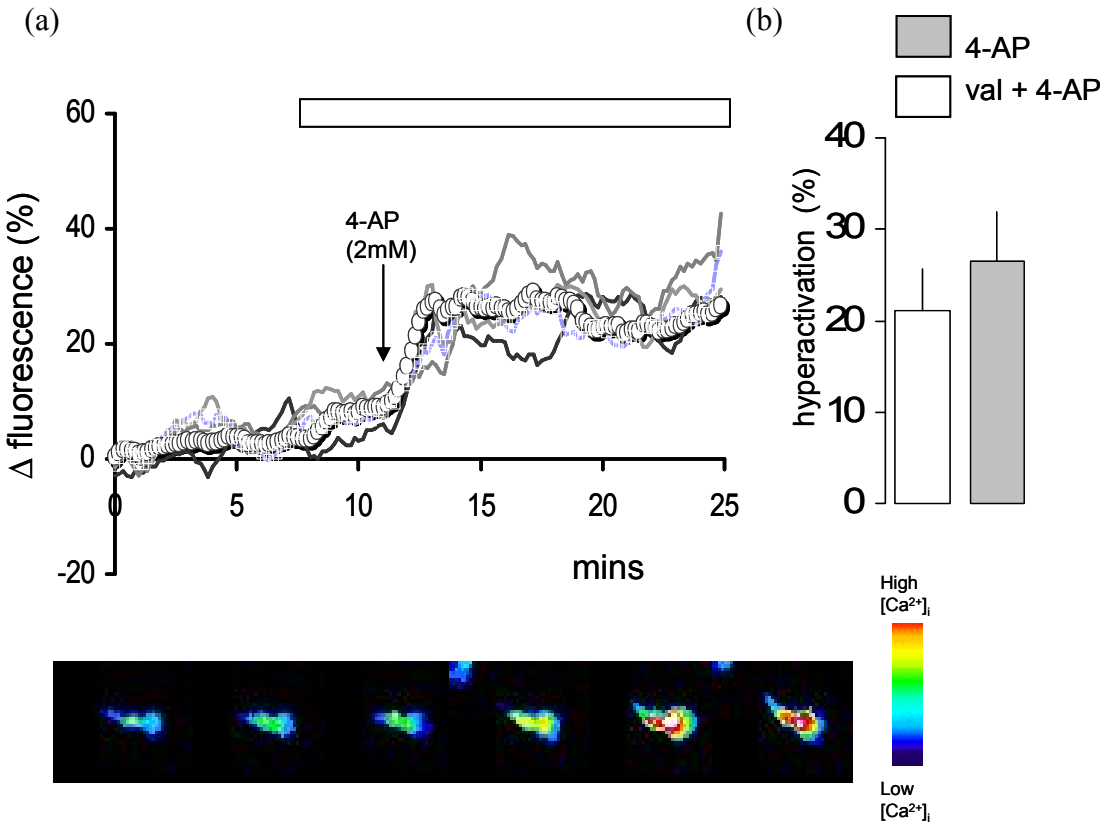

Figure 2.4. Investigation whether modulation of E_m (due to K^+ channel blockade) might underlie the action of 4-aminopyridine. **(a)** E_m was clamped at E_k (-83mV) by pre-incubating cells with the K^+ -ionophore valinomycin (1µM, white bar) and incubation with 2mM 4-aminopyridine (black arrow) on $[Ca^{2+}]_i$ (Oregon Green BAPTA-1 fluorescence) in capacitated human sperm bathed in standard saline. Traces show 6 responses of representative cells and also mean response (o-o) of all 76 cells in this experiment. Lower panel shows pseudo-colour images (warm colours show high $[Ca^{2+}]_i$) of Oregon Green-1 BAPTA fluorescence in sperm cell at various points in the experiment. First two images are prior to treatment, images 3 and 4 are during valinomycin superfusion and images 5 and 6 are during valinomycin and 4-aminopyridine superfusion. **(b)** Exposure to valinomycin and 4-aminopyridine (open bar) reduced slightly the % of hyperactivation (paired *t*-test, $P>0.05$, n=7 experiments).

2.5 Discussion

Hyperactivation is essential for sperm detachment from oviductal epithelium and penetration of the outer vestments of the oocyte (Stauss *et al.*, 1995; Carlson *et al*., 2003; Ho and Suarez, 2001a; Ho *et al.*, 2009). Hyperactivated motility occurs when the flagellum develops high amplitude waves in the proximal rather than the distal region (Mortimer *et al.,* 1997). When the wave is propagated its movement away from the proximal region of the tail results in the sperm head being moved back toward the average path at a high velocity much like a whiplash. To identify hyperactivated sperm CASA thresholds for human sperm analyzed at 60Hz are VCL ≥ 150µm/s and Lin ≤ 50% and ALHmax ≥ 7.0µm (section 1.11.1) (Mortimer *et al.,* 1997). This is an indirect measure of flagellar bend amplitude, because the head is displaced from side-to-side by the developing principal and reverse bends and it gets wagged further by deeper flagellar bends. Because ALH is not a direct measure of flagellar bends it is imperfect however CASA compensates by precision and acquisition of data from hundreds of sperm.

4-aminopyridine induced hyperactivation in capacitated human spermatozoa

Previously our group demonstrated the potent effect of 4-aminopyridine on human sperm motility. Gu *et al*. (2004) found that application of 4-aminopyridine (2mM) induced a dramatic increase in hyperactivated motility in capacitated human spermatozoa (Gu *et al*., 2004; Barfield *et al.,* 2005). The proportion of hyperactivated sperm increased from 3-5% in control experiments to 40-50%.following stimulation with 4-aminopyridine (Gu *et al.,* 2004). The potency of 4-aminopyridine is strongly dependent upon pH_o which explains the discrepancy between the results presented here and those reported by Gu *et al*., (2004). At elevated pH_o (>8.0) the proportion of the compound which in a non-ionised form and therefore membrane permeable is greatly enhanced (Howe and Richie, 1991; Stephens *et al.*, 1994). 4-aminopyridine is a weak organic base (pKa 9.2, Albert *et al*., 1948; Babiak and Testa, 1976) and at physiological pH exists in equilibrium between the neutral (figure 1.8a) and protonated

forms (figure 1.8b) that strongly favors the charged form. By maintaining extracellular pH at 7.3, membrane permeability of the compound is lowered, resulting in a lower proportion of spermatozoa displaying hyperactivated motility (3.0 ±2.5 in control experiments to 15.6 ±4.0% following stimulation with 4-aminopyridine (n=9; P<0.001; figure 2.1a). Furthermore throughout these experiments we have utilized 20µm depth CASA chambers rather than 50µm depth chamber often used in hyperactivation experiments. Use of these chambers may result in some constraint of flagellar movements and further explain the lower proportion of 4-aminopyridine induced hyperactivation rates. Despite this, application of 4-aminopyridine increased the proportion of hyperactivated cells three fold in comparison to parallel controls.

4-aminopyridine increased $[Ca^{2+}]_i$ fluorescence in human spermatozoa

Control of motility and hyperactivation is dependent on $[Ca^{2+}]_i$ signalling in the principal piece and midpiece of the flagellum. Application of 2mM 4-aminopyridine to cells loaded with OGB induced a sustained increase in $[Ca^{2+}]_i$ in ≈75% human spermatozoa. Fluorescence increased within 10-30 seconds and stabilised at an elevated level 1-5mins after application (figure 2.2). Upon washout of 4-aminopyridine $[Ca^{2+}]_i$ immediately falls to levels at or near those observed before treatment. It is unclear from these initial experiments as to the source of 4-aminopyridine induced sustained increase in $[Ca^{2+}]_i$ since human spermatozoa contain both plasma membrane calcium channels and an intracellular calcium store localised at the proximal head/midpiece. These results are consistent with other studies which demonstrate 4-aminopyridine induced Ca^{2+} mobilisation in a wide variety of cell types including astrocytes, neurons, and skeletal muscle cells (Grimaldi *et al*., 2001).

Examination of OGB emission spectra in the presence and absence of 4-aminopyridine indicated a degree of fluorescent quenching and decreased fluorescent signal. However, the effect of 4-aminopyridine will lead to an underestimate not an exaggeration of effect on $[Ca^{2+}]_i$. Increased intracellular calcium is pivotal for the induction of hyperactivation and therefore

must be examined. Furthermore, the varying nature of human sperm $[Ca^{2+}]_i$ response justified the examination of individual imaging traces, not visible in population responses.

4-aminopyridine and K^+ channel blockade

4-aminopyridine is a broad spectrum K^+ channel blocker. Human spermatozoa express K^+ channels and blocking these channels could cause E_m depolarisation induced calcium influx. Therefore, 4-aminopyridine may be modulating E_m resulting in Ca^{2+} influx and hyperactivated motility. Spermatozoa were pre-treated with the K^+ ionophore, valinomycin prior to examination of the effects of 4-aminopyridine on $[Ca^{2+}]_i$ and hyperactivation. 4-aminopyridine induced hyperactivation was not significantly affected by valinomycin. Imaging experiments show that valinomycin caused a significant sustained increase in $[Ca^{2+}]_i$ in single cell responses however this may be due to modulation of sperm mitochondria. In somatic cells mitochondria play an important role in regulation of intracellular Ca^{2+} signalling. Mitochondria sequester increased $[Ca^{2+}]_i$ through the mitochondrial Ca^{2+} uniporter (MCU), which is driven by the large negative potential across the inner mitochondrial membrane (Parekh, 2003; Breitbart *et al.,* 1983; 1996). Valinomycin has previously been shown to uncouple oxidative phosphorylation and dissipate mitochondrial membrane potential (Salviolo *et al.,* 2000; Amaral and Ramalho-Santos, 2009) and inhibit mitochondrial Ca^{2+} uptake in rat primary bone marrow stromal cells (Foreman *et al.,* 2006). However, the response to 4-aminopyridine was essentially unchanged, the mean increase in fluorescence 5 min after exposure to 4-aminopyridine was slightly higher than parallel controls. These results, are in agreement with Xia and Ren (2009b) who used a similar approach to show that the recorded $[Ca^{2+}]_i$ influx was not dependent on E_m and activation of voltage gated Ca^{2+} channels. In summary, the 4-aminopyridine induced $[Ca^{2+}]_i$ increase is not dependent on membrane voltage change which persists when the voltage is "clamped" with valinomycin.

In whole cell patch clamp, a constitutively active, weakly outwardly rectifying K^+ current was measured (I_{KSper}), localised to the principal piece of the flagellum (Navarro *et al*., 2007). This current activates in response to intracellular alkalinisation, leading to hyperpolarisation and an increased driving force for calcium entry through CatSper channels. Navarro *et al*., (2008) have suggested that Slo3 channels are the best candidate for the K^+ current (I_{KSper}) principally because of Slo3 pH sensitivity although other disagree due to key differences between KSper and Slo3 (Martínez-López *et al*., 2009). Martínez-López *et al.,* (2009) report that Slo3 K^+ currents were inhibited by Ba^{2+} and stimulated by 8 Br-cAMP whereas I_{KSper} was weakly inhibited by Ba^{2+} and insensitive to cAMP (Navarro *et al.,* 2007). Although K^+ channel activity was not measured directly in this study, recent data on the pharmacological properties of Slo3 channels (expressed in *Xenopus* oocytes) demonstrated that extracellular 4-aminopyridine has little effect on the K^+ current (Tang *et al*., 2010). The authors conclude that any accumulation of 4-aminopyridine in the cytosol would elevate pH to promote Slo3 activity (act in opposition to any channel blocking effects).

In summary, exposure of capacitated human spermatozoa to 2mM 4-aminopyridine induced hyperactivation in a proportion of sperm and a sustained increase in $[Ca^{2+}]_i$ in the majority of cells. The 4-aminopyridine induced increase in $[Ca^{2+}]_i$ was independent of K^+ fluxes however the source of calcium is not clear from these experiments.

CHAPTER THREE

EFFECT OF pH ON 4-AMINOPYRIDINE INDUCED HYPERACTIVATION AND ELEVATION OF $[Ca^{2+}]_i$

3.1 Abstract

Various studies have shown that a pH-stimulated Ca^{2+} increase in sperm, triggers hyperactivation (Mújica *et al*., 1994; Marquez and Suarez, 2004, 2007). Navarro *et al*., (2007) demonstrated that 4-aminopyridine (pKa9.2) induced cytoplasmic alkalinisation enhanced the pH-sensitive Ksper and CatSper currents in mouse sperm. Assessment using BCECF confirmed that 2mM 4-aminopyridine caused an immediate increase in pH_i of ≈0.2 units in human sperm. To investigate whether cytoplasmic alkalinisation of human sperm might underlie the effect of 4-aminopyridine on $[Ca^{2+}]_i$ and hyperactivation NH_4Cl was used directly to increase pH_i. Exposure of sperm to NH_4Cl had an almost identical effect on pH_i and also caused a sustained increase in $[Ca^{2+}]_i$ only slightly less than that seen with 2mM 4-aminopyridine. However, there was negligible induction of hyperactivation compared to cells treated with 4-aminopyridine. Treatment of sperm with propionate (to decrease pH_i and inhibit CatSper channel) did not significantly effect 4-aminopyridine induced hyperactivation but enhanced the effect of 4-aminopyridine on $[Ca^{2+}]_i$. In summary the 4-aminopyridine induced hyperactivation response in human sperm is not solely pH or CatSper channel dependent.

3.2 Introduction

Human spermatozoa are quiescent in the male reproductive system and become activated upon ejaculation (Acott and Carr, 1984; Carr and Acott, 1989; Giroux-Widemann *et al.,* 1991). Intracellular pH is a critical regulator of sperm function and cytoplasmic alkalinisation is essential for the initiation and maintenance of motility, capacitation, chemotaxis, hyperactivation and the acrosome reaction (Acott and Carr, 1984; Parrish *et al*., 1989; Cook and Babcock, 1993; Jones and Barvister, 2000; Ho *et al.,* 2002, Böhmer *et al*., 2005; Darszon *et al*., 2006; Marquez and Suarez, 2007; Shiba *et al.,* 2008). Despite the importance of intracellular alkalinisation to sperm motility the mechanisms enabling or enhancing transition from activated to hyperactivated motility are unclear.

Transition from activated to hyperactivated motility also requires an increase in $[Ca^{2+}]_i$. Influx through calcium channels (localised in the flagellum) or release from intracellular stores have been shown to play a role in supporting and sustaining hyperactivation. Calcium channels include transient receptor potential channels, cyclic nucleotide gated calcium channels and voltage-gated Ca^{2+} channels and the pH sensitive sperm specific CatSper calcium. Male mice lacking functional CatSper1 or CatSper2 genes are sterile (Ren *et al*., 2001; Quill *et al*., 2001; Carlson *et al*., 2003, 2005). . It has been shown that intracellular alkalinisation potentates an inward Ca^{2+} current through CatSper-associated channels (Kirichok *et al*., 2006; Navarro *et al.,* 2007; Xia and Ren, 2009b). The weak base 4-aminopyridine (pKa9.2) induced cytoplasmic alkalinisation enhanced I_{KSper} induced hyperpolarisation resulting in an increased driving force for Ca^{2+} entry through CatSper channels (Navarro *et al*., 2007). In addition, the weak base procaine has been shown to induce hyperactivation in bovine sperm which further implicates pH as a component in the signalling pathway that control Ca^{2+} entry and hyperactivation (Mújica *et al*., 1994; Marquez and Suarez, 2004).

However, studies in rodent and bovine sperm has provided strong evidence that the elevation of $[Ca^{2+}]_i$ that occurs upon mobilisation of Ca^{2+}, from a store in the sperm neck/midpiece

region also contribute to regulation of flagellar activity, including (or at least facilitating) aspects of hyperactivation (Ho *et al*., 2001b; 2003). It is unclear if store mobilisation contributes or interacts with the CatSper channels (Olson *et al*., 2010).

Since the efficacy of 4-aminopyridine (Howe and Ritchie, 1991; Stephens *et al*., 1994) is strongly dependent upon pH, this report examines the effects of intracellular and extracellular pH modulation on 4-aminopyridine induced responses in human sperm.

3.3 Experimental Procedures

3.3.1 Materials

Supplemented Earle's balanced salt solution (sEBSS) was prepared as in chapter two. For maintenance of pH at 8.5, HEPES was replaced with 10mM [(2-hydroxy-1,1-bis(hydromethyl)ethyl)amino]-1-propanesulfonic acid (TAPS). For maintenance of pH at 6.5, HEPES was replaced with 10mM N-(2-acetamido)iminodiacetic acid (ADA). Ammonium supplemented sEBSS (25mM) and proprionate supplemented sEBSS (15mM, pH 8.5) was prepared in the laboratory. Non-capacitating saline was prepared as for standard saline with the omission of $NaHCO_3$ and BSA. Osmotic strength was maintained (285-295mOmol) by adjusting NaCl in all saline prepared.

3.3.2 Spermatozoa preparation and capacitation

As described in chapter two with the following modifications.

Spermatozoa were harvested by direct swim-up into non-capacitating saline without $NaHCO_3$ and BSA.

3.3.3 Fluorimetry

For fluorimetric studies on pH_i, 2ml aliquots (6×10^{-6} cells.ml^{-1}) were labelled with 1µM 2'-7'-bis-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for 30 min at 37^{o}C and 5.5% CO_2 then centrifuged (300g for 5 min). The pellet was re-suspended in sEBSS with pH adjusted and buffered as required. Excitation wavelengths alternated between 440 and 495nM. Emission was recorded at 535nM with slit width was set at 15nM and a sampling rate of 12.5Hz. The pH_i values for control and 4-aminopyridine or NH_4Cl were determined using a calibration procedure similar to that described by Fraire-Zamora and Gonzalez-Martinez (2004). Briefly, cells suspended in sEBSS at pH 7.35 were permeabilised with Triton X-100 (final concentration 0.12%) then a series of additions of HCl were made. At

each step (including the one obtained after detergent addition) the pH was determined with a conventional pH electrode to obtain a calibration curve. BCECF ratio values were plotted as a function of pH. As the ratio was linear in the pH range between 6.5 and 7.35 (figure3.4a) a simple transformation using a straight line equation was performed to estimate pH_i values from experimental data.

For fluorimetric studies on sperm $[Ca^{2+}]_i$ 2ml aliquots (6 x 10^{-6} cells.ml^{-1}) were labelled with 1μM fura-2 acetoxymethyl ester (fura-2/AM) for 12 min at 37°C and 5% CO_2, centrifuged (300 g for 5 min) and re-suspended in sEBSS for a further 17 min (37°C) to allow for further de-esterification of the dye. Experiments were carried out using a Perkin-Elmer LS50B fluorescence spectrofluorimeter running FLWinlab version 2.01. Fluorimetry was performed in a magnetically stirred methylacrylate cuvette maintained at constant temperature by a water jacket (25°C or 37°C). Excitation wavelengths alternated between 340 and 380nM. Emission was recorded at 510 nM with slit width was set at 15nM with a sampling rate of 12.5Hz. Calibration of $[Ca^{2+}]_i$ was carried out using digitonin (50μM) to generate a maximal response and subsequent addition of EGTA (20mM) to provide a minimum. A Kd value of 224nM was assumed and calibration was performed according to the equation of Grynkiewicz *et al.* (1985).

3.3.4 Evaluation of sperm hyperactivation

For experiments involving TAPS-buffered medium, cells were centrifuged (300 g for 5 min) and the pellet re-suspended in 1ml TAPS buffered saline immediately prior to the experiment. 200μl aliquots were treated with either saline (control) or 4-aminopyridine and immediately introduced into a 20μM depth chamber (Microcell, Conception Technologies Ltd.) at a concentration of ~ 6 million/ml on a Hamilton Thorne HTM IVOS semen analyzer (37°C). Hyperactivation was defined as in chapter two.

3.3.5 Evaluation of extracellular pH on degree of 4-aminopyridine ionisation

The Henderson-Hasselbalch equation for a weak base is as follows

$$pH = \frac{[B]}{[BH^+]} + pKa \qquad (1)$$

Using this equation the % ionisation of the weak base can be calculated from the ratio of the ionised to neutral compound, multiplied by 100.

$$\%\text{ionisation} = \frac{I \bullet 100}{I + N} \qquad (2)$$

I = ionised 4-aminopyridine
N = neutral 4-aminopyridine

Rearranged as $$\frac{N}{I} = \text{antilog}(pH - pKa) \qquad (3)$$

Solved for $$N = I \bullet \text{antilog}(pH - pKa) \qquad (4)$$

Upon substitution of (4) into (2) the equation for % ionisation for a weak base is as follows:

$$\%\text{ionisation (base)} = \frac{100}{1 + \text{antilog}(pH - pKa)}$$

3.3.6 Single Cell Imaging

As in chapter two.

3.3.7 Imaging Data Processing

As in chapter two.

3.3.8 Statistical analysis

As in chapter two.

3.4 Results

3.4.1 Ionisation state of 4-aminopyridine was dependent on pH_o

The potency and membrane permeability of 4-aminopyridine is dependent upon the ionisation state of the compound. A modified Henderson-Hasselbach equation was used to calculate % ionisation of 4-aminopyridine (pKa9.2 at 25°C, Albert *et al*., 1948; Babiak and Testa, 1976) over a range of pH values (see methods 3.3.5). At physiological pH, the basic nature of 4-aminopyridine leads to an equilibrium between the ionised (I) and neutral (N) form that strongly favors the protonated form (~99%) (figure 3.1). At pH_o7.3 the majority of the compound is charged and membrane impermeable (Howe and Richie, 1991; Zhang *et al*., 1998), whereas at pH_o8.5 approximately 20% of the compound is in a neutral, membrane permeable form.

Figure 3.1

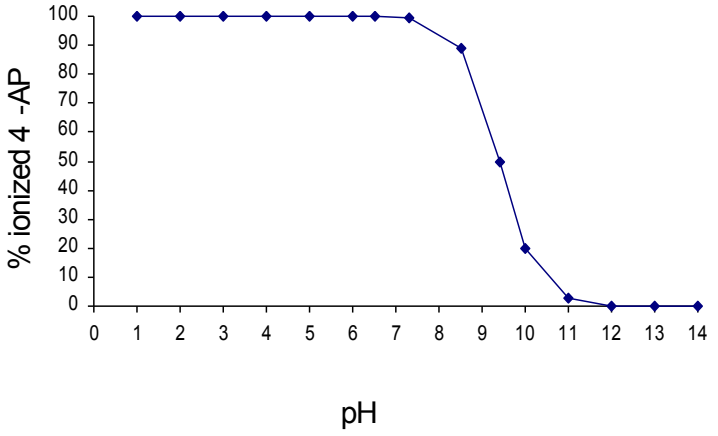

Figure 3.1: 4-aminopyridine % ionisation was calculated (see text) for a range of pH values from the 4-aminopyridine pKa value of 9.2 at 25°C (Albert *et al*., 1948; Babiak and Testa, 1976)

3.4.2 *4-aminopyidine induced hyperactivation was significantly increased at extracellular pH8.5*

Initially we investigated the efficacy of 4-aminopyridine at a pH_o of 8.5 (sEBSS with HEPES replaced by 10mM TAPS) (figure 3.2a). Elevation of pH_o had no significant effect on the level of hyperactivation, increasing the proportion of hyperactivated cells assessed by CASA from 5.1 ± 1.2 to 8.1 ± 2.2% (n=14, p=0.09, paired *t*-test, $P>0.05$). However, upon application of 2mM 4-aminopyridine at pH_o8.5 the proportion of hyperactivated cells increased to 33.1± 3.1% (n=14; $P<10^{-6}$ compared to control, paired *t*-test) more than double that which was seen at pH_o7.35 (unpaired *t*-test, $P<0.0005$). This action of 4-aminopyridine was clearly dose dependent (figure 3.2a), application of 0.5mM 4-aminopyridine induced hyperactivation in only ≈ 7.5 of cells and no effect was detectable at a dose of 0.1mM (pH_o8.5, TAPS buffered media). At 10mM 4-aminopyridine induced hyperactivation in 26.3 ± 5.3% of cells (pH 8.5, TAPS buffered media). To facilitate observation of the effect of 4-aminopyridine, all subsequent experiments (unless stated otherwise) were carried out at pH_o 8.5.

The ability of 4-aminopyridine to induce hyperactivation was independent of capacitation. Highly motile sperm cells were harvested by direct swim-up into non-capacitating sEBSS (NCsEBSS, lacking BSA and HCO_3^-) or standard saline as previously described. Motility was immediately assessed by CASA in the presence and absence of 4-aminopyridine (2mM). The proportion of hyperactivated cells did not differ significantly between treatments (NCsEBSS cells= 27.2±8.0%, standard sEBSS 31.5±2.6%, n= 6experiments, paired *t*-test, $P>0.05$) (figure 3.1b). Similarly, cells capacitated for ~1 hour (time taken to swim-up and assess) did not differ significantly from cells capacitated for 5-6 hours in the response to 4-aminopyridine (t=1 hour capacitation 31.5±2.6%, t=5-6 hours capacitation 30.8±5.6%, n=6 paired *t*-test, $P>0.05$) (figure 3.2b).

Since inhibition of K^+ channels has been shown to have a deleterious effect on intracellular volume regulation and motility in human sperm (Yeung and Cooper, 2001), the effects of prolonged exposure to 2mM 4-aminopyridine was investigated (figure 3.2c). Sperm were capacitated in standard saline for 4/5 hours and 4-aminopyridine induced hyperactivation assessed by CASA (37.3±8.2% hyperactivation after 2 minutes -minimum time taken to load and assess sample). The effects of 2mM 4-aminopyridine showed only a minor decay when the same aliquot of cells was tested 45 minutes later (30.0±7.5%) (n=3exps, paired *t*-test, $P>0.05$). Similarly, % motility was not significantly altered despite prolonged exposure to 4-aminopyridine (82.8±3.6% t=2mins, 82.2 ± 3.7% t=45mins, n= 3experiments, paired *t*-test, $P>0.05$).

Figure 3.2

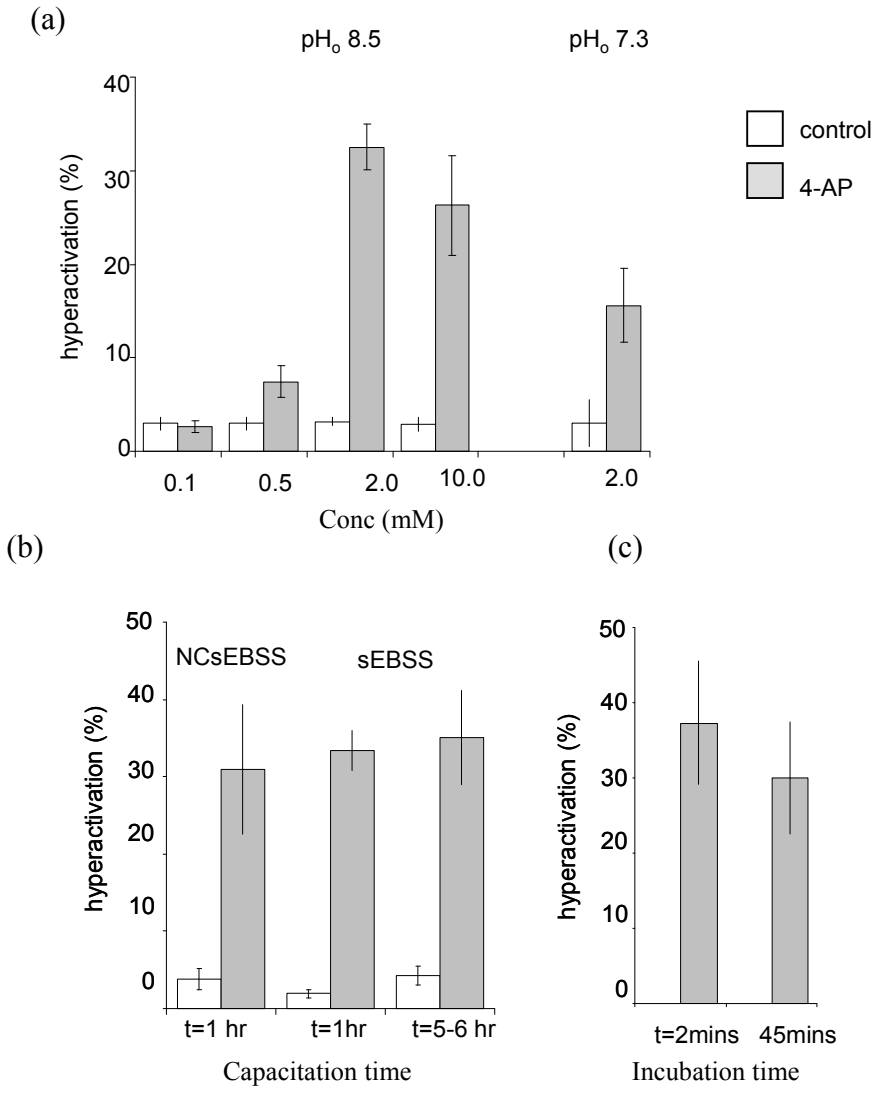

Figure 3.2. 4-aminopyridine (2mM) induced hyperactivation in non-capacitated and capacitated human spermatozoa (assessed by CASA) is enhanced at pH$_o$ 8.5. **(a)** The efficacy of (2mM) 4-aminopyridine to induce hyperactivation was greatly enhanced at pH$_o$ 8.5 in comparison to pH$_o$ 7.3. This action of 4-aminopyridine was clearly dose dependent with maximal effect at 2mM, each bar shows mean ± s.e.m. of 9 experiments (P<0.001; paired t-test). **(b)** The ability of 4-aminopyridine to induce hyperactivation did not significantly differ between non-capacitated cells (NCsEBSS, n=6 exps) and capacitated cells (1hr or 5-6hrs) (n=6 exps, paired t-test, P>0.05). **(c)** The proportion of 4-aminopyridine induced hyperactivated cells remained constant over a prolonged exposure period (n=3, 45 mins) in capacitated cells.

3.4.3 Extracellular pH effects 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response in human spermatozoa

Initially the effect of lowering pH_o (from 7.35 to 6.5), prior to application of 2mM 4-aminopyridine was investigated. Superfusion of cells with saline (pH_o6.5) decreased $[Ca^{2+}]_i$ fluorescence in ~30% of cells (decrease of ~10%) but in many cells there was no discernible effect (figure 3.3 a). Application of 2mM 4-aminopyridine resulted in an increase in $[Ca^{2+}]_i$ fluorescence in 37% of cells (a transient response of 21.2 ± 1.0% in 10% of cells and a sustained increase of 8.5 ± 0.3% in 27% of cells (n=4 experiments, 264 cells)).

Increasing pH_o (from 7.35 to 8.5) caused a detectable increase in $[Ca^{2+}]_i$ fluorescence in ≈95% of cells, fluorescence of OGB stabilising within 2-3 min at 10-15% above control levels (figure 3.3b) (pH_i increased from 7.08 ± 0.10 (n=23) to 7.2 ± 0.10 (n=17; paired *t*-test, P>0.05). Application of 2mM 4-aminopyridine caused a further significant increase in $[Ca^{2+}]_i$ fluorescence in 75% of cells, a similar proportion to that observed at pH 7.35. The amplitude of this response (24.5 ± 0.66% of fluorescence intensity at pH8.5; 8 experiments, 580 cells) was greater than at pH7.35 (15.0±1.4%, paired *t*-test, P=0.01; figure 2.2). It was not necessary to sensitise the cells to high pH_o with TAPs buffered saline (pH_o8.5), addition of 2mM 4-aminopyridine (to non-Hepes buffered saline) resulted in a significant increase in $[Ca^{2+}]_i$ fluorescence (amplitude of the response was 25.3 ± 4.4% in ~85% of cells 5 mins after exposure (n=3 experiments, 311 cells, unpaired *t*-test, P<0.05) (figure 3.3c), a response similar to that of cell pre-treated with TAPS buffered saline (figure 3.3b), Upon 4-aminopyridine washout $[Ca^{2+}]_i$ fluorescence dropped rapidly to pre-application levels and the response could be repeated by re-application of the drug (amplitude of second response 30.3 ±2.8% 5 mins after exposure, figure 3.3 c). Similar to the effect of 4-aminopyridine on hyperactivation, elevation of $[Ca^{2+}]_i$ by 4-aminopyridine applied at pH_o8.5 showed a marked dose sensitivity over the range 0.1-2.0mM (figure 3.3d).

In some cells an initial peak was clearly discernible upon application of the drug, followed by a plateau phase. In single cell imaging experiments this was detectable in mean responses (R_{tot}) as a 'notch' occurring 2-3 minutes after application of 4-aminopyridine (figure 3.3c). Fluorimetric (population) records of $[Ca^{2+}]_i$ response to 2mM 4-aminopyridine in cells incubated at 25^{o}C and 37^{o}C (pH_o8.5, figure 3.3de) was investigated. Application of 4-aminopyridine to cells at 25^{o}C resulted in a similar response to that of single cell imaging experiments. At both temperatures the response appears to comprise an initial transient response followed by a plateau (consistent with store mobilisation followed by Ca^{2+} influx) but separation of the phases is more clearly visible at 37^{o}C.

At 37^{o}C an initial transient response peaked at ≈30 s after application, followed by a plateau (figure 3.3e). At 25^{o}C the response rose more slowly, taking up to 100 s to reach maximum and then decayed slowly to a plateau (figure 3.3e). The estimated amplitude of the response (Δ $[Ca^{2+}]_i$ at peak) was 112.1 ± 16.3nM (n=6 exps) at 37^{o}C and 187.2 ± 19.2nM (n=7 exps) at 25^{o}C.

Figure 3.3

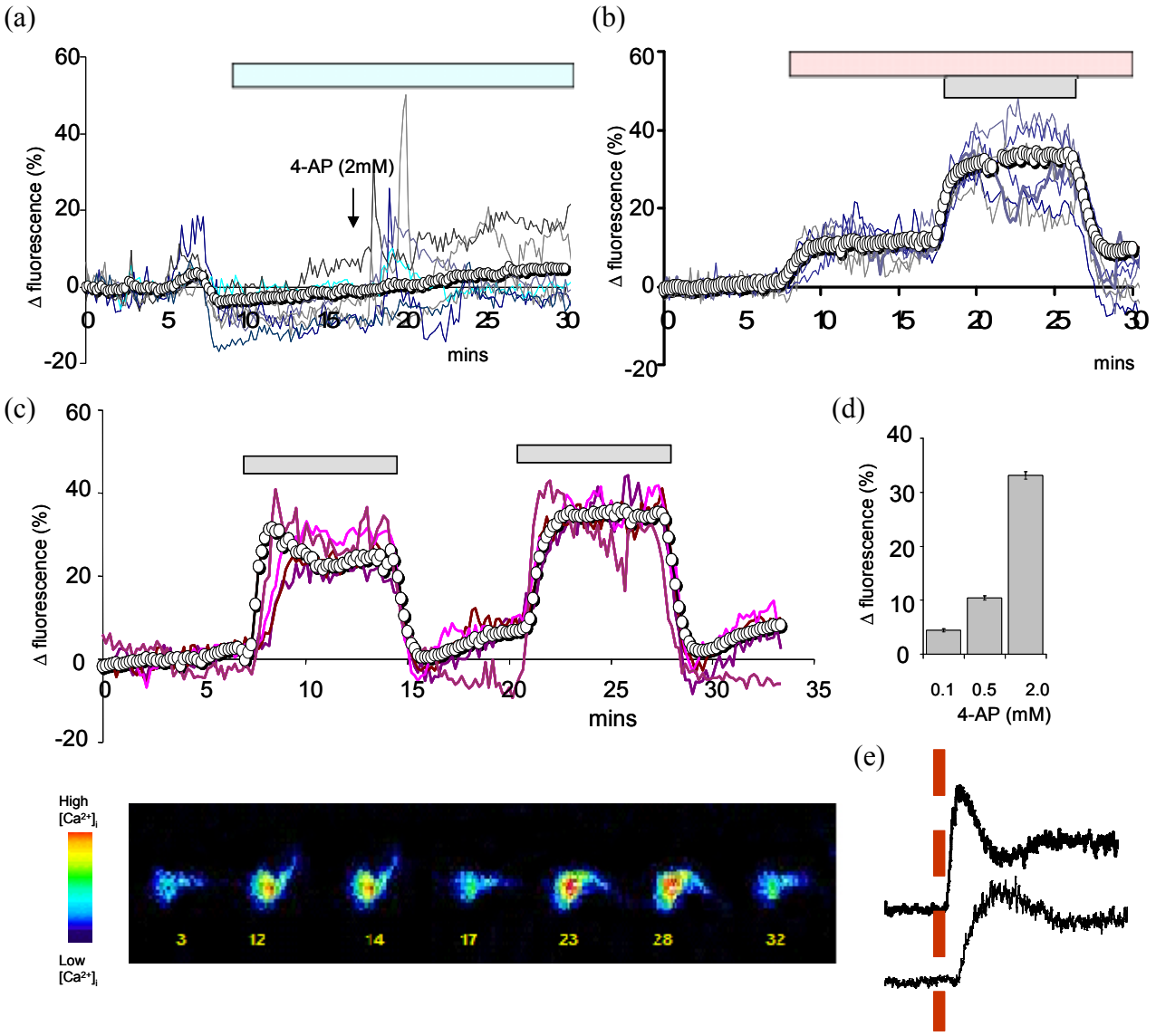

Figure 3.3. The effect of extracellular pH on 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response in human sperm. **(a)** Effect of 4-aminopyridine on $[Ca^{2+}]_i$ fluorescence at pH_o6.5. Cells were initially superfused with standard sEBSS (~6mins, pH_o7.35). Superfusion with ADA-buffered saline (pH_o6.5, blue bar) caused a slight decrease in population $[Ca^{2+}]_i$ fluorescence. Upon application of 4-aminopyridine (also pH_o6.5, arrow) there was a transient increase in $[Ca^{2+}]_i$ fluorescence in a proportion of cells. Responses of 8 representative cells are shown and also mean response (o-o) of all the 66 cells in the experiment. **(b)** Effect of 4-aminopyridine on $[Ca^{2+}]_i$ (OGB fluorescence) at pH_o8.5. Cells were initially superfused with standard sEBSS (pH_o7.35, ~6 mins). Superfusion with TAPS-buffered saline (pH_o8.5, pink bar) caused a sustained rise in $[Ca^{2+}]_i$ fluorescence. Upon application of 4-aminopyridine (also pH_o8.5, grey bar) there was a further and much larger rise in $[Ca^{2+}]_i$ which reversed immediately upon washout. Responses of 5 representative cells are shown and also mean response (o-o) of all the 70 cells in the experiment. **(c)** 4-aminopyridine enhanced $[Ca^{2+}]_i$ fluorescence did not require prior sensisation with TAPS buffered saline. Since 4-aminopyridine can act as a weak base (pKa9.2) application in non-buffered

saline (grey bar) caused an increase in pH_o and increased $[Ca^{2+}]_i$ fluorescence. This effect reversed immediately upon washout and re-application of 4-aminopyridine (grey bar) caused a second significant sustained rise in $[Ca^{2+}]_i$ fluorescence which again reversed upon washout. Responses of 10 representative cells are shown and also mean response (o-o) of all the 77 cells in the experiment). Lower panel shows pseudocolour images (warm colours show high $[Ca^{2+}]_i$) of OGB fluorescence in a sperm cell at various times points (figure in each image shows the time in minutes from start of experiment). **(d)** Elevation of $[Ca^{2+}]_i$ by 4-aminopyridine applied at pH_o8.5 showed a marked dose sensitivity over the range 0.1-2.0mM **(e)** Fluorimetric (population) records of fura-2/AM labelled $[Ca^{2+}]_i$ cell response to 2mM 4-aminopyridine incubated at 37^{o}C (top trace) and 25^{o}C (lower trace). Application of 4-aminopyridine to cells at 25^{o}C resulted a similar response to that of Oregon Green Bapta1 single cell imaging experiments. The response to 4-aminopyridine at 37^{o}C compromised an initial transient response followed by a plateau. Traces have been aligned by the time of 4-aminopyridine application (shown by red dotted line).

3.3.4 Effects of NH_4Cl on intracellular pH and 4-aminpyridine induced hyperactivation and $[Ca^{2+}]_i$ fluorescence response

As a weak organic base (pKa9.2), 4-aminopyridine on entering the cell may directly elevate pH_i, potentially leading to increased Ca^{2+} influx and hyperactivation through activation of pH-regulated CatSper channels. To investigate, we used BCECF directly to monitor intracellular pH in suspensions of human sperm. Fluorimetric experiments on suspended cells (loaded with fura-2) showed that the $[Ca^{2+}]_i$ response to 4-aminopyridine was similar to that of immobilised cells (compare figure 3.3c with 3.3e). In capacitated cells (5-6 hours), the resting pH_o (calibrated as described in methods, figure 3.4b) was 7.08 ± 0.10 (n=23). Suspension in TAPS-buffered medium (pH_o8.5) resulted in a slightly increased pH_i to 7.2 ± 0.10 (n=17; NS). Application of 2mM 4-aminopyridine to cells bathed in TAPS-buffered medium caused an immediate rise in pH_i, which stabilized within 10-20 s at a value 0.20 ± 0.06 units (n=9) above control level (P<0.01; paired t-test; figure 3.4b; grey trace).

To determine whether this effect on pH_i might be sufficient to explain the observed effects of 4-aminopyridine, we used cell permeant NH_4Cl to increase pH_i. In cells suspended in sEBSS held at pH 7.35 (15mM HEPES), 25mM NH_4Cl rapidly increased pH_i by 0.26 ± 0.06 units (n=6, figure 3.4b- black trace), consistent with previous studies on human and bovine sperm (Fraire-Zamora and Gonzalez-Martinez, 2004; Marquez and Suarez, 2007). Addition of 4-aminopyridine to cells pre-treated with NH_4Cl resulted in a further increase of ~0.10 (n=4).

In cells suspended in TAPS-buffered (pH_o8.5) medium, NH_4Cl increased pH_i by 0.18 ± 0.05 units (n=9; P<0.01; paired t-test), an effect very similar to that caused by 4-aminopyridine (P=0.17; paired t-test) figure 3.4b, grey trace).

A similar response occurred in Ca^{2+} imaging experiments., upon exposure to 25mM NH_4Cl we observed a sustained increase in fluorescence in ~70% of cells (mean response after 5mins exposure 19.5 ±2.6%, 3 mins after exposure; 3 experiments, 168 cells; figure 3.4c) apparently

reflecting CatSper channel activation. However, pretreatment with NH_4Cl did not occlude a subsequent response to 2mM 4-aminopyridine in ~50% of cells (41% of cells responded with 20% increase in $[Ca^{2+}]_i$ fluorescence and 9% responded with a transient type response amplitude 32%) (figure 3.4c).

Previous studies have shown that NH_4Cl induced cytoplasmic alkalinisation enhanced Ca^{2+} influx and hyperactivation in bovine sperm (Marquez and Suarez, 2007). Surprisingly, the effect of 25mM NH_4Cl (at pH_o7.3 or 8.5, figure 3.4d, table 3.1) treatment on hyperactivation was negligible, the proportion of hyperactivated cells increasing from 3.9±0.7 to just 6.4±1.3% (at pH_o 7.3) and from 2.4±0.8 to 7.8±1.9% (pH_o 8.5) (P>0.05, n=7; paired t-test, figure 3.4d table 3.1). In parallel incubations, 4-aminopyridine increased the proportion of hyperactivated cells from 3.9±0.7% to 15.6±3.9% (pH_o 7.3) and 2.4±0.8% to 35.6±1.6% (pH_o 8.5) (paired t-test, P<0.005 compared to NH_4Cl; figure 3.4d, table 3.1). This discrepancy may reflect different criteria for defining hyperactivation. Throughout this report, hyperactivation was defined by the following criteria (Mortimer and Swan, 1995; Mortimer, 2000) - curvilinear velocity ≥150 μm/s and linearity <50% and lateral head displacement ≥7 μm (analyzed at 60Hz). Examination of overall mean VCL, ALH and LIN (table 3.1) motion parameters demonstrate that addition of NH_4Cl increased ALH and VCL and had a slight effect of %LIN but not to the same extent as parallel 4-aminopyridine treated cells. The application of 4-aminopyridine to NH_4Cl pre-treated cells (at pH_o 8.5) significantly increased the proportion of hyperactivated cells (NH_4Cl only 7.8±1.9%, turquoise bar to 24.7±7.2% NH_4Cl and 4-aminopyridine, stripped bar, figure 3.4d) (paired t-test, P<0.05). Pre-treatment with NH_4Cl reduced the hyperactivation rates compared to cells treated with 4-aminopyridine only at pH_o8.5 (figure 3.4d, table 3.1- NH_4Cl and 4-aminopyridine 24.7±7.2% (stripped bar), 4-aminopyridine only 35.6±1.6% (grey bar), paired t-test, P>0.05), apparently due to lowered overall motility rates (table 3.1, TAPS buffered saline % motile = 90.6 ± 2.7%, NH_4Cl and 4-

aminopyridine in TAPS buffered saline % motile= 78.2 ± 4.3%, paired *t*-test, $P<0.05$, 7 paired experiments), with some cells observed to arrest with angulated flagellum and became motionless (Gibbons and Gibbons, 1980; Sale, 1986).

Figure 3.4

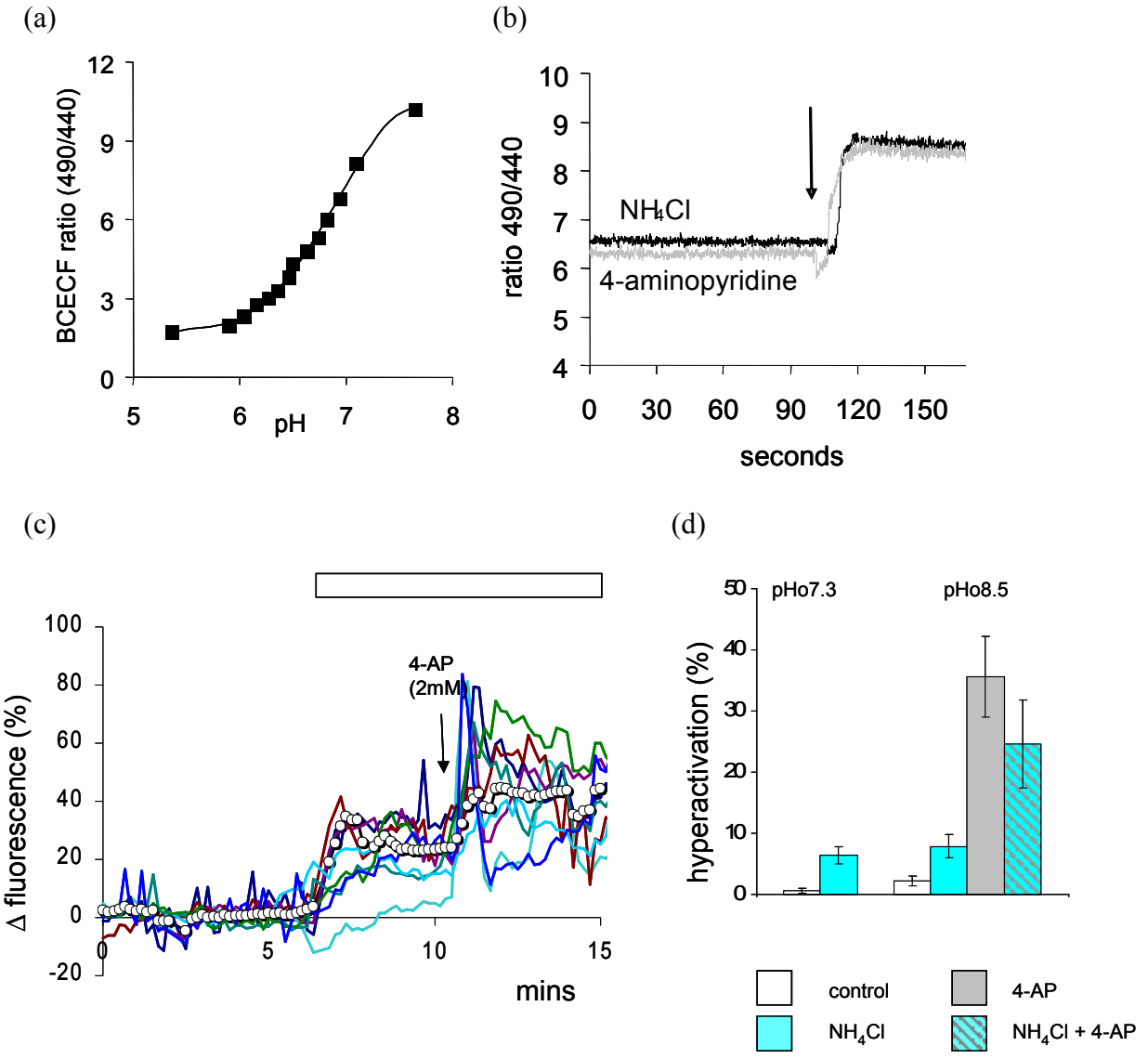

Table 3.1. Effect of NH_4Cl and/or 4-aminopyridine on human sperm motility parameters at pH_o 7.3 or 8.5 (mean ± s.e.m., n=7 experiments).

	Motile(%)	VCL (µm/sec)	ALH (µm)	LIN (%)	% hyperactivation
Control (pH 7.3)	81.1±3.5	106.6±5.0	4.3±0.2	58.0±1.0	3.9±0.7
+ NH_4Cl (25mM, pH 7.3)	75.5±4.2	119.5±5.1	5.1±0.2	57.5±0.9	6.4±1.3
+ 4AP (2mM, pH 7.3)	83.6±4.0	121.1±9.4	5.3±0.3	49.6±1.3	15.6±3.9
Control (pH 8.5)	90.6±2.7	117.4±7.3	4.1±0.3	65.1±2.3	2.4±0.8
+ NH_4Cl (25mM, pH 8.5)	86.3±3.6	137.0±8.1	5.2±0.3	69.0±2.2	7.8±1.9
+ 4-AP (2mM, pH 8.5)	81.4±3.7	190.8±10.2	7.5±0.5	64.7±1.2	35.6±1.6
+ NH_4Cl (25mM) + 4-AP(2mM) (pH 8.5)	78.2±4.3	168.5±10.1	6.7±0.3	67.0±1.7	24.7±7.4

Figure 3.4. The effects of increased intracellular pH on hyperactivation and $[Ca^{2+}]_i$ response in cells exposed to 4-aminopyridine and NH_4Cl **(a)** an example of calibration plot for BECEF-loaded human sperm. BCECF-loaded sperm suspended in standard sEBSS (pH_o7.35) were permeabilized with Triton X-100 and the medium was than acidified by serial additions of HCL. After each addition the fluorescence ratio (490/440) was recorded and pH measured with a standard electrode to produce the calibration curve. **(b)** BCECF ratio traces obtained from suspensions of human sperm (from the same sample) exposed to 2mM 4-aminopyridine (grey trace) and 25mM NH_4Cl (black trace). Additions are marked by the arrow. **(c)** Elevation of $[Ca^{2+}]_i$ fluorescence in response to application of 25mM NH_4Cl (white bar). Responses of 7 representative cells are shown and also mean response (o-o) of all the 65 cells in the experiment. Application of 2mM 4-aminopyridine (arrow) after exposure to NH_4Cl induced a further elevation of $[Ca^{2+}]_i$ in 50% of cells (see text). **(d)** The proportion of cells showing hyperactivated motility in TAPS-buffered medium (pH8.5) under control conditions and 2-3 min after addition of 2mM 4-aminopyridine (grey bar) or 25mM NH_4Cl (turquoise bar) or both compounds (stripped bar). Each bar shows mean ± s.e.m. of 7 experiments. **Table 3.1** CASA motility parameters (% motility, VCL, ALH, LIN and % hyperactivation) for cell treated with NH_4Cl and/or 4-aminopyridine at pH_o 7.3 and 8.5 (n=7 exps).

3.4.5 Effect of propionate pre-treatment on 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response

Previous studies have demonstrated that the ionised form of 4-aminopyridine is most effective intra-cellularly as a K^+ channel blocker (Howe and Richie, 1991; Stephens *et al.,* 1994). Sperm cells were treated with propionate (15mM for 5 mins) to induce intracellular acidification (Cross and Razy-Faulkner, 1997; Sandoval *et al.,* 2007) and enhance ionisation of intracellular 4-aminopyridine. Following the initial control period in standard saline (pH_o7.3 ~6mins), pH_o was maintained at 8.5 for the remainder of the experiment (figure 3.5a grey bar). As mentioned previously raising pH_o, caused a detectable increase in $[Ca^{2+}]_i$ in ~80% of cells, fluorescence of OGB stabilising ~15-20% above control levels. Subsequent addition of propionate (to reduce pH_i) caused a slight increase in $[Ca^{2+}]_i$ calcium (figure 3.5a, blue bar), although in many cells a response was barely discernible. Addition of 2mM 4-aminopyridine (figure 3.5a, black arrow) enhanced $[Ca^{2+}]_i$ in ~44% of cells (of these cells 31% responded with a sustained increase of 43.5 ± 3.6% and 13% responded with a transient response of 82.7± 11.0 % (n= 4 experiments 220 cells figure 3.5b). In paired, non-propionate treated experiments, 82% of cells responded to 4-aminopyridine with an increase of 23.3±0.7% (n=4 experiments, 315 cells, figure 3.5b). The frequency distribution of % increase in $[Ca^{2+}]_i$ amplitude illustrated that although the proportion of propionate pre-treated cells (44%) that responded to 4-aminopyridine was lower in comparison to non-propionate treated control cells (82%), the amplitude of the response was significantly higher (paired *t*-test, $P<0.05$, figure 3.5b).

Finally, pre-treatment with propionate enhanced 4-aminopyridine induced hyperactivation rates (at pH_o8.5) although the effect was not significant (figure 3.5c) (41.4 ± 4.9% (stripped bar, figure 3.5c) in comparison to 4-aminopyridine only treated cells 33.1 ± 3.6 % (grey bar, figure 3.5c) (n=7 experiments, paired *t*-test, $P>0.05$). Motility did not differ significantly between treatments (4-aminopyridine % motility (pH_o 8.5) = 82.4±3.2% compared with propionate and 4-aminopyridine % motility (pH_o) = 83.0±3.6%, paired *t*-test, $P>0.05$).

Figure 3.5

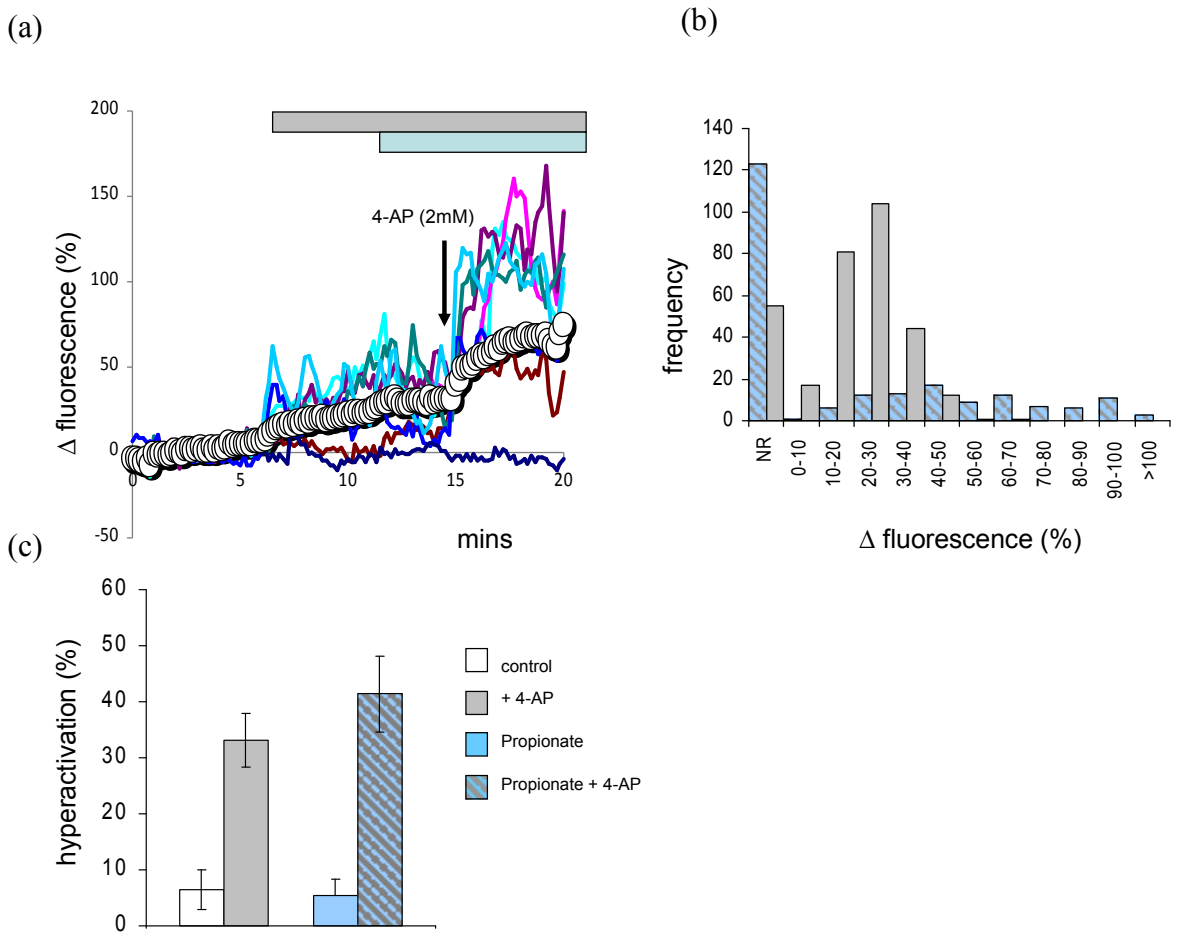

Figure 3.5 The effect of extracellular alkalinisation and intracellular acidification on the 4-aminopyridine induced hyperactivation and $[Ca^{2+}]_i$ fluorescence response in human sperm **(a)** Elevation of $[Ca^{2+}]_i$ (OGB) in response to superfusion with TAPS buffered saline (pH_o8.5, grey bar) followed by addition of 15mM propionate (~ 5mins, blue bar). Application of 4-aminopyridine (black arrow) resulted in a further significant increase in $[Ca^{2+}]_i$ fluorescence in ≈ 40% of cells. Responses of 8 representative cells are shown and also mean response (o-o) of all the 99 cells in the experiment. **(b)** Although a lower proportion of propionate pre-treated cells responded to 4-aminopyridine (44% versus 82%) the amplitude of the response was significantly increased and is reflected by the frequency distribution shift to higher amplitude of response values. **(c)** Pretreatment of cells with propionate enhanced the proportion of hyperactivated cells in response to 4-aminopyridine at pH_o8.5 (n=7 paired *t*-test, $P>0.05$)

3.5 Discussion

Intracellular pH is an important regulator of sperm function reaction (Acott and Carr, 1984; Parrish *et al.,* 1989; Ho *et al.,* 2002, Marquez and Suarez, 2007). During capacitation intracellular pH gradually increases and is associated with flagellar activation, acquisition of progressive motility and transition to hyperactivated motility.

Modulation of pH_o effected 4-aminopyridine induced hyperactivation and $[Ca^{2+}]_i$ response in human sperm

Membrane permeability of the weak organic base 4-aminopyridine (pKa 9.2, Albert *et al*., 1948; Babiak and Testa, 1976) was strongly dependent upon pH_o, consistent with previous reports that the action of the drug depends upon the proportion of the compound that is in the non-ionised (membrane permanent) form (Howe and Richie, 1991; Stephens *et al*., 1994). Therefore, application of 4-aminopyridine in alkaline saline resulted in an increased proportion of the neutral form of the compound and induced a dose-dependent increase in the proportion of hyperactivated cells (figure 3.2a). 4-aminopyridine induced hyperactivation was independent of capacitation; the response was similar in non-capacitated and capacitated cells. These results agree with Marquez and Suarez (2004) who demonstrated that hyperactivation in bovine cells can occur independently of capacitation.

Sperm, like all mammalian cells, must regulate their volume and treatment with K^+ channel blockers can cause sperm cells to swell significantly effecting motility (Kulkarni *et al*., 1997; Yeung and Cooper, 2001; Yeung *et al*., 2004, 2006; Cooper *et al.*, 2005). Human spermatozoa exposed continuously to 4-aminopyridine (2mM) did not show significantly altered % motility over 45 minutes (mean motility t=0 min, 83.3%, and t=45 min 81%, n=4 experiments, paired *t*-test, $P > 0.05$). These results are in agreement with Barfield *et al*., (2005) who found that human spermatozoa exposed to 4-aminopyridine (1mM) for 30 minutes did not significantly

effect cell volume regulation whereas exposure to higher concentrations of 4-aminopyridine (4mM) decreased motility and increased cell volume.

Increased pH_o (8.5) enhanced $[Ca^{2+}]_i$ in the majority of cells apparently reflecting activation of the pH sensitive CatSper channels. Additionally alkaline pH_o affects Ca^{2+} clearance, the plasma membrane Ca^{2+} ATPase (PMCA) plays a significant role in maintaining basal $[Ca^{2+}]_i$ in human sperm and activity is significantly reduced in alkaline pH_o but unaffected by cytoplasmic alkalinisation (Xu *et al.,* 2000; Wennemuth *et al*., 2003b). The sperm sodium calcium exchanger (NCX) also plays a role in Ca^{2+} clearance but activity is not significantly affected by high pH_o (Linck *et al.,* 1998). Application of 4-aminopyridine in TAPs buffered saline increased the membrane permeable neutral form of the compound, resulting in enhanced $[Ca^{2+}]_i$ fluorescence in the majority of cells. The time-course, dose-sensitivity and dependence upon pH_o of this effect resembled those for induction of hyperactivation (figure 3.3d). Furthermore since 4-aminopyridine is a weak base (pKa9.2) addition to non-Hepes buffered saline induced an immediate reversible $[Ca^{2+}]_i$ fluorescence response, cells therefore did not require pre-treatment with alkaline saline to enhance sensitivity to the compound.

Cross (2007) report that BCECF loaded human sperm cells are pH compliant; low pH_o reflected by lowered pH_i. The sperm specific CatSper channels contribute to resting $[Ca^{2+}]_i$ levels (Xia and Ren, 2009a) and are effectively inhibited at low pH_i (Carlson *et al.,* 2009). The reduction of $[Ca^{2+}]_i$ fluorescence in cells superfused with saline maintained at 6.5 may reflect CatSper channel inactivation. Furthermore application of 4-aminopyridine to saline maintained at pH6.5 would result in the majority of the compound been charged and therefore membrane impermeable. However, a proportion of cells responded to 4-aminopyridine with a transient increase in $[Ca^{2+}]_i$ fluorescence apparently reflecting store mobilisation.

Modulation of pHi effected 4-aminopyridine induced hyperactivation and $[Ca^{2+}]_i$ response in human sperm

Studies on capacitated human sperm have shown that NH_4Cl induced cytoplasmic alkalinisation caused a dose-dependent increase in $[Ca^{2+}]_i$ and enhanced depolarisation-induced Ca^{2+} influx (Fraire-Zamora and Gonzalez-Martinez, 2004; Neri-Vidaurri Pdel *et al*., 2006; Publicover *et al.*, 2008). 4-aminopyridine can act as a weak organic base (pKa9.2) and it has been demonstrated recently that cytoplasmic alkalinisation (with 4-aminopyridine or NH_4Cl) greatly enhances activity of the pH-sensitive sperm K^+ current (I_{KSper}) in the principal piece of mouse sperm by cytoplasmic alkalinisation (Navarro *et al,* 2007). Since the sperm-specific, pH-sensitive Ca^{2+}-permeable CatSper channels are similarly localised (Ren *et al,* 2001; Kirichok *et al,* 2006), it is possible that 4-aminopyridine induces Ca^{2+}-influx in the sperm tail (and sustained hyperactivation) may reflect (at least in part) activation of $I_{CatSper}$.

Assessment using BCECF confirmed that 2mM 4-aminopyridine caused an immediate increase in pH_i of ≈0.2 units in human sperm. Exposure of sperm to 25mM NH_4Cl had an almost identical effect on pH_i and also caused a sustained increase in $[Ca^{2+}]_i$ only slightly less than that seen with 2mM 4-aminopyridine. Surprisingly, there was negligible induction of hyperactivation compared to cells treated with 4-aminopyridine (figure 3.4d). In contrast, Marquez and Suarez (2007) showed that Ca^{2+}-influx induced by elevation of pH_i (with 25mM NH_4Cl) was sufficient to induce hyperactivation in bovine sperm. Marquez and Suarez reported population mean values for the various hyperactivation motility parameters (enhanced VCL and ALH (by ~40%) and decreased % LIN (~50%)). In comparison NH_4Cl induced effects on human sperm motility are muted with enhanced VCL and ALH (by 12% and 19% respectively) and a very slight effect on % LIN. Furthermore, hyperactivation was rescued in NH_4Cl pre-treated cells with the addition of 4-aminopyridine (stripped bar, figure 3.4d, table 3.1). Similarly, cytoplasmic alkalinisation (with NH_4Cl) induced $[Ca^{2+}]_i$ fluorescence increase

did not occlude the 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response in many cells (figure 3.4c). The negligible effect of NH_4Cl on human sperm motility may reflect species variation in the mechanisms that control hyperactivation (and sensitivity to hyperactivating stimuli) due to differences in the behavioural requirements for sperm activity to achieve fertilisation in different species (discussed in Chapter 7). These results demonstrate that 4-aminopyridine induced response in human sperm is not solely dependent on cytoplasmic alkalinisation and CatSper activation.

Finally cytoplasmic acidification significantly enhanced the $[Ca^{2+}]_i$ response in a proportion of cells (44%) exposed to 4-aminopyridine and of these (~25%, the response was altered from a sustained to a transient response indicating Ca^{2+} store mobilisation. In agreement with other studies these results confirm that the charged form of 4-aminopyridine has a more potent intracellular effect. Furthermore intracellular acidification would reverse the effects of the sodium hydrogen exchanger (NHE) and proton pump (Hv1) (Sandoval *et al*., 2007) to effectively inhibit $I_{CatSper}$ (Kirichok *et al*., 2006; Carlson *et al.,* 2009) providing further evidence that 4-aminopyrine induced $[Ca^{2+}]_i$ response is pH/CatSper independent. Although intracellular acidification did not significantly enhance the proportion of 4-aminopyridine induced hyperactivation (nor effect % motility) these results suggest the presence of a population of cells that respond to 4-aminopyridine induced hyperactivation independent of CatSper channel activation.

Summary

These results indicate that the 4-aminopyridine induced Ca^{2+}-influx may not be solely pH/CatSper dependent. CatSper channel activation (NH_4Cl induced cytoplasmic alkalinisation) and inhibition (propionate induced cytoplasmic acidification) did not occlude the 4-aminopyridine induced hyperactivation or $[Ca^{2+}]_i$ fluorescence response in human sperm.

We conclude that hyperactivation of human sperm that occurs upon exposure to 4-aminopyridine may, in a number of ways, parallel the induction of hyperactivation in bovine sperm induced by manoeuvres designed to mobilise stored Ca^{2+} (Ho and Suarez, 2001b; 2003).

CHAPTER FOUR

4-AMINOPYRIDINE INDUCED HYPERACTIVATION IS NOT SOLELY CATSPER DEPENDENT

4.1 Abstract

Transition from activated to hyperactivated motility requires an increase in cytoplasmic Ca^{2+}, to amplify flagellar bend amplitude and asymmetry of flagellar beat. Increased calcium entry via the sperm specific pH-sensitive flagellar CatSper channels has been shown to be an important contributor to hyperactivation in mouse spermatozoa (Ren *et al*., 2001; Quill *et al*., 2001). The effects of calcium channel inhibition on 4-aminopyridine induced hyperactivation was investigated. The divalent metal ions (300µM Ni^{2+} and 200µM Cd^{2+}) and ruthenium red (10µM) have been shown to reversibly inhibit CatSper channel activity in mice sperm (Kirichok *et al*., 2006). Inhibition of CatSper channels did not occlude the 4-aminopyridine induced hyperactivation in a population of human spermatozoa. The application of 4-aminopyridine to cells briefly pre-treated with Ni^{2+} triggered a transient increase in $[Ca^{2+}]_i$ fluorescence indicative of stored Ca^{2+} mobilisation in a small proportion of cells. These results provide further evidence that 4-aminopyridine induced hyperactivation is not solely dependent on CatSper dependent calcium influx. The presence of a population of 4-aminopyridine responsive cells suggest that store mobilisation and channels resistant to Ni^{2+} and Cd^{2+} play a significant role in human sperm hyperactivation.

4.2 Introduction

Sperm motility is essential for natural conception, with successful cells altering motility pattern quickly and appropriately, according to extracellular cues as they move through the female reproductive system (chemotaxis, thermotaxis, activated and hyperactivated motility). Intracellular Ca^{2+} regulates activated and hyperactivated motility (Ho and Suarez, 2001a; Carlson *et al*., 2005; Suarez, 2008a), capacitation (Visconti, 2009) and the acrosome reaction (Kirkman-Brown *et al.,* 2003). Spermatozoa generate Ca^{2+} signals to selectively regulate specific activities in response to extracellular cues. Increased intracellular Ca^{2+} concentration is the central regulator of hyperactivation, required both for initiation and maintenance of hyperactivation, probably by strongly enhancing flagellar bending. (Lindemann and Goltz 1988; Ho *et al*., 2002, Ishijima *et al*., 2006; Marquez *et al*., 2007). Ca^{2+} signals are produced by the opening of plasma membrane Ca^{2+} channels, mobilisation of intracellular stored Ca^{2+} or both, acting together (Berridge, 2005; Felix, 2005; Jimenez-Gonzalez *et al.,* 2006). Increased calcium entry via plasma membrane channels is known to play a role in hyperactivation, the sperm specific pH-sensitive CatSper channels have been shown to be important for mouse fertility (Ren *et al*., 2001; Quill *et al.,* 2003,Carlson *et al*., 2005; Kirichok *et al.,* 2006; Qi *et al.,* 2007; Xia *et al.,* 2007). Human spermatozoa can be described as "a stripped down but refined machine" (Barratt *et al*., 2009), but unlike somatic cells lack an identifiable intracellular calcium storage organelle. Evidence suggests the redundant nuclear envelope (RNE) located at the posterior head functions are a calcium store. Crucially, Marquez *et al.,* (2007) demonstrated that store mobilisation was sufficient to initiate hyperactivation in CatSper null mice, we have examined the importance of CatSper channels in human sperm hyperactivation.

The divalent metal ions (cadmium (200μM) and nickel (300μM)) and the dye ruthenium red (10μM) were found to reversibly block $I_{CatSper}$ (Kirichok *et al.,* 2006). The effects of the putative CatSper channel blockers (nickel, cadmium and ruthenium red) on the 4-

aminopyridine induced hyperactivation were investigated. Since 4-aminopyridine has been shown to potentiate $I_{CatSper}$ current in mouse sperm (Kirichok *et al*., 2006) the effects of nickel (300µM) pre-treatment on the to 4-aminopyridine induced increase in $[Ca^{2+}]_i$ fluorescence was examined.

4.3 Materials and Methods

4.3.1 Materials

As described in chapter two, with the following modifications.

To limit precipitation, 300uM nickel chloride ($NiCl_2$) was prepared in a simplified media (4.5mM KCl, 9.0 mM $C_6H_{12}O_6$, 2.0mM $CaCl_2.2H_2O$, 1mM $MgCl_2.6H_2O$, 132.0mM NaCl and 15mM HEPES (pH 7.3, 285-295 mOsm)), supplemented with 0.3% (w/v) fatty acid free BSA. Osmotic strength (285-295mOsmol) was maintained by adjusting NaCl. Standard saline (sEBSS) was maintained at pH7.3 (15mM Hepes buffer).

All chemicals referred to in the text were from Sigma-Aldrich (Poole, Dorset, UK). All chemicals were cell-culture-tested grade where available.

4.3.2 Spermatozoa preparation and capacitation

As in chapter two.

4.3.3 Single cell Imaging

Single cell fluorescence plots in response to Ni^{2+} (300μM) were sorted as described in chapter two. Visual examination of fluorescence-time plots for individual sorted cells confirmed that a proportion of cells responded to 4-aminopyridine. Quantification of 4-aminopyridine induced responses was carried out by direct observation of individual time-fluorescence intensity plots.

4.3.4 Evaluation of Ni^{2+} fluorescence enhancement

As described in chapter two (section 2.3.5). Emission spectrum was recorded in the presence and absence of nickel (0.1-0.5mM final concentration) and the fluorescence enhancement

factor calculated as described in Rai and Kasturi, (1994). The fluorescence enhancement factor Q was defined as

$Q=F/F_0 - 1$

where F and F_0 are the fluorescence intensities in the presence (F) and absence (F_0) of Ni^{2+}. The enhancement factor (1/Q) can be obtained from the intercept of the double reciprocal plot $F_0/(F-F_0)$ vs $1/[Ni^{2+}]$.

OGB emission spectra were recorded for 4-aminopyridine (0.0 to 5.0mM) in the presence of 300µM nickel. Fluorescence quenching was measured quantitatively using the Stern-Volmer equation as described in chapter two.

4.3.5 Motility Analysis.

As in chapter two.

4.3.6 Statistical Analysis

As in chapter two.

4.4 Results

4.4.1 The putative CatSper channel blockers (nickel, cadmium and ruthenium red) reduced 4-aminopyridine induced hyperactivation in human spermatozoa

As described previously 4-aminopyridine (2mM) induced an immediate transition to hyperactivated motility in a proportion of human sperm. In capacitated cells (3-4 h) the proportion of hyperactivated sperm (as assessed by CASA) increased from 3.5± 1.4% to 36.0 ± 4.3% (figure 4.1a, n= 6 experiments, paired *t*-test, $P<0.001$).

Brief treatment (~2 mins, minimum loading time) of cells with nickel (300µM), cadmium (200µM) and ruthenium red (10µM) did not significantly effect the proportion of hyperactivated cells (figure 4.1) or affect % motility or hyperactivation motility parameters (VCL, ALH and LIN - table 4.1).

The proportion of 4-aminopyridine induced hyperactivated cells was diminished in the presence of the heavy metal cations (figure 4.1). Pre-treatment with Ni^{2+} and Cd^{2+} (~2 mins) reduced 4-aminopyridine induced hyperactivation rate by 54.9% and 64.4% respectively. However, a proportion of cells remained responsive to 4-aminopyridine induced hyperactivation in the presence of Ni^{2+} (45.1%) and Cd^{2+} (35.6%). Pre-treatment of Ni^{2+} significantly effected the 4-aminopyridine induced VCL response (decreased by 23%, from 171.2±13.0 µm/sec to 131.2±16.8 µm/sec, paired *t*-test, $P<0.05$) but had no significant effect on ALH (decreased by 21% from 6.8±0.5µm to 5.4±0.6µm, paired *t*-test, $P>0.05$) and % LIN (increased by 7% from 41.3±1.7% to 44.0±6.5%, paired *t*-test, $P>0.05$) (table 4.1, n=6 experiments). Pre-treatment with Cd^{2+} did not significantly effect 4-aminopyridine induced VCL response (reduced by 15% from 171.2±13.0 µm/sec to 143.0±15.1 µm/sec), ALH (reduced by 10% from 6.8±0.5µm to 6.1±0.5µm,) and % LIN (increased by 5% from 41.3±1.7% to 44.9±3.6%) (n=6 experiments; paired *t*-test, $P>0.05$). Finally, pre-treatment of cells with ruthenium red decreased the proportion of hyperactivated cells in respond to 4-

aminopyridine by ~30% from 32.5±4.0% to 22.2±6.8% and did not significantly effect other motility parameters (n=6 experiments; paired *t*-test, $P>0.05$).

Figure 4.1

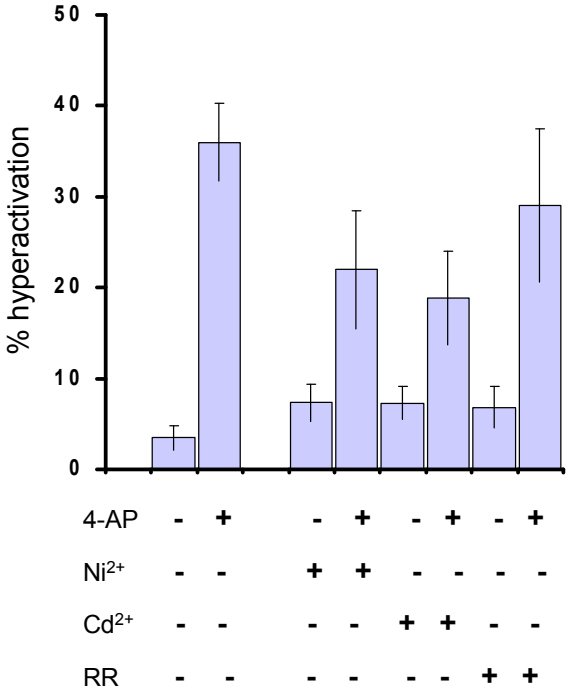

Figure 4.1 4-aminopyridine (2mM) induced hyperactivation on capacitated human spermatozoa (assessed by CASA) in the presence of Ni^{2+} (300µM), Cd^{2+} (200µM) and Ruthenium Red (10µM). **(a)** 4-AP causes an increase in the % of hyperactivated sperm from control conditions. Pre-incubation of cells with Ni^{2+} (300µM) or Cd^{2+} (200µM) significantly decreased the 4-AP induced hyperactivation by 54.9-64.4% (paired *t*-test, $P<0.05$). Pre-incubation with RR (10µM) decreased the 4-AP induced hyperactivation by 31.8% (NS). Each bar shows mean ± SEM. of 6 experiments

Table 4.1. Effect of Ni^{2+} (300µM), Cd^{2+} (200µM) and RR (10µM) ± 4-aminopyridine (2mM) on sperm motility, VCL, ALH and LIN (mean±SEM; n=6donors)

	Motile(%)	VCL (µm/sec)	ALH (µm)	LIN (%)
Control	79.0±4.0	126.8±8.3	4.8±0.4	62.7±2.2
+ 4AP	78.0 ±7.1	171.2±13.0	6.8±0.5	41.3±1.7
+ Ni^{2+}	79.2±2.8	122.6±8.2	4.9±0.3	58.7±1.9
+ Ni^{2+} and 4AP	80.2±2.9	131.2±16.8	5.4±0.6	44.0±6.5
+ Cd^{2+}	78.9±3.7	118.2±8.2	5.0±0.2	55.4±1.6
+ Cd^{2+} and 4AP	73.3±5.2	143.0±15.1	6.1±0.5	44.9±3.6
+ RR	73.8±4.1	121.2±8.0	5.1±0.2	56.0±2.4
+ RR and 4AP	75.0±4.6	159.8±20.2	6.9±0.7	42.1±2.5

Table 4.1 Effects of Ni^{2+}(300µM), Cd^{2+} (200µM) and RR (10µM) ± 4-AP on human sperm motility, VCL, ALH and LIN. Sperm were treated with saline only (Cntrl), Ni^{2+}(300µM), Cd^{2+} (200µM) and RR (10µM) ± 4-AP (mean ± SEM, n=6 donors).

4.4.2 Effect of Ni^{2+} and 4-aminopyridine on OGB fluorescence emission

To investigate the effect of Ni^{2+} on OGB fluorescence emission, de-esterified OGB was prepared as described in previously (section 2.3.5) ($[Ca^{2+}]_o$ = 3.6 X10^{-4}M, 25^{o}C, pH 7.3) and steady state fluorescence emission (λ_{em}=522nm) was recorded in the presence and absence of Ni^{2+} (0.1-0.5mM at 25^{o}C) (figure 4.2 a). Addition of Ni^{2+} enhanced the emission signal and enhancement factor of 0.6 (1/Q) was obtained from the intercept of the double reciprocal Stern-Volmer plot (figure 4.2 b).

Steady state emission spectra were also recorded for OGB containing 300μM Ni^{2+} with additions of 4-aminopyridine (0.5-5mM) (figure 4.2 c). Addition of 4-aminopyridine (0.5-5mM) (at 25^{o}C, pH 7.3, $[Ca^{2+}]_o$ = 3.6 X10^{-4}M) had a slight quenching effect on fluorescence emission (Stern-Volmer constant= 0.0012). 4-aminopyridine in the presence of Ni^{2+} (300μM) did not significantly effect OGB emission (figure 4.2c).

Figure 4.2

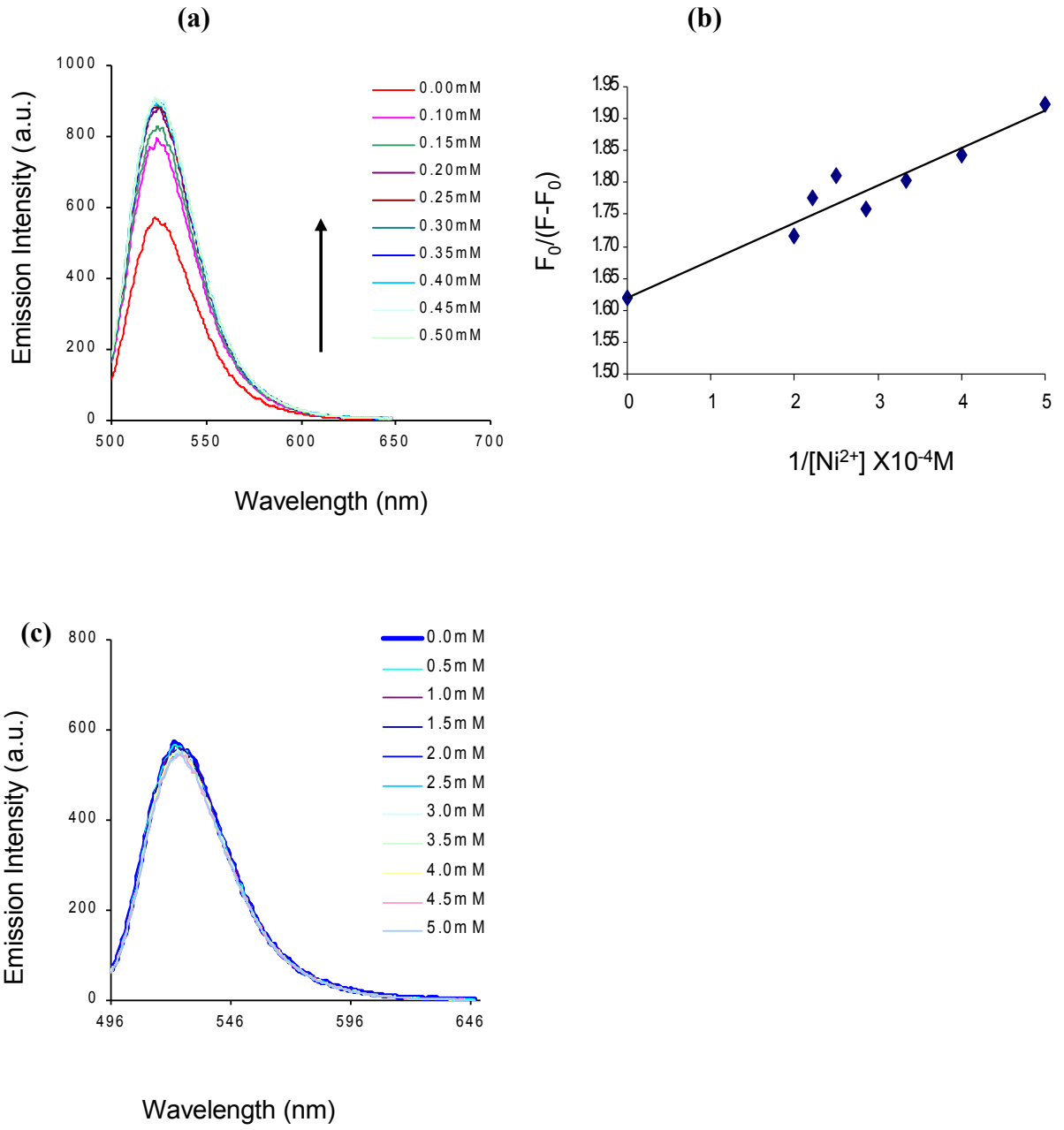

Figure 4.2: The effect of Ni^{2+} (± 4-aminopyridine) on Oregon Green Bapta1 (OGB) emission (irradiation λ_{ex}= 488nm, emission at λ_{em}=522nm) (a) Emission spectra of OGB (red trace) was enhanced with the addition of Ni^{2+} (0.10-0.50mM). (b) The enhancement factor Q for Ni^{2+} was obtained from the intercept of the linear double reciprocal plot ($F_0/(F-F_0)$ vs $1/[Ni^{2+}]$) (as described in Rai and Kasturi, 1994) (c) Emmision spectra for de-esterified OGB and Ni^{2+} (300µM) in the absence (blue trace) and presence of 4-aminopyridine in saline ($[Ca^{2+}]_o$ 3.6 X 10^{-4}M).

4.4.3. Effect of nickel on 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response

Nickel is a non-specific inhibitor of numerous Ca^{2+} channels including CatSper channels (300µM) (Kirichok *et al.,* 2006). The application of Ni^{2+} in standard media resulted in immediate transient fluorescent signal of ~35% in the majority of cells, indicating uptake and binding of Ni^{2+} to a fraction of the fluorescent indicator (figure 4.3a). However, pre-treatment with Ni^{2+} did not necessarily occlude a subsequent transient $[Ca^{2+}]_i$ fluorescence response to 4-aminopyridine in a proportion of cells. Visual examination of individual single cell traces pre-treated with Ni^{2+} followed by 4-aminopyridine showed that ~20% of cells responded with a transient increase in $[Ca^{2+}]_i$ fluorescence of 25.9±2.1% (n=4 experiments, 291 cells; figure 4.3b). The kinetics of this response was significantly different from the 4-aminopyridine induced sustained response in paired control experiments (in the absence of Ni^{2+}, 70% of cells respond with a tonic increase of 17.8±2.2% (pH 7.3, n=5 experiments, 401 cells, paired *t*-test, $P<0.05$).

In order to minimize Ni^{2+} precipitation a further set of experiments were carried out with simplified saline lacking bicarbonate and phosphates. Cells were initially superfused with standard saline to remove excess dye and unattached cells before switching to simplified saline. However, transition to simplified saline resulted in an increase in $[Ca^{2+}]_i$ of ~ 20% in the majority of cells. Once a control period had been established in simple saline the cells were briefly treated with Ni^{2+} (300µM). As before, the majority of cells responded to Ni^{2+} with a increase in fluorescent signal indicating Ni^{2+} uptake and interaction with the dye (figure 4.3c). Although, the response to 4-aminopyridine was not discernible from the mean population response, visual examination of individual traces demonstrated that in a proportion of cells (12.0%) responded with a transient increase in $[Ca^{2+}]_i$ fluorescence (20.5±1.7% (n=3 experiments, 259 cells, figure 4.3 c). The response to 4-aminopyridine only in simplified saline was lower in comparison to standard saline, (30.7% of cells responded with a sustained response of 6.6±1.6% (pH 7.3, n=3 experiments, 127 cells; figure 4.3d).

Figure 4.3

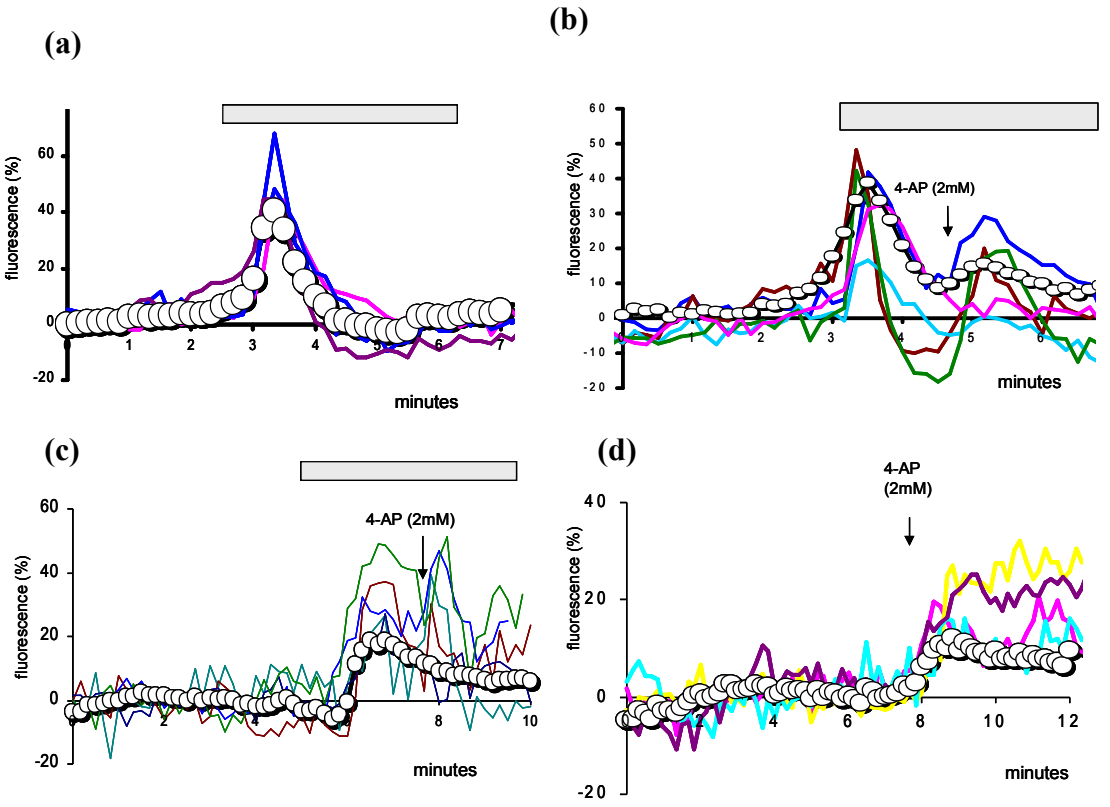

Figure 4.3 Pre-treatment of human spermatozoa with Ni^{2+} (300µM; grey bar) in standard or simplified saline altered the kinetics of the 4-aminopyridine elevation of $[Ca^{2+}]_i$ in a proportion of OGB loaded human sperm. **(a)** Superfusion with Ni^{2+}(300µM) only in standard saline caused an immediate transient fluorescent artifact in the majority of cells Responses of 5 representative cells are shown and also mean response (o-o) of all the 87 cells in the experiment. **(b)** The application of 4-aminopyridine (2mM) in the continued presence of Ni^{2+} caused a transient rise in $[Ca^{2+}]_i$ in ~20% of cells. Coloured lines show responses of 8 representative cells. Open circles (o-o) show mean response of 4 experiments (291 cells). **(c)** The application of 4-aminopyridine (2mM) in the continued presence of Ni^{2+} caused a transient rise in $[Ca^{2+}]_i$ in ~12% of cells (in simplified saline). Coloured lines show responses of 6 representative cells. Open circles (o-o) show mean response of 259 cells in 3 experiments. **(d)** Control experiment where the application of 4-aminopyridine (2mM) caused a sustained response in simplified saline. Coloured lines show responses of 4 representative cells. Open circles (o-o) show mean response of 97 cells in the experiment.

4.5 Discussion

There is little doubt that increased intracellular calcium is essential in sperm, enabling transition from activated to hyperactivated motility in mammalian sperm. $[Ca^{2+}]_i$ is increased as Ca^{2+} enters the cytoplasm through plasma membrane calcium channels or as Ca^{2+} is released from intracellular stores (Marquez *et al.*, 2007; Navarro *et al.,* 2008). Plasma membrane Ca^{2+} channels identified in mammalian sperm include voltage-gated Ca^{2+} channels (section 1.6.1), cyclic nucleotide gated Ca^{2+} channels (1.6.2), canonical transient receptor potential channels (section 1.5.1) and the sperm specific pH sensitive CatSper channels (section 1.5.1) (Wennemuth *et al.,* 2000; Ren *et al.,* 2001; Castellano *et al.,* 2003; Quill *et al.,* 2003; Trevino *et al.,* 2001, 2004; Kirichok *et al.,* 2006; Xia *et al.,* 2007). The CatSper channels have been shown to be important for hyperactivated sperm motility, CatSper proteins (1-4) mutations result in a loss of function phenotype and sterility in mice (Carlson *et al.,* 2005; Marquez *et al.,* 2007; Qi *et al.,* 2007). Indeed many recent papers (examining mouse sperm) have implicated CatSper dependent Ca^{2+} influx with a BSA induced Ca^{2+} influx (Xia and Ren, 2009a), maintenance of basal Ca^{2+} levels for activated motility (Marquez *et al.,* 2007: Qi *et al.,* 2007), ATP homeostasis (Xia *et al*., 2007) and ZP3 induced acrosome reaction (Xia and Ren, 2009b).

Treatment with Ni^{2+}, Cd^{2+} or ruthenium red did not occlude 4-aminopyridine induced hyperactivation in human sperm

The divalent metal ions cadmium (Cd^{2+},200µM) and nickel (Ni^{2+},300µM) and the ruthenium red dye (10µM) were found to reversibly block $I_{CatSper}$ in mouse sperm (Kirichok *et al.,* 2006). Although pre-treatment with Ni^{2+} and Cd^{2+} decreased 4-aminopyridine induced hyperactivation rates in human spermatozoa approximately ~35-40% of cells were uneffected. Pre-treatment with ruthenium red did not significantly affect 4-aminopyridine induced hyperactivation response or motility parameters. These results demonstrate that 4-aminopyridine induced

hyperactivation is not solely CatSper dependent and store-mobilisation and Ca^{2+} influx through channels resistant to Ni^{2+}, Cd^{2+} and ruthenium red may play a significant role in human sperm hyperactivation. These results are in agreement with Marquez *et al*., (2007) who reported that in the presence of 1mM Ni^{2+} approximately 30% of mice sperm hyperactivate in response to thimerosol induced store mobilisation (Marqeuz *et al.,* 2007).

Intracellular calcium response to 4-aminopyridine in OGB loaded human spermatozoa pre-treated with Nickel or Cadmium

Divalent metal ions have relatively high affinities for ion channel binding sites and typically inhibit Ca^{2+} influx by competing for the channel site. Since metal ions can move through the channel slowly, endogenous metal cations may interact with fluorescence dye to quench or enhance fluorescence signal (Snitsarev *et al*., 1996). Superfusion of spermatozoa with Ni^{2+} in standard saline or simplified saline resulted in a fluorescence artifact although it is unclear how Ni^{2+} entered the cell. Addition of Ni^{2+} (100µM) was found to enhance de-esterified OGB fluorescence emission whereas 4-aminopyridine had a negligible quenching effect on OGB emission signal in the presence of Ni^{2+} (300µM).

Despite the presence of a nickel induced fluorescent artifact, a 4-aminopyridine induced transient response was evident in a significant proportion of cells (18% in standard saline and 12% in simplified saline). We have previously demonstrated that both 4-aminopyridine and NH_4Cl have a similar effect on pH_i (figure 3.4b) and cytoplasmic alkalinisation should activate available CatSper channels. Inhibition by Ni^{2+} would not only block CatSper dependent Ca^{2+} influx, but various other channels including voltage-operated channels (VOC) (Fox *et al.,* 1987; Zamponi *et al*., 1996), receptor operated channels (ROC) (Hughes and Barritt, 1989) and capacitative calcium entry (CCE) channels (Hoth and Penner, 1993; Kerschbaum and Cahalan 1998). 4-aminopyridine induced a transient $[Ca^{2+}]_i$ fluorescence response in a population of cells which reflects intracellular Ca^{2+} store mobilisation however the sustained response is lost

apparently due to inhibition of store-operated channels (SOC) or CatSper dependent Ca^{2+} influx. These results indicate that release of Ca^{2+} from an internal store is sufficient to initiate 4-aminopyridine induced hyperactivation. Similar results from the Suarez group demonstrated that hyperactivation could also be induced in CatSper null mice (Marquez *et al*., 2007). This group demonstrated that addition of Ca^{2+} releasing agents (thimerosal and high dose thapsigargin) induced hyperactivation in bovine sperm in the absence of extracellular Ca^{2+} (Ho and Suarez, 2001b). Suarez *et al.,* (1993) demonstrated the sustained increase in $[Ca^{2+}]_i$ in the post-acrosomal head was greater than the proximal/distal midpiece of hyperactivated hamster sperm. Intracellular calcium store mobilisation and consequential sustained CCE in sperm has been observed by a number of groups (Blackmore, 1993; Dragileva *et al*, 1999; Williams and Ford, 2003; Espino *et al,* 2009).

Conclusion

The presence of a population of 4-aminopyridine responsive cells suggest that store mobilisation and channels resistant to Ni^{2+}, Cd^{2+} and ruthenium red play a significant role in human sperm hyperactivation. Furthermore application of 4-aminopyridine induced a transient $[Ca^{2+}]_i$ response in a similar proportion of cells indicating a role for store mobilisation in human sperm hyperactivation.

CHAPTER FIVE

4-AMINOPYRIDINE INDUCED INTRACELLULAR Ca^{2+} STORE MOBILISATION

5.1 Abstract

4-aminopyridine caused a robust and persistent hyperactivation of motility in human sperm. Furthermore Ca^{2+} imaging showed that treatment with 4-aminopyridine induced a parallel elevation of $[Ca^{2+}]_{i,}$ which initiated at the sperm neck/midpiece and was associated with asymmetric flagellar bending in this region. In low Ca^{2+} saline ($\leq$5 μM Ca^{2+}) the ability of 4-aminopyridine to cause an elevation of $[Ca^{2+}]_i$ was similar to that seen in standard saline. Application of 4-aminopyridine to cells briefly (4-5min) exposed to EGTA-buffered saline ($<10^{-7}$ M Ca^{2+}) induced a $[Ca^{2+}]_i$ transient. Hyperactivation induced by 4-aminopyridine was similarly transient (10-15min) in EGTA-buffered saline, though motility persisted for 20-30 min. 4-aminopyridine acted as a non-specific intra-calcium store agonist however it remains unclear how this compound triggers store mobilisation in microsomal vesicles. In conclusion 4-aminopyridine induced hyperactivation is not solely pH or CatSper channel dependent and intracellular Ca^{2+} store mobilisation contributes to the induction of hyperactivation in human sperm.

5.2 Introduction

Despite their small size and apparent structural simplicity, it is evident that sperm are capable of generating and regulating complex, compartmentalised Ca^{2+} signals directly controlling a range of activities including the acrosome reaction, chemotaxis and hyperactivation (Jimenez-Gonzalez *et al*., 2006; Publicover *et al.,* 2007). Various studies from a number of species demonstrated the presence of a Ca^{2+} store in the neck region of mammalian sperm (Kuroda *et al.,* 1999; Ho and Suarez, 2001b, 2003; Naaby-Hansen *et al.,* 2001; Harper *et al.,* 2004; Lefièvre *et al.,* 2007; Marquez *et al.,* 2007). Investigations in rodent and bovine sperm has provided strong evidence that the elevation of $[Ca^{2+}]_i$ that occurs upon mobilisation of this store regulates flagellar activity, including (or at least facilitating) aspects of hyperactivation (Ho *et al*., 2001b; 2003). Importantly hyperactivation can be induced independently of CatSper channels with store mobilisation induced hyperactivation occurring in mouse sperm lacking CatSpers (Marquez *et al.,* 2007). Since capacitative calcium entry (CCE) has been observed in sperm (Blackmore, 1993; Dragileva *et al*, 1999; Williams and Ford, 2003; Espino *et al,* 2009; Costello *et al*., 2009), store mobilisation might even recruit this mechanism to supplement Ca^{2+} influx through CatSper channels (Olson *et al.,* 2010). This second pathway for induction of hyperactivation may play a separate role from that mediated by CatSper channels enabling sperm to switch hyperactivated motility on and off in response to *in vivo* triggers (e.g. nitric oxide, low dose progesterone). Mobilisation of stored Ca^{2+}, which is rapidly reversible and localised, may be better suited to such transient regulation of motility whereas pH regulated activation of CatSpers could support the prolonged hyperactivation that permits 'permanent' detachment from the cells of the female tract (Ho *et al.,* 2009) and penetration of the zona pellucida.

4-aminopyridine disrupts intra-cellular calcium homeostasis in a number of cell types (Ishida and Honda, 1993; Guse *et al*., 1994; Gobet *et al.,* 1995, Grimaldi *et al.,* 2001) including mammalian sperm (Navarro *et al.,* 2007). 4-aminopyridine induced hyperactivation and

$[Ca^{2+}]_i$ fluorescence response in human spermatozoa is not solely dependent on cytoplasmic alkalinisation and concomitant CatSper channel activation. In this report, the effects of 4-aminopyridine on $[Ca^{2+}]_i$ fluorescence and rates of hyperactivation in Ca^{2+} free condition ($[Ca^{2+}]_o$ $<10^{-7}$ M) are examined. Furthermore, the effects of 4-aminopyridine on Ca^{2+} uptake and Ca^{2+} release channels in microsomal vesicles (sacroplasmic reticulum (SR) and brain microsomes) are investigated so that results are independent of cytoplasmic alkalinisation, K^+ channel inhibition and CatSper channel activation.

5.3 Materials and Methods

5.3.1 Materials

Ca^{2+}-free sEBSS (no added Ca^{2+} , ($[Ca^{2+}]_o \leq 5\mu M$)) and EGTA buffered medium (5mM Ca^{2+}, 6mM EGTA) were prepared in the laboratory ($[Ca^{2+}]_o < 10^{-7}$ M). Osmotic strength was maintained by adjusting NaCl. The calcium concentration calculator Maxchelator (V2.2) written by Chris Patton from Stanford University (http://www.stanford.edu/~cpatton/maxc.html) was used to calculate the calcium concentration in saline containing EGTA.

Creatine Kinase was purchased from Boerhinger, Manheim. D-myo-Inositol-1,4,5-triphosphate hexapotassium salt (IP_3 – 2mM stock made up in H_2O) was purchased from Enzo Life Sciences (Exeter). All other materials were purchased from Sigma Aldrich (Poole, Dorset) including 1-[2-Amino-5-(2,7-dichlooro-6-hydroxy-3-oxy-9-xanthenyl)phenoxyl]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid ammonium potassium salt (fluo-3 ammonium salt - 0.5mM stock concentration made up in H_2O), 2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester (Rhodamine123 - 15mM stock concentration dispersed with Pluronic F-127), progesterone [4-pregnene-3,20-dione] (6mM stock concentration prepared in DMSO), thapsigargin (0.1mM stock concentration prepared in DMSO), 2-(dimethylamino)ethyl 4-(butylamino)benzoate (tetracaine hydroxide, 50mM stock in H_2O), phosphocreatine from bovine heart, adenosine 5'-triphosphate disodium salt (ATP – made up with $MgSO_4$ in water, pH 7.0) and calcimycin A32187 (1mM stock in DMSO) . All chemicals were cell culture-tested grade were available.

5.3.2 Spermatozoa preparation and capacitation

As in chapter two.

5.3.3 Single Cell Imaging

Investigation of 4-aminopyridine on Oregon Green Bapta1 loaded cells were as previously described (chapter two). All experiments were performed at 25±0.5°C in a continuous flow of medium (sEBSS, Ca^{2+}-free' sEBSS ($[Ca^{2+}]_o \leq 5$ μM) or EGTA-buffered saline ($[Ca^{2+}]_o < 10^{-7}$ M). For high speed fluorescence imaging (9 Hz) the protocol was as described previously (chapter two) but cells were viewed using a 60x oil immersion objective and images were captures with an EMCCD camera controlled by a PC running iQ software.

To investigate the effect of 4-aminopyridine on mitochondrial membrane potential, 200μl aliquots of capacitated spermatozoa at 3-6 x 10^6 cells/ml were loaded with Rhodamine123 (6.5μM final concentration dispersed with Pluronic F-127) and transferred to an imaging chamber (volume 180 μl) in which the lower surface was a 1% (w/v) poly-$_D$-lysine coated coverslip and allowed to adhere during a 30 min incubation at 37^{o}C and 5.5% CO_2. The chamber was connected to the perfusion apparatus (peristaltic pump and header tank; superfusion rate 0.4ml min^{-1}) and cells were superfused with approximately 10 ml of fresh medium to remove unattached cells and excess dye. All experiments were performed at 25±0.5°C in a continuous flow of medium. Spermatozoa were imaged on a Nikon TE200 inverted fluorescence microscope. Images were obtained at 10s intervals using a 60x oil objective and a Andor iXon EM^+ camera controlled by a PC running iQ software (Andor Technology, Belfast, UK). To minimize photobleaching EM gain was set to 70 (to improve signal to noise ratio), exposure was reduced to 1ms.

5.3.4 Evaluation of sperm hyperactivation

For motility assessment in nominal Ca^{2+} free medium (no added Ca^{2+}), 200μl aliquots of spermatozoa ($\geq$ 6 x 10^{-6} cells.ml^{-1}) were prepared by direct swim and incubated for ~1-1.5 hours. For experiments involving EGTA-buffered medium, cells were centrifuged (300 g for 5 min) and the pellet re-suspended in 1ml EGTA buffered saline immediately prior to the

experiment. 200µl aliquots were treated with either saline (control) or 4-aminopyridine and immediately (or after an appropriate period of incubation for time-course experiments) introduced into a chamber (depth 20µm) (Microcell, Conception Technologies Ltd.) at a concentration of 6-7 million/ml on a Hamilton Thorne HTM IVOS semen analyzer (37^{o}C). Hyperactivation was defined as previously described (chapter two).

5.3.5 Evaluation of Ca^{2+} uptake and release in microsomal vesicles

Evaluation of 4-aminopyridine induced fluo-3 fluorescence quenching

Fluoresence quenching experiments were performed with 250nM fluo-3 (free acid) in 2ml of 40mM Tris, 100mM KCL saline (pH 7.2) in a magnetically stirred methylacrylate cuvette maintained at 25^{o}C or 37^{o}C by a water jacket. Emission spectra were recorded with 4-aminopyridine (0-4mM final concentration) with fixed excitation wavelength held at 506nm, slit width set at 3nM. Emission was scanned at 1000 nm/min between 506 and 650nm. Fluorescence quenching was measured quantitatively using the Stern-Volmer equation as described in chapter two.

Microsomal membrane purification

Sarcoplasmic reticulum microsomes (SR) and porcine brain microsomes were kindly donated by Dr F. Michelangeli (Department of Biosciences, University of Birmingham).

Calcium uptake and release assay

Experiments were carried out using a Perkin-Elmer LS50B fluorescence spectrofluorimeter running FLWinlab version 3.0. Fluorimetry was performed with 2ml of 40mM Tris, 100mM KCL (pH 7.2) in a magnetically stirred methylacrylate cuvette maintained at 37^{o}C by a water jacket. Rabbit sacroplasmic reticulum (SR) (2µg) (Michelangeli and Munkonge, 1991) or porcine brain vesicles (40-50µg) were added in the presence of 250nM Fluo-3 (free acid),

10µg/ml creatine kinase and 10mM phosphocreatine. Ca^{2+} uptake was initiated by the addition of Mg-ATP. To examine the effect of 4-aminopyridine on Ca^{2+}-ATPases, Ca^{2+} uptake was measured by addition of the drug prior to the start of the experiment. Ca^{2+} release was measured by addition of 4-aminopyridine (0.5-4mM) following ATP uptake in the presence of thapsigargin (~1-1.5µM). Fluorescence changes were monitored using excitation and emission wavelengths of 506nm and 526nm, respectively. Calcium concentrations were determined using the following equation:

$$[Ca^{2+}]_i = K_d \text{ X } [(F-F_{min})/(F_{max}-F)]$$

where K_d is the dissociation constant for Ca^{2+} binding to fluo-3 (900nM at 37°C, pH 7.2, in 40mM Tris and 100mM KCL), F is the fluorescence intensity of the sample and F_{min} and F_{max} are the fluorescence intensities of 1.25mM EGTA and 2mM $CaCl_2$, respectively (Michelangeli, 1991; Menza and Michelangeli, 1996). Percentage Ca^{2+} release (%) was determined by comparing the amount of Ca^{2+} released by 4-aminopyridine with that observed with 12.5µg/ml A23187.

5.3.6 Evaluation of acrosome reaction

After capacitation (in sEBSS + 0.3% BSA, incubated at 37°C and 5.5% CO_2. for 5-6 hours), 200 µl aliquots of spermatozoa were then stimulated with 4-aminopyridine (2 mM), progesterone (final concentration of 3.2 µM) or A23187 (10 µM) or solvent control (0.05% DMSO). After an incubation period of 1 hour (37°C) cells were centrifuged (500 g for 5 min). The supernatant was removed and the spermatozoa re-suspended in 0.5 ml of hypo-osmotic swelling (HOS) medium (0.74% sodium citrate, 1.35% fructose in double-distilled H_2O). After 45 min cells were centrifuged (500 g for 5 min), supernatant was removed and pellets were smeared on poly-L-lysine coated slides and air-dried. The cells were permeabilised in methanol

for ≈30 seconds. 15µl of FITC-labelled *Pisum sativum* agglutinin (FITC-PSA) in PBS was spread on each slide and incubated for 45 min in a humid chamber at 37^{o} C. Slides were then washed in a constant flow of tap water for 5 min before air drying and mounting with hydromount. Acrosomal status of viable (curly tailed) spermatozoa was assessed by fluorescence microscopy (Cross *et al.,* 1988). A total of 200 cells were scored for each treatment (100 each from 2 slides).

5.3.7 Statistical Analysis

As in chapter two.

5.4 Results

5.4.1 The response to 4-aminopyridine initiates at the sperm neck.

To localise the point(s) of initiation of the $[Ca^{2+}]_i$ signal in cells exposed to 4-aminopyridine, we collected images for a brief period (2 min) at 9 Hz. In many cells it was impossible to identify the point of origin of the Ca^{2+} signal, intensity rising rapidly in both the anterior flagellum (which often remained in focus despite flagellar beating) and the posterior head/neck (not the anterior head). However, in a minority of cells a focus of Ca^{2+} mobilisation was clearly visible at the sperm neck, the signal spreading rapidly (≈5s) into the posterior head, midpiece and anterior principal piece (fig. 5.1b). In a very small number of cells (<1%) a gradual rise in fluorescence in the neck and posterior sperm head apparently 'triggered' a $[Ca^{2+}]_i$ response in the midpiece (figure 5.1c). A common observation in 4-aminopyridine treated cells was the occurrence of a unilateral bend at the midpiece, which developed as $[Ca^{2+}]$ increased. The example in fig. 5.1b is particularly striking, bending by ≥45^o, but most cells in which this occurred showed a more conservative response with a bend of 10-20^o. The principal piece of the flagellum continued to beat but was displaced laterally. Upon washout of 4-aminopyidine bending of the flagellum 'relaxed' with the fall in $[Ca^{2+}]_{i..}$ Re-application of the drug restored both $[Ca^{2+}]_i$ elevation and bending of the proximal flagellum repeated.

Figure 5.1

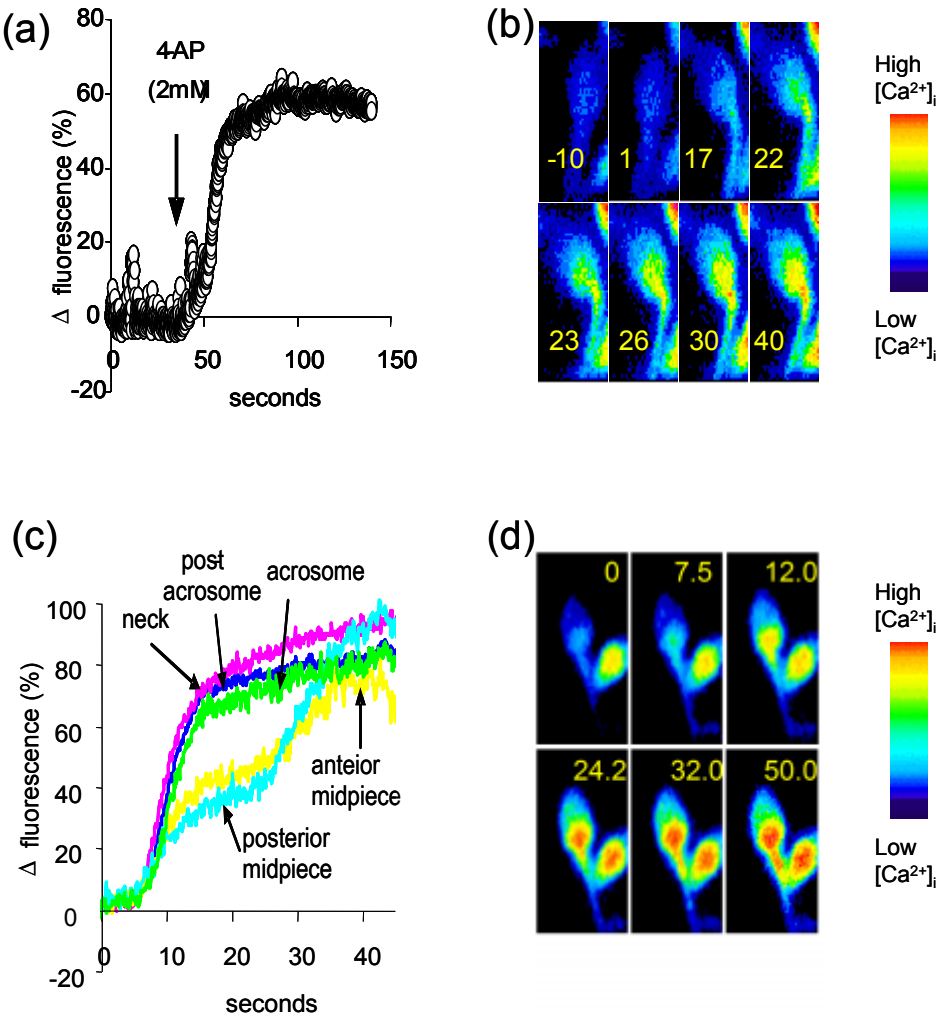

Figure 5.1. Imaging of 4-aminopyridine-induced hyperactivation and Ca^{2+} mobilisation. **(a)** plot showing 4-aminopyridine-induced (arrow) increase in fluorescence of Oregon Green BAPTA-1 in the cell shown in **(b)** Pseuocolour image series showing $[Ca^{2+}]_i$ (Oregon Green-1 BAPTA fluorescence) in a cell (bathed in sEBSS) exposed to 2mM 4-aminopyridine. The $[Ca^{2+}]_i$ response originates at the sperm neck and anterior midpiece then spreads into the posterior head and flagellum. Figures in each image shows the time in seconds with respect to application of 2mM 4-aminopyridine. **(c)** Delayed mobilisation of Ca^{2+} in the sperm neck/midpiece. Plot shows $[Ca^{2+}]_i$ (normalized Oregon Green BAPTA-1 fluorescence) at various points in a sperm stimulated with 2mM 4-aminopyridine (applied at 0 seconds). An initial rise in fluorescence increases fastest in the sperm neck (cerise), preceding the other areas by <1 s. In the midpiece (yellow and turquoise) $[Ca^{2+}]_i$ initially rises with similar kinetics but after 20-25 s a second mobilisation of Ca^{2+} is triggered. **(d)** Pseudocolour images of Oregon Green-1

BAPTA fluorescence) in the same cell (left hand cell in each picture) at various times points after stimulation. Figure in each image shows the time in seconds with respect to application of 2mM 4-aminopyridine.

5.4.2 4-aminopyridine mobilises stored Ca^{2+}

Previous studies on excitable cells have shown that 4-aminopyridine can mobilise stored Ca^{2+}. To investigate whether stored Ca^{2+} is involved in the action of 4-aminopyridine in human sperm we imaged cells superfused with modified bathing medium. To reduce any contribution of Ca^{2+} influx, we used sEBSS with no added Ca^{2+} (no added Ca^{2+} sEBSS; measured contaminating $[Ca^{2+}] < 5\mu M$) (Harper *et al*., 2004). When Ca^{2+} was simply omitted from the medium, 4-aminopyridine still induced a clear rise in $[Ca^{2+}]_i$ in >75% of cells. Fluorescence increased by 29.4±0.7% within 5 min (5 experiments; 353 cells; figure 5.2b) an effect slightly greater than that seen in standard sEBSS (24.5 ±0.7%; paired *t*-test, $P>0.05$). Analysis of single cell records showed that, within the same experiment, responses to 4-aminopyridine in 'Ca^{2+}-free' medium were of two types. $[Ca^{2+}]_i$ either peaked within 1-2 min (≈20% of responsive cells) or rose more slowly but reached a similar level after ≈5 min exposure to 4-aminopyridine (figure 5.2a). When Ca^{2+} was reintroduced to the recording >90% of cells showed a clear increase in $[Ca^{2+}]_i$. 15-20% of cells generated $[Ca^{2+}]_i$ oscillations, this occurring almost exclusively in those cells that had responded to application of 4-aminopyridine with a rapid rise in $[Ca^{2+}]_i$ (figure 5.2a; $P<10^{-120}$, chi square test).

Exposure of human sperm to EGTA-buffered medium causes an immediate fall in $[Ca^{2+}]_i$ which rapidly depletes intracellular Ca^{2+} stores (Harper *et al.,* 2004; Bedu-Addo *et al.,* 2007). Cells incubated in EGTA-buffered saline ($[Ca^{2+}] <10^{-7}$ M) for 10-15 min showed no response to 4-aminopyridine. However, when the drug was applied after exposure to EGTA-buffered saline for ≤10 min a clear transient increase in $[Ca^{2+}]_i$ was detectable in a proportion of the cells (23.8±8.2%; n=7; figure 5.2c). In cells showing a detectable response the size of the transient (expressed as change compared to fluorescence intensity immediately before

application of 4-aminopyridine) was highly variable, amplitude ranging from 2.5-55% of control fluorescence (figure 5.2c; mean =8.7±1.4%) and duration was typically 5-10 min. In some cells 4-aminopyridine caused a slowly developing elevation of $[Ca^{2+}]$ followed by a 'spike', suggesting the occurrence of Ca^{2+}-induced Ca^{2+} release (fig. 5.2c). Upon re-introduction of Ca^{2+} to the imaging chamber there was a large increase in $[Ca^{2+}]_i$ in most cells, consistent with activation of capacitative Ca^{2+} influx.

Figure 5.2

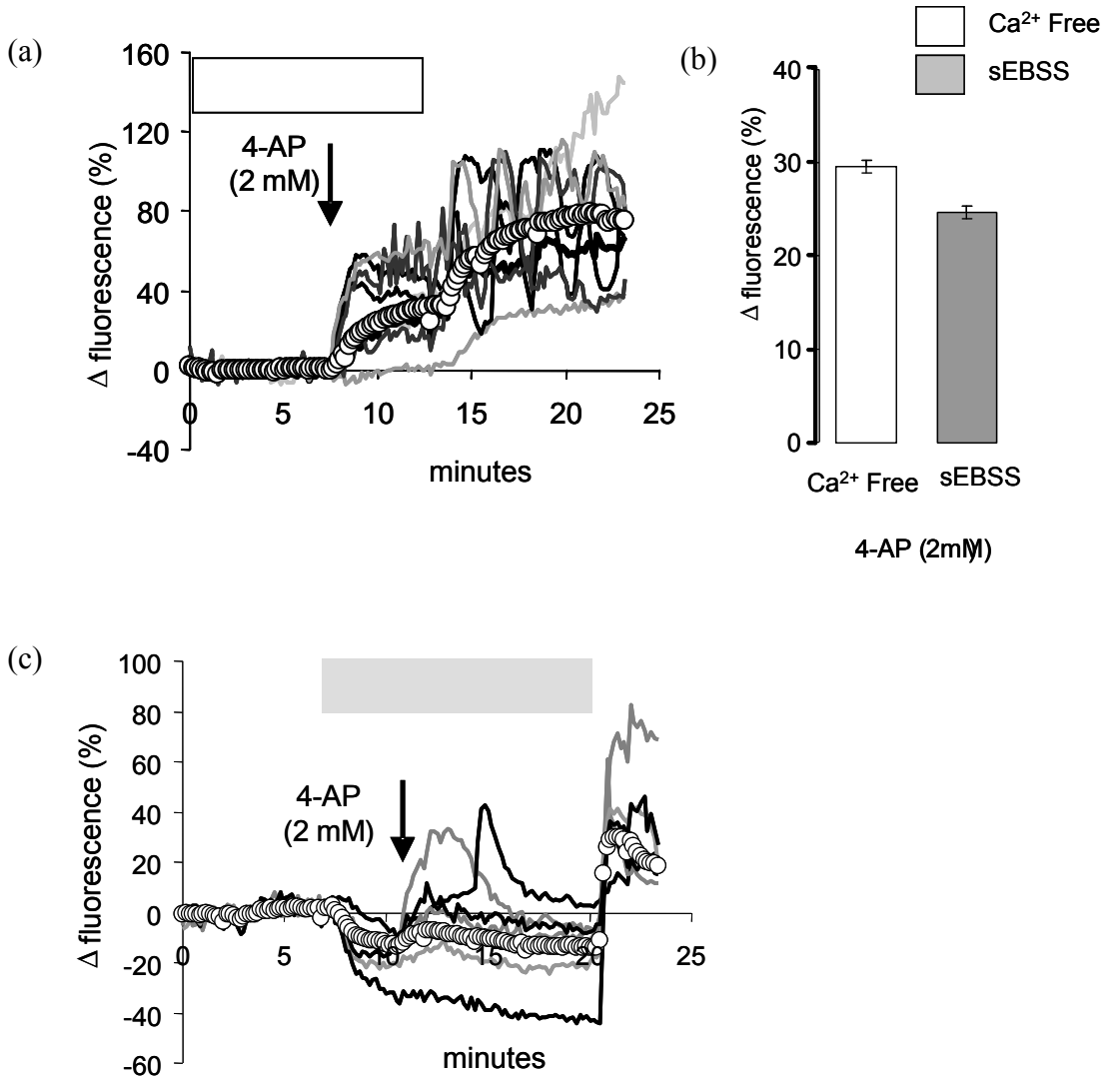

Figure 5.2. 4-aminopyridine mobilises stored Ca^{2+}. **(a)** Effect of 2 mM 4-aminopyridine on $[Ca^{2+}]_i$ in cells superfused with nominally Ca^{2+}-free sEBSS (no added Ca^{2+}; $[Ca^{2+}] \leq 5$ μM; shown by white bar). Upon application of 4-aminopyridine (arrow) most cells responded to 4-aminopyridine. Responses of 7 representative cells are shown and also mean response (○-○) of all the 234 cells in the experiment. In most cells $[Ca^{2+}]_i$ rose slowly, similarly to the mean response, but in ≈20% there was a more rapid rise, comprising an initial transient and a plateau. Upon introduction of standard sEBSS (1.8mM Ca^{2+}) to the recording chamber there was a further increase in $[Ca^{2+}]_i$ (including some cells that showed no initial response) and most of those cells that had showed an initial transient response to 4-aminopyridine generated $[Ca^{2+}]_i$ oscillations. **(b)** Elevation of $[Ca^{2+}]_i$ by 4-aminopyridine in cells superfused with Ca^{2+}-free sEBSS and standard sEBSS . Each bar shows mean increase in fluorescence (at plateau) of 144-364 cells from 4 experiments. **(c)** Effect of 4-aminopyridine on cells superfused

with EGTA-buffered sEBSS ($[Ca^{2+}]<10^{-7}$ M). Responses of 6 representative cells are shown and also mean response (○-○) of all the 95 cells in the experiment. Superfusion with EGTA-buffered saline ($[Ca^{2+}] <10^{-7}$M; shown by grey bar) caused a rapid fall in $[Ca^{2+}]_i$. Application of 4-aminopyridine ≈5 min after EGTA caused a transient rise in $[Ca^{2+}]_i$, lasting 5-10 min, in a subset (24%) of cells. Upon re-introduction of standard sEBSS $[Ca^{2+}]_i$ in all cells rose rapidly.

5.4.3 4-aminopyridine induced Ca^{2+} mobilisation and hyperactivation in sperm

Experiments on sperm bathed in EGTA-buffered saline show that the action of 4-aminopyridine on $[Ca^{2+}]_i$ in human sperm involves both Ca^{2+} influx and an initial mobilisation of stored Ca^{2+} in the neck/midpiece region of the cell. To investigate whether stored Ca^{2+} contributes to the strong hyper-activating action of 4-aminopyridine, we assessed the motility of cells exposed to the drug in medium with modified $[Ca^{2+}]$. The ability of 4-aminopyridine to dramatically increase the proportion of hyperactivated cells was not inhibited by simple omission of Ca^{2+} from the medium. In 12 paired experiments in which cells from the same sample were exposed to 4-aminopyridine both in standard medium and medium from which Ca^{2+} had been omitted, ($[Ca^{2+}] \leq 5$ μM) the mean proportion of hyperactivated cells was not significantly reduced (4-aminopyridine induced hyperactivation in standard saline= 21.3.2±4.4% (grey bar) and in nominal calcium free saline = 33.2±5.2% (open bar), paired *t*-test, $P>0.05$, figure 5.3a).

4-aminopyridine-induced elevation of $[Ca^{2+}]_i$ in cells suspended in EGTA-buffered medium ($<10^{-7}$ M), which reflects mobilisation of stored Ca^{2+}, is transient (figure 5.2c). We therefore examined the persistence of the hyperactivating effect of 4-aminopyridine in cells suspended in EGTA-buffered medium compared to that in cells in standard sEBSS. Whereas the action of 4-aminopyridine on cells in sEBSS was stable over a period of 45 min, when cells were re-suspended in EGTA-buffered medium 2 min before stimulation with 4-aminopyridine hyperactivation decayed to zero within 10-15 min (figure 5.3b). Incubation in EGTA-buffered saline also caused gradual loss of motility but a significant proportion of the cells remained motile for >30 min (figure 5.3c), almost twice as long as the persistence of 4-aminopyridine-induced hyperactivation.

Figure 5.3

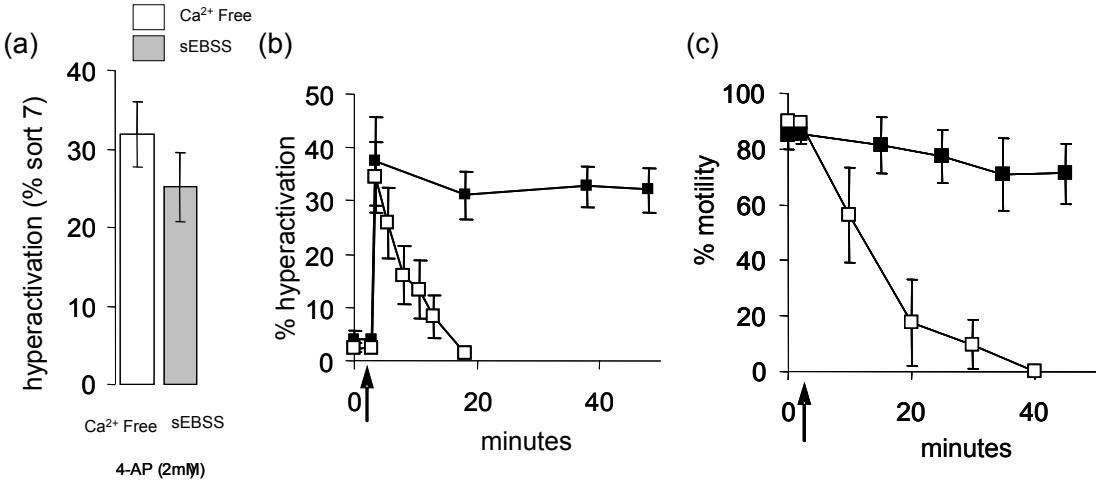

Figure 5.3 Stored Ca^{2+} and 4-aminopyridine induced hyperactivation. (**a)** Induction of hyperactivation (% cells) by 2 mM 4-aminopyridine in cells suspended in standard sEBSS (1.8 mM Ca^{2+}; grey bar) and in saline with no added Ca^{2+} ($[Ca^{2+}]_o \leq 5$ μM; Harper *et al*, 2004; open bar). Each bar shows mean ±sem of 12 experiments. Hyperactivation was measured ≈2 min after application of 4-aminopyridine ($P>0.05$; paired t-test). The effect of suspension in EGTA-buffered medium on motility and hyperactivation was also assessed. Cells were suspended in EGTA-buffered medium $<10^{-7}$ M at 0 min and aliquots were withdrawn at intervals to assess motility (c) and induction of hyperactivation by 4-aminopyridine hyperactivation (b). Each point shows mean sem of 4 experiments. **(b)** Decay of 4-aminopyridine-induced hyperactivation. After suspension of cells in appropriate medium an aliquot was immediately assessed for hyperactivation. 2 mM 4-aminopyridine was then applied to the cells (arrow) and time-dependence of hyperactivation was assessed by withdrawing aliquots at a series of time points as shown. Filled squares (■) shows cells suspended in sEBSS (mean ±sem of 4 experiments). Open squares (□) shows cells re-suspended in EGTA-buffered medium 2 min before application of 4-aminopyridine (mean ±sem of 4 experiments). **(c)** Decay of 4-aminopyridine-induced motility. After suspension of cells in appropriate medium an aliquot was immediately assessed for motility 2 mM 4-aminopyridine was then applied to the cells (arrow) and time-dependence of motility was assessed by withdrawing aliquots at a series of time points as shown. Filled squares (■) shows cells suspended in sEBSS (mean ±sem of 4 experiments). Open squares (□) shows cells re-suspended in EGTA buffered medium 2 min before application of 4-aminopyridine (mean ±sem of 4 experiments).

5.4.4 Effects of 4-aminopyridine on intracellular Ca^{2+} transport and release proteins

The elevation of $[Ca^{2+}]_i$ caused by 4-aminopyridine in EGTA buffered saline reflected mobilisation (figure 5.2c) of intracellular stored Ca^{2+} located at the posterior head. Although the structure of this putative store is not fully elucidated, evidence suggests the presence of calreticulin (a Ca^{2+} storage protein) (Naaby-Hansen *et al.,* 2001; Ho and Suarez, 2003), inositol 1,4,5-trisphosphate receptors (IP_3Rs) (Kuroda *et al*., 1999; Ho and Suarez, 2001b, 2003) and ryanodine receptor (Giannini *et al*., 1995; Harper *et al*., 2004). There is also some evidence of Ca^{2+}-ATPase localised to this area including SERCA (sacroplasmic endoplasmic reticulum Ca^{2+}-ATPase) and SPCA (secretory pathway Ca^{2+}-ATPase) (Harper *et al.,* 2005; Lawson *et al*., 2007). In an attempt to isolate the effects of 4-aminopyridine on individual components of intracellular store calcium uptake pumps and release channels uptake and release of Ca^{2+} by sacroplasmic reticulum (SR) and brain microsome vesicles suspended in medium containing the calcium indicating dye fluo3 (acid free) was examined.

5.4.4.1 Effects of 4-aminopyridine fluo3 emission

Since previous data demonstrated that 4-aminopyridine had a quenching effect on OGB fluorescent signal (chapter two), the quenching effects of this compound on fluo3 was investigated. Fluorescence emission spectra were recorded in the presence and absence of 4-aminopyridine (0.5–4.0mM). As expected, there was a decrease in fluorescence signal in the presence of 4-aminopyridine however neither the emission spectrum shape nor wavelength were affected. The Stern-Volmer quenching constant was 0.05 at 25^{o}C (figure 5.4).

Figure 5.4

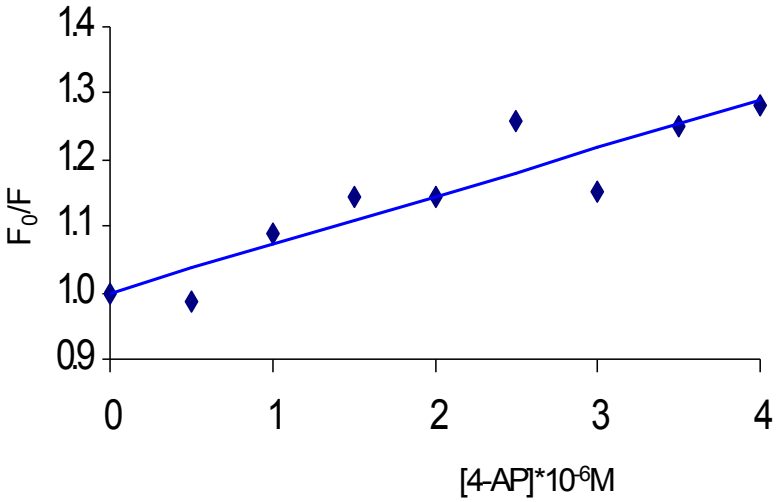

Figure 5.4: Stern-Volmer plot of fluorescence lifetime of fluo-3 (250nM in 40mM Tris and 100mM KCL, pH 7.2) containing increasing concentrations of 4-aminopyridine (0.5-4mM) at 25°C. Irradiation wavelength was held at 506nm.

5.4.4.2 Effects of 4-aminopyridine on Ca^{2+} uptake and Ca^{2+} ATP-ase activity

4-aminopyridine has previously been shown to inhibit the ATP-driven Ca^{2+} uptake in guinea pig sacroplasmic reticulum (Ishida and Honda, 1993). Figure 5.5 (a) shows typical traces of ATP-dependent Ca^{2+} uptake in rabbit skeletal muscle sacroplasmic reticulum (SR) (SERCA isoform 1 predominantly expressed) in the absence (blue trace) and presence (pink trace) of 4-aminopyridine. The membranes were pre-incubated with 4-aminopyridine (2mM) for ~2 mins prior to the addition of 1.5mM ATP to initiate Ca^{2+} uptake (arrow). The rate of ATP-dependent Ca^{2+} uptake did not differ significantly different in the presence of 4-aminopyridine (2mM) (n=3 experiments, paired *t*-test, $P>0.05$).

5.4.4.3 Effects of 4-aminopyridine on the IP_3 Ca^{2+} release channels

Porcine cerebellar microsomes are relatively enriched in intracellular pumps (predominantly SERCA2) and channels (predominantly IP_3R). As described in the methods, brain microsomes were loaded with Ca^{2+}, initiated by the addition of 1.5mM Mg-ATP (at 100s). Adequate Ca^{2+} accumulation required a prolonged period due to the age of the microsomal preparation

(~1000s). Additions of 2mM 4-aminopyridine to the cuvette (at ~1050s) caused a clear mobilisation of Ca^{2+} (figure 5.5b insert). Application of IP_3 (10µM at 1155s) resulted in further release. Total accumulated Ca^{2+} within the brain microsomes was measured by the addition of 12.5µg/ml A23187 and the degree of 4-aminopyridine induced Ca^{2+} release was calculated as a % of the maximum. Average 4-aminopyridine induced Ca^{2+} release was 6.9% followed by IP_3 induced release of 3.8% (mean n=3 experiments, 3 different microsomal aliquots). These results demonstrate that 4-aminopyridine can trigger partial store release probably by IP_3R activation but may not occupy or is displaced from the IP_3 binding site since the application of IP_3 triggered further Ca^{2+} release.

5.4.4.4 Effects of 4-aminopyridine on the ryanodine receptor Ca^{2+} release channel

Immunolocalisation studies from our laboratory demonstrate the presence of ryanodine receptors proteins (isoforms 1 and 2) expressed in the posterior head of human sperm (Harper *et al.,* 2004; Lefièvre *et al.,* 2007). Since both IP_3R and RyR have similar structures 4-aminopyridine-induced Ca^{2+} release (0.1 to 3.0mM) on ryanodine receptor1 predominantly expressed in rabbit skeletal muscle heavy sacroplasmic reticulum was investigated. A typical experimental trace is presented in figure 5.5(c). Ca^{2+} uptake was initiated by the addition of 1.5mM Mg-ATP (at ~185s) and once sufficient Ca^{2+} had accumulated further Ca^{2+} uptake was inhibited with thapsigargin (1-1.5µM, at ~200s) prior to the addition of 4-aminopyridine (at ~400s) (figure 5.5c insert). Total accumulated Ca^{2+} within the SR microsomes was measured by the addition of 12.5µg/ml A23187 and the degree of 4-aminopyridine induced Ca^{2+} release was represented as a % of the maximum. Figure 5.5(d) shows the dose-dependent mobilisation of Ca^{2+} release from skeletal muscle SR induced by 4-aminopyridine. Average 4-aminopyridine (2mM) induced Ca^{2+} release was 5.0±1.1% slightly lower than brain microsomal vesicles (IP_3R) (n=12 experiments, mean±s.e.m.).

Figure 5.5

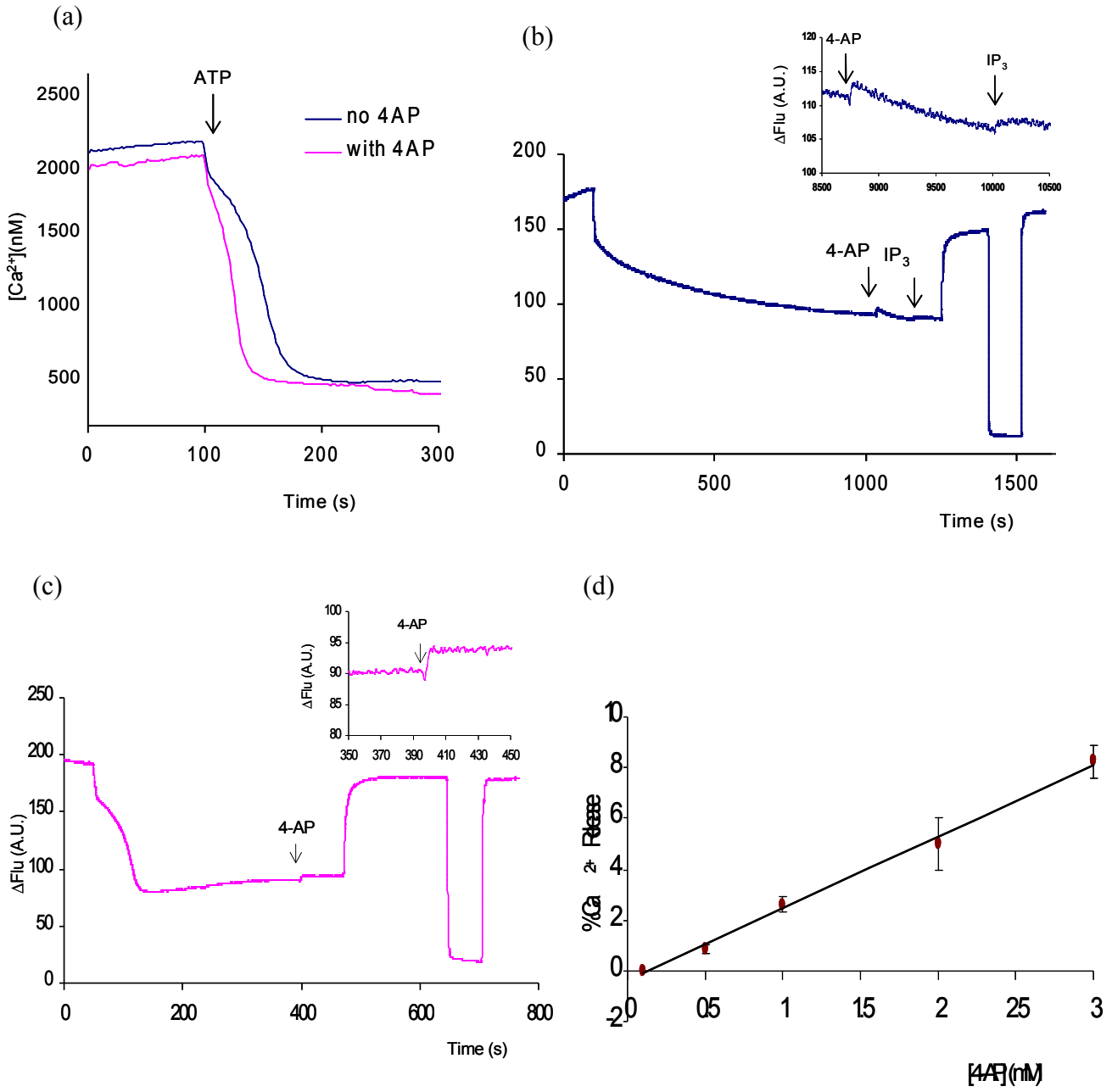

Figure 5.5 Effect of 2mM 4-aminopyridine on Ca^{2+} uptake and release in microsomal vesicles (rabbit skeletal muscle vesicles or brain microsomes) at pH7.2 and 37°C **(a)** Typical traces showing Ca^{2+} uptake initiated by addition of 1.5mM Mg-ATP (indicated by arrow) in SR vehicles in the presence (pink line) and absence (blue line) of 4-aminopyridine (2mM) (representative of 3 experiments). **(b)** Effect of 2mM 4-aminopyridine on Ca^{2+} release in brain microsomal vesicles. Typical traces showing Ca^{2+} uptake initiated by addition of 1.5mM Mg-ATP (at ~100s) followed by 4-aminopyridine (2mM, arrow at 1050s) and IP3 (arrow at 1155s) resulting in Ca^{2+} release (insert). Maximal store release was achieved by addition of the calcium ionophore A23187 (~1250s). **(c)** Effect of 2mM 4-aminopyridine on Ca^{2+} release in rabbit skeletal muscle microsomal vesicles. Typical traces showing

Ca^{2+} uptake initiated by addition of 1.5mM Mg-ATP (at ~185s) followed by thapsigargin (1μM) at ~200s. 4-aminopyridine (2mM arrow at 400s, insert) resulting in Ca^{2+} release. Maximal store release was achieved by addition of the calcium ionophore A23187 (~475s). **(d)** Dose-dependency of Ca^{2+} release induced by 4-aminopyridine presented as a percentage of Ca^{2+} release compared to that released by A23187 (vertical bars indicate s.e.m.).

5.4.5 Effect of low dose P_4 on the 4-aminopyridine induced $[Ca^{2+}]_i$ response

Previous studies from this laboratory indicate that exposure of human spermatozoa to very low concentrations of progesterone produced a low tonic 'background' influx of Ca^{2+} that allowed the store in the neck/midpiece to fill up which was mobilised when cells were exposed to nitric oxide donors (Machado-Oliveira *et al*., 2008). Exposure of sperm to 100pM progesterone in standard saline for approximately 10mins increased resting $[Ca^{2+}]_i$ by 10.7± 0.7% (figure 5.6, 5mins after exposure) in the majority of cells. Furthermore the subsequent 4-aminopyridine induced $[Ca^{2+}]_i$ response was altered in many cells with approximately 50% of cell responding with a transient increase (amplitude 34.5 ± 2.4%), followed by oscillations in ~15% of cells. Approximately 40% of cells responded with a sustained elevation $[Ca^{2+}]_i$ of 17.0 ± 0.9%.

Figure 5.6

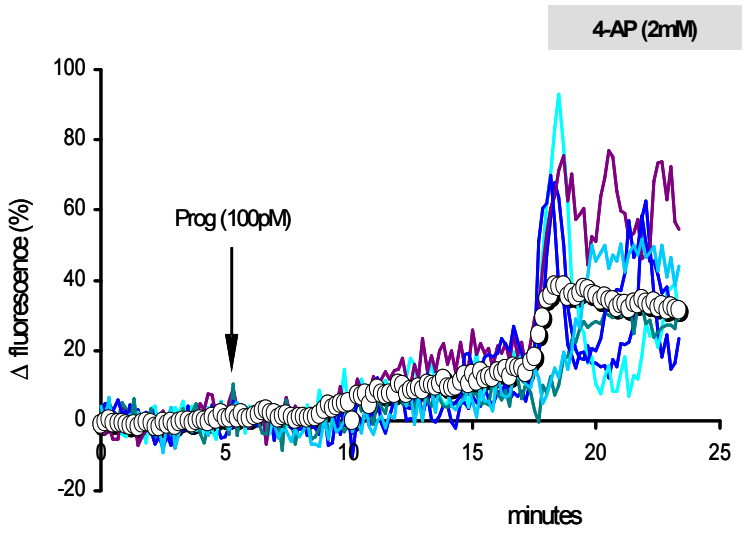

Figure 5.6 Effect of progesterone (100pM) pre-treatment on the response to 4-aminopyridine. $[Ca^{2+}]_i$ responses of 7 representative cells are shown and also mean response (○-○) of all the 135 cells in the experiment. Cells were exposed to 100pM progesterone for ≈10 min, causing a sustained elevation of $[Ca^{2+}]_i$. Application of 2mM 4-aminopyridine (grey bar) caused a further rise in $[Ca^{2+}]_i$.

5.4.6 Effects of tetracaine on 4-aminopyridine induced $[Ca^{2+}]_i$ response

Harper *et al*., (2004) demonstrated that application of tetracaine inhibited progesterone (3µM) induced oscillations (Harper *et al*., 2004). Tetracaine has been shown to inhibit the ryanodine receptor/Ca^{2+} release channel in SR microsomal membranes (Martin *et al.,* 1993; Tovey *et al.,* 1998). In these experiments, sperm have been pretreated to 1mM tetracaine for approximately 10mins which in 66% of cells resulted in a no change or a decrease in resting $[Ca^{2+}]_i$ of 9.8± 0.7% (5 mins after exposure) and the remaining cells (34%) responded with a tonic increase $[Ca^{2+}]_i$ of 7.8 ± 0.7%. The overall kinetics (response) of the subsequent 4-aminopyridine induced $[Ca^{2+}]_i$ response was similar to controls (non-tetracaine experiments) as the majority of cells responded with a sustained increase in $[Ca^{2+}]_i$ of 23.8±1.2%. However upon washout of tetracaine, the 4-aminopyridine response was altered to a transient response (amplitude 34.9 ± 1.7%) in the majority of cells (figure 5.7a). This transient was absent in parallel experiments upon washout of 4-aminopyridine (figure 5.7b).

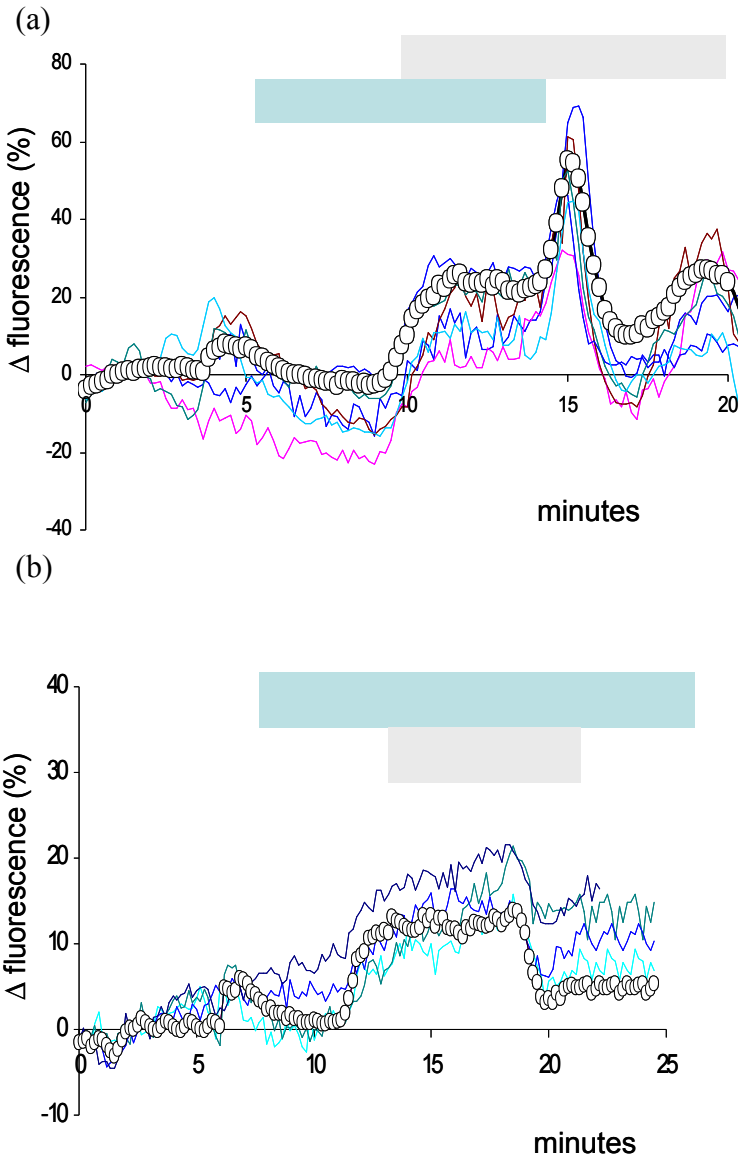

Figure 5.7 Effect of tetracaine (1mM, blue bar) pre-treatment on the response to 4-aminopyridine (2mM, grey bar).

(a) $[Ca^{2+}]_i$ responses of 7 representative cells and mean response (○-○) of all the 55 cells in the experiment are shown. Cells were exposed to 1mM tetracaine (blue bar) for ≈10 min prior to the application of 2 mM 4-aminopyridine (grey bar), resulting in transient rise in $[Ca^{2+}]_I$. Washout of tetracaine altered the 4-aminopyridine response to a transient $[Ca^{2+}]_i$ signal in >90% of cells.

(b) This transient response is lost upon washout of 4-aminopyridine (grey bar). Responses of 6 representative cells and mean response (○-○) of all the 60 cells in the experiment.

5.4.7 4-aminopyridine and sperm mitochondrial Ca^{2+}

To investigate whether mitochondria are involved in the generation of 4-aminopyridine induced calcium increase, we pre-treated cells with the mitochondrial uncoupler 2,4-dinitrophenol (100μM for ~ 6 mins), to collapse the mitochondrial inner membrane potential, prior to the application of 4-aminopyridine. In the majority of cells (~ 60%) application of 2,4-dinitrophenol caused a tonic increase in $[Ca^{2+}]_i$ of 5.6 ± 0.4% (n= 2 experiments, 145 cells, figure 5.8a), probably due to release of mitochondrial Ca^{2+}. However, the 4-aminopyridine induced response was not significantly altered in comparison to non-pretreated controls (figure 5.8b)- amplitude of 4-aminopyridine response in pretreated cells (21.4 ± 0.4%) and non-pretreated cells (20.5 ± 0.7, n=2 experiments; paired *t*-test, $P>0.05$). A further convenient method of measuring mitochondrial membrane depolarisation and membrane integrity was to record any decrease in fluorescence of the mitochondrial compartmentalizing probe, Rhodamine 123 (Rh123) (Ogunbayo *et al*., 2008). Rh123 is a cationic, lipophilic species that accumulates in the matrix of the polarised mitochondria. Upon depolarisation or loss of mitochondrial membrane integrity (release of pro-apoptotic factors through the permeability transient pore (PTP)) Rh123 fluorescent signal is lost. Rh123 is light sensitive and photobleaching results in a gradual slow decrease of signal. Figure 5.8(c) shows the fluorescence intensity of Rhodamine 123-loaded sperm cells. 4-aminopyridine (2mM) was added to the cells at 100 sec after the start of the recording. Although a small degree of fluorescence decrease could be observed in some cells which was attributed to photobleaching, the mean rate of fluorescence decrease was not enhanced upon exposure to 4-aminopyridine.

Figure 5.8

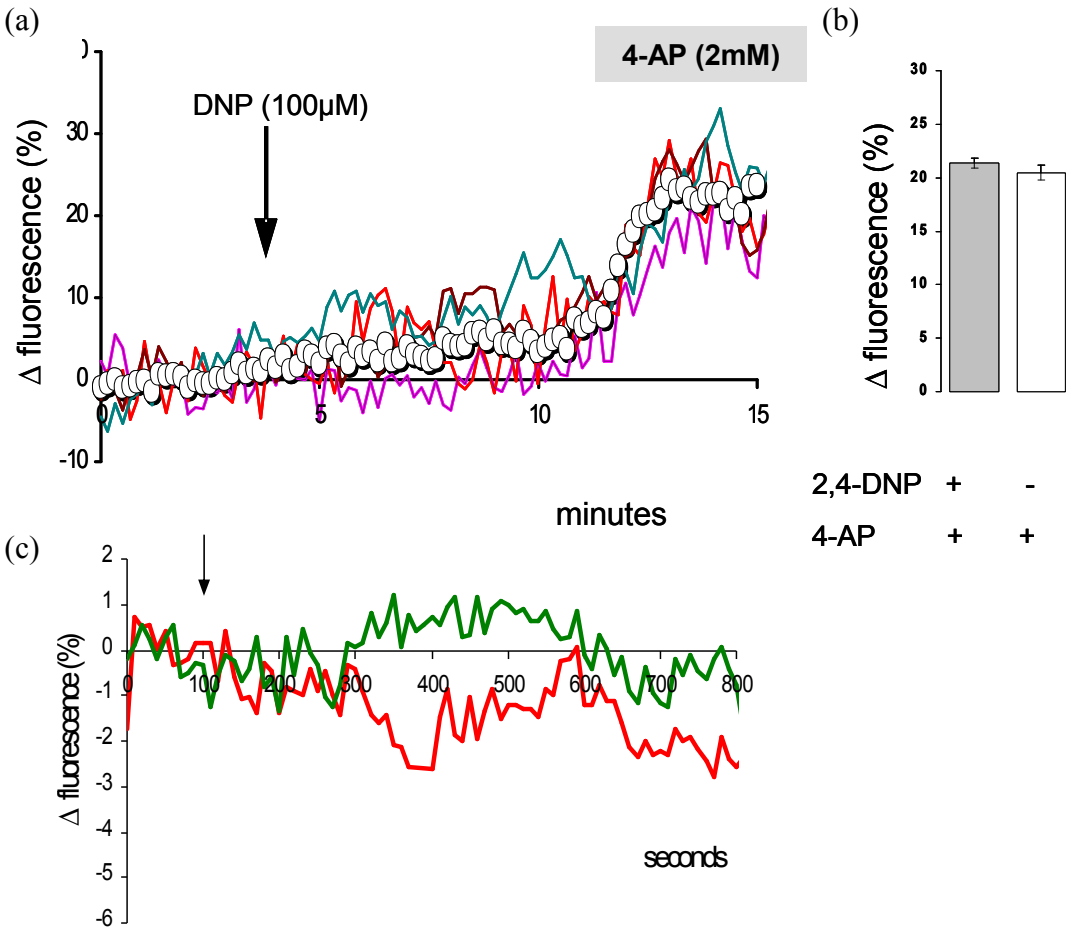

Figure 5.8 Effect of 4-aminopyridine on mitochondrial Ca^{2+} (a) $[Ca^{2+}]_i$ responses of 6 representative cells are shown and also mean response (○-○) of all the 92 cells in the experiment. Cells were exposed to 100µM 2,4-dinitrophenol for ≈5 min, causing a sustained elevation of $[Ca^{2+}]_i$. Application of 2 mM 4-aminopyridine (grey bar) caused a further large rise in $[Ca^{2+}]_i$ (b) Elevation of $[Ca^{2+}]_i$ by 4-aminopyridine (2mM) in cells pre-treated with 2,4-DNP (grey bar, n=2 exps, 145 cells) and non-pretreated control (open bar, n= 2exps, 150 cells) . Each bar shows mean increase in fluorescence (at plateau) ± s.e.m. (c) The effects of 4-aminopyridine (2mM) on mitochondrial membrane potential in human sperm cells loaded with Rhodamine 123. Green trace is a combination of 16 cells exposed to 4-aminopyridine (black arrow) and red trace is a combination of 15 cells exposed to saline only. Downward drift due to photobleaching.

5.4.8 4-aminopyridine and the acrosome reaction

Mammalian spermatozoa are relatively hard wired cells. We have shown previously that Ca^{2+} signals in human sperm are compartmentalised, permitting the sperm separately to regulate different activities (e.g. hyperactivation and acrosome reaction) even through both are activated by Ca^{2+}-mediated signalling cascades (Bedu-Addo *et al.,* 2007). 4-aminopyridine induces hyperactivation in human sperm, apparently through prolonged elevation of $[Ca^{2+}]_i$. To investigate whether the sperm were capable of restricting the effects of this signal to regulation of flagellar activity, we exposed sperm, suspended in standard saline, to 4-aminopyridine (2mM). After one hour the sperm were processed for assessment of acrosomal status. Whereas A23187 (10 μM) increased the proportion of acrosome reacted cells from 6.6±1.7% to 67.6±5.4% (paired *t*-test, $P<5x10^{-5}$; paired-t, n=7), 4-aminopyridine had no effect on the rate of acrosome reaction (5.0±1.2%; paired *t*-test, $P>0.4$; n=7; figure 5.8).

Figure 5.9

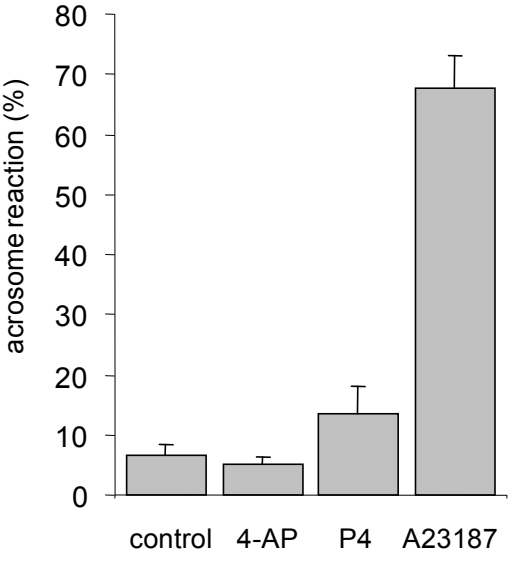

Figure 5.9. 4-aminopyridine does not induce acrosome reaction. Histogram shows proportion of cells which had undergome acrosome reaction (failure to stain with *Pisum sativum* agglutinin; see methods). A23187 increases the rate of acrosome reaction to >65%. Progesterone (P_4) also increased the proportion of acrosome reacted cells but 2 mM 4-aminopyridine failed to cause any stimulation.

5.5 Discussion

Although sperm are specialised cells they have a limited number of intracellular organelles (in comparison to somatic cells) there is evidence to suggest they contain at least two separate intracellular calcium stores. While the Ca^{2+} content of any putative intracellular stores would be small and difficult to quantify, mobilisation could induce capacitative calcium entry (CCE) and the opening of plasma membrane Ca^{2+} channels to prolong Ca^{2+} influx (Blackmore, 1993; Williams & Ford, 2003). Calcium channel opening and depletion of Ca^{2+} from acrosomal stores results in elevation of calcium which drives exocytosis and the acrosome reaction (Florman *et al*., 1989; Florman, 1994; O'Toole *et al,* 2000; Jungnickel *et al*, 2001). Furthermore, mobilisation of the neck/midpiece store and resulting CCE may contribute significantly to regulation of motility (Castellano *et al*, 2003; Yoshida *et al*, 2003). The neck/midpiece region of mammalian and sea urchin sperm Ca^{2+} store is composed of the redundant nuclear envelope (RNE), which is retained following nuclear condensation and located behind the nucleus of mammalian sperm. Membrane vesicles have been observed closely opposed to the plasmalemma, in the cytoplasmic droplet of human sperm. Both these structures have calreticulin (a Ca^{2+} storage protein) and IP_3 receptors and Ca^{2+} mobilisation in this region is sensitive to ryanodine (a Ca^{2+} activated Ca^{2+} channel) (Ho and Suarez, 2001b. 2003; Naaby-Hansen *et al.,* 2001; Harper *et al.,* 2005). Accumulation and mobilisation of Ca^{2+} from one or both these structures is believed to initiate and or control hyperactivation in mouse and bovine sperm and regulation of flagellar beat mode in human sperm (Ho and Suarez, 2001a, 2003; Harper *et al.,* 2007; Bedu-Addo *et al.,* 2008; Machado-Oliveira *et al*., 2008).

In the previous chapters results have demonstrated that in a population of human sperm 4-aminoopyridine induced hyperactivation and increased $[Ca^{2+}]_i$ is not solely pH or CatSper dependent.

Ca^{2+} store mobilisation and asymmetric bending of the midpiece.

In imaging experiments of 4-aminopyridine-treated cells (both fluorescence imaging of $[Ca^{2+}]_i$ and high speed acquisition of phase contrast images), a common observation was a strong bending of the sperm flagellum in the midpiece and (sometimes) proximal part of the principal piece. In fluorescence image series it was clear that this bending was tightly associated with elevation of $[Ca^{2+}]_i$ at the neck/midpiece (Suarez and Dai, 1992; Carlson *et al*, 2003; Quill *et al*, 2003; Harper *et al.*, 2004; Marquez *et al.*, 2007; Bedu-Addo *et al*., 2007, 2008). The most extreme examples of this occurred in cells showing very strong $[Ca^{2+}]_i$ responses and 'relaxation' of the bend occurred upon the return of $[Ca^{2+}]_i$ to resting levels. Similar reversible effects on bending of the proximal flagellum are observed during $[Ca^{2+}]_i$ oscillations induced by progesterone (Harper *et al.*, 2004, Bedu-Addo *et al.*, 2007, Machado-Oliveira *et al.*, 2008) and NO (Machado-Oliveira *et al.*, 2008). Increased bending of the midpiece and anterior flagellum is characteristic of hyperactivated motility and will lead to exaggerated lateral movement of the sperm head (Suarez and Ho, 2003a, 2003b; Ishijima *et al.*, 2006; Ohmuro and Ishijima, 2006). Bedu-Addo *et al*., (2007) suggested that mobilisation of Ca^{2+} at the sperm neck/midpiece and consequent bending in this region is an important contribution to hyperactivation.

Stored Ca^{2+} and 4-aminopyridine-induced $[Ca^{2+}]_i$ response

Assessment of $[Ca^{2+}]_i$ in 4-aminopyridine stimulated cells showed a sustained elevation of $[Ca^{2+}]_i$ originating in the neck/midpiece of the sperm. Omission of Ca^{2+} from the bathing medium had little, if any, effect on the ability of 4-aminopyridine either to elevate $[Ca^{2+}]_i$ (figure 5.2 a,b) or to induce hyperactivation of the cells (figure 5.3a). However, when cells were superfused with EGTA-buffered medium ($[Ca^{2+}]_o<10^{-7}$M) the response to 2mM 4-aminopyridine (applied within a few minutes of exposure to EGTA) was a transient elevation of $[Ca^{2+}]_i$ which decayed completely within 5-10 min (figure 5.2c). It appears unlikely that the

action of 4-aminopyridine in human sperm was to inhibit ATPases since, when cells were treated with the drug in low-Ca^{2+} saline, subsequent addition of Ca^{2+} evoked large oscillations of $[Ca^{2+}]_i$, (indicating repeated accumulation and release of stored Ca^{2+}) which occurred almost exclusively in those cells that had previously shown a 4-aminopyridine-induced $[Ca^{2+}]_i$ transient (figure 5.2 a). Thus an action actively to mobilise Ca^{2+}, or to sensitise Ca^{2+} release, is more likely.

Exposure of human sperm to EGTA-buffered, 'Ca^{2+} free' medium causes an immediate and large fall in $[Ca^{2+}]_i$ and rapidly depletes intracellular Ca^{2+} stores (Harper *et al*, 2004; Bedu-Addo *et al,* 2007). Cells incubated in EGTA-buffered saline for 10 -15 min showed no response to 4-aminopyridine, but application of the drug 4-5 min after exposure to EGTA induced a clear response in almost 25% of cells . Under these circumstances the rise in $[Ca^{2+}]_i$ was transient and was of diminished amplitude. Thus it appears that at least part of the elevation in $[Ca^{2+}]_i$ that occurs in human sperm exposed to 4-aminopyridine reflects the mobilisation of stored Ca^{2+}, though the prolonged effect is probably dependent upon Ca^{2+}-influx.

Effect of 4-aminopyridine on microsomal Ca^{2+} uptake and release

The asynchronous behaviour and minute size of human sperm presents many technical difficulties. As discussed above, single cell imaging experiments in Ca^{2+} free conditions indicated that 4-aminopyridine was mobilising stored calcium however it is difficult to isolate and identify the channels or pumps involved. Furthermore, 4-aminopyridine has potentially numerous other effects on human sperm including blocking K_v channels, cytoplasmic alkalinisation and activation of KSper and CatSper channels. In an attempt to isolate the components of the calcium signalling 'toolkit' targeted by 4-aminopyridine, microsomes (brain and sacroplasmic reticulum) were utilized to examine calcium uptake and mobilisation. Application of 2mM 4-aminopyridine to rabbit SR did not significantly alter the rate or kinetics

of Ca^{2+}-ATPase uptake, although an increased dose of 4-aminopyridine may have an effect. These results are in agreement with Ishida and Honda (1993) who examined the effects of 4-aminopyridine on guinea pig SR Ca^{2+}-ATPase activity. High doses of 4-aminopyridine (10mM) were required to inhibit guinea pig SR Ca^{2+}-ATPase uptake whereas lower dose (<3mM) 4-aminopyridine did not have a significant effect.

As yet there is no consensus to the identity of the Ca^{2+} channel localised in the neck/midpeice region of mammalian sperm. Suarez and co report the presence of IP_3R however high, non-specific doses of thapsigargin are required to mobilise this store. Despite the slow Ca^{2+} uptake by brain microsomes, Ca^{2+} release occurred in response to 4-aminopyridine (~7% of total accumulated) but did not occlude a subsequent response to IP_3 indicating that 4-aminopyridine and IP_3 act at different sites on the channels. These results are contrary to Palade and co-workers (1989) who reported that 4-aminopyridine inhibited IP_3 induced Ca^{2+} release from brain microsomes by direct interaction with the Ca^{2+} channel. However these results are in agreement with Guse *et al*., 1994 who report 4-aminopyridine induced a dose-dependent IP_3R calcium release followed by a sustained elevation of $[Ca^{2+}]_i$ in Jurkat T-lymphocytes. Furthermore, Grimaldi and co-workers (2001) demonstrated that 4-aminopyridine enhanced phospholipase C (PLC)/IP_3 pathways in neurons and astrocytes resulting in store mobilisation. It is possible that 4-aminopyridine induced store mobilisation in intact human spermatozoa directly or indirectly by enhanced IP_3 production.

Immunolocalisation studies from our laboratory demonstrate the presence of ryanodine receptors (RyR) proteins (isoforms 1 and 2) expressed in the posterior head of human sperm (Harper *et al*., 2004; Lefièvre *et al*., 2007). The ryanodine receptors are large ion channels, positively regulated by S-nitrosylation and the recent report by Machado-Oliveira *et al.,* (2008) demonstrated that nitric oxide donors and progesterone converge and act synergistically to enhance calcium mobilisation in human sperm. Ryanodine receptors have high conductance (>100pS; Zalk *et al.,* 2007) so it is likely that any sperm RyRs are expressed at extremely low

levels (Costello *et al.,* 2009). We have examined the dose-dependent effects of 4-aminpoyridine on RyR1, the predominant isoform expressed in rabbit skeletal muscle (figure 5.5c). Following Ca^{2+} uptake Ca^{2+}-ATPase activity was inhibited with thapsigargin prior to the addition of 4-aminopyridine. In the majority of experiments a definite increase in fluorescence signal was observed reflecting Ca^{2+} release. This data demonstrates that 4-aminopyridine is a relatively effective ryanodine receptor activator of Ca^{2+} release (~5% release, figure 5.5d). For comparision the commonly used ryanodine recptor activator, 4-chloro-m-cresol (4CmC) was recently shown to release ~10% of SR vesicle stored calcium (Al-Mousa and Michelangeli, 2009). These results agree with Ishida *et al*., (1992) who demonstrated that 4-aminopyridine (1mM) induced a transient Ca^{2+} release from guinea pig SR.

Treatments which induce Ca^{2+} influx can 'switch on' cyclical mobilisation of this store (causing cytoplasmic $[Ca^{2+}]_i$ oscillations) apparently due to a form of calcium induced calcium release (CICR) (Kirkman-Brown *et al*, 2004; Harper *et al,* 2005; Bedu-Addo *et al*, 2007). Previous work in this laboratory has provided evidence for participation of stored Ca^{2+} in complex $[Ca^{2+}]_i$ signals that occur in human sperm stimulated with progesterone or NO, both products of the female tract and cumulus-oocyte complex (COC) (Publicover *et al*, 2007; Machado-Oliveira *et al.,* 2008). Pre-treatment of sperm with low dose progesterone (100pM) induced a small rise in $[Ca^{2+}]_i$ and altered the NO induced response from a tonic increase in $[Ca^{2+}]_i$ to a large transient which was often followed by oscillations (Machado-Oliveira *et al*., 2008). Similar results are reported in this study, low dose progesterone caused a modest background (tonic) increase in Ca^{2+} 'leak' into the sperm leading to elevation of resting $[Ca^{2+}]_i$ and possible accumulation of Ca^{2+} into the neck/midpiece store. The response to 4-aminopyridine is altered in cells pre-treated with low dose progesterone to a transient increase in $[Ca^{2+}]_i$ which is followed by oscillations in ~15% of cells, providing further evidence of 4-aminopyridine induced store mobilisation in human sperm. 4-aminopyridine induced Ca^{2+}

release from brain and SR microsomal vehicles provides evidence that this compound can activate intracellular Ca^{2+} stores mobilisation.

Stored Ca^{2+} and 4-aminopyridine-induced hyperactivation.

CASA was used to examine whether mobilisation of stored Ca^{2+} might contribute to the strong hyperactivating action of 4-aminopyridine on human sperm. The ability of 4-aminopyridine dramatically to increase the proportion of hyperactivated cells was not inhibited by simple omission of Ca^{2+} from the medium, the ability of the drug to induce hyperactivation being, if anything, enhanced (figure 5.3a). Furthermore, following brief re-suspension in EGTA-buffered saline (~2 mins), application of 2mM 4-aminopyridine induced hyperactivation in a proportion of cells only slightly lower than that in controls (standard medium; figure 5.3b). Induction of hyperactivation by 4-aminopyridine was truncated in cells re-suspended in EGTA-buffered medium, lasting for a maximum of 12 min (figure 5.3b) even though motility of the cells persisted for 20-30 min (figure 5.3c). The decay of 4-aminopyridine-induced elevation of $[Ca^{2+}]_i$ in the presence of EGTA appeared to be more rapid than the decay of hyperactivation in 'parallel' CASA experiments (figure 5.3b), but this probably reflects the fact that, for CASA, the cells were pelleted and re-suspended in EGTA buffered medium. Though this procedure was done with care to minimize carry over of sEBSS in the pellet, $[Ca^{2+}]_o$ may well have been slightly less stringently buffered in these experiments. It appears that mobilisation of Ca^{2+} from a labile, EGTA-sensitive store, contributes to the ability of 4-aminopyridine to induce hyperactivation. These findings are consistent with previous reports that extra-cellular calcium is not a requirement for initiation of hyperactivation in bovine and murine sperm. Suarez and colleagues concluded that mobilisation of stored Ca^{2+} (possibly from the redundant nuclear envelope) is sufficient to initiate but cannot sustain hyperactivated motility (Ho and Suarez 2001b; Marquez *et al*, 2007; Olson *et al.*, 2010; Chang and Suarez, 2010).

Inhibition of sperm intracellular Ca^{2+} store channels and 4-aminopyridine-induced $[Ca^{2+}]_i$ response

In an attempt to inhibit intracellular Ca^{2+} store release cells were treated with a local anesthetic tetracaine (1mM for 10mins) prior to application of 4-aminopyridine (2mM) Tetracaine (1mM) has previously been shown to inhibit ryanodine receptor calcium release in brain microsomal membranes (Martin *et al.,* 1993) and diminish motility in human sperm (2mM, tetracaine) (Teves *et al*., 2009).

Tetracaine contains an aromatic amine (pKa8.3) and like 4-aminopyridine its activity and membrane permeability are dependent on the ionisation state of the compound. At higher extracellular pH, the compound is membrane permeable but the charged compound is active intracellular. Pre-treatment of cells with tetracaine did not significantly effect the response to 4-aminopyridine, the majority of cells responded with a sustained increase in $[Ca^{2+}]_i$ fluorescence. However removal of the inhibitor altered the 4-aminoyridine response to a transient increase in $[Ca^{2+}]_i$ fluorescence (figure 5.7a), possibly due to store mobilisation. This response was absent in experiments where cells continue to be exposed to tetracaine (figure 5.7b). However, tetracaine does not specifically inhibit intracellular Ca^{2+} release channels, this compound also affects Ca^{2+}-ATPases, sodium potassium ATPase (NaK-ATPases), mitochondrial membrane integrity and estrogen receptor all of which are present in mammalian sperm cells.

4-aminopyridine induced $[Ca^{2+}]_i$ response is not due to mobilisation of mitochondrial Ca^{2+} in human sperm

The mitochondria of mammalian sperm can accumulate Ca^{2+} in the matrix compartment *in situ* (Wennemuth *et al*., 2003b) through the mitochondrial Ca^{2+} uniporter (MCU) and controlled release occurs through the Na^+ Ca^{2+} exchanger (NCX) (Bernardi *et al.,* 1999). Excessive Ca^{2+} accumulation can lead to activation of the permeability transition pore, which permits release

of apoptotic factors leading to cell death (Jeong and Seol, 2008). Previously, we have shown that mitochondrial Ca^{2+} uptake is not involved in store-mediated calcium oscillations (stimulated with low dose progesterone or NO) (Machado-Oliveira *et al.,* 2008). We report that uncoupling of mitochondrial respiration (with 2,4 dinitrophenol) did not inhibit the 4-aminopyridine induced tonic increase in $[Ca^{2+}]_i$ (figure 5.8b). These results are in agreement with other studies which have demonstrated that mitochondrial function did not contribute significantly to store mobilisation (Ho and Suarez, 2003; Machado-Oliveira *et al*., 2008). Evidence suggests that excessive K^+ efflux and intracellular K^+ depletion are key early stages in apoptosis. Mitochondrial viability (retention of rhodamine 123 as described in Ogunbayo *et al*., 2008), did not differ significantly in the presence or absence of 4-aminopyridine (figure 5.8c). Furthermore recent investigations on cerebellar granule neurons demonstrated that 4-aminopyridine did not significantly depolarise the resting mitochondrial membrane potential but enhanced cell viability. In summary 4-aminopyridine does not significantly mobilise mitochondrial Ca^{2+} or induce cell death (apoptosis).

4-aminopyridine does not induce the acrosome reaction in human sperm

The acrosomal store is strongly implicated in regulation of exocytosis of the acrosomal vesicle itself (acrosome reaction) (O'Toole *et al*., 2000; Herrick *et al*, 2005). Despite the sustained effect of 4-aminopyridine on $[Ca^{2+}]_i$, we observed no increase in the proportion of acrosome reacted cells (figure 5.9) further illustrating the ability of sperm to maintain specific 'targeting' of responses mediated through $[Ca^{2+}]_i$ signalling (Publicover *et al.*, 2007).

Conclusion

4-aminopyridine caused reversible, repeatable mobilisation of Ca^{2+} stored in the neck/midpiece accompanied by parallel, reversible and repeatable asymmetric bending of the flagellum. Investigations of the Ca^{2+} dependence of 4-aminopyridine-induced hyperactivation clearly

show that, as in mouse and bovine sperm, mobilisation of stored Ca^{2+} is sufficient to initiate hyperactivation, but also suggest that store-operated Ca^{2+} influx contributes to maintenance of this mode of motility.

Previous studies on the effects of 4-aminopyridine on cellular Ca^{2+}-homeostasis have shown that the drug mobilises stored Ca^{2+} in other cell types, though the mechanism by which this may be achieved is not clear (Guse *et al.*, 1994; Ishida and Honda, 1993; Gobet *et al*., 1995; Grimaldi *et al*., 2001). These results demonstrate that 4-aminopyridine induced store mobilisation is non-specific having a similar effect on IP_3R and RyR in microsomal preparations. It seems likely that 4-aminopyridine induced Ca^{2+} mobilisation (in Ca^{2+} free conditions) of the neck/midpiece store would involve activation of the ryanodine receptor channel or IP_3R channel although the exact mechanism and target remains unclear. Store depletion may also trigger Ca^{2+} influx via flagellar store operated calcium mechanisms to refill the depleted neck region store.

CHAPTER SIX

4-AMINOPYRIDINE AND CAPACITATIVE Ca^{2+} ENTRY

6.1 Abstract

4-aminopyridine, at concentrations comparable with those used here, strongly potentates capacitative Ca^{2+} entry (CCE) in astrocytes and skeletal muscle cells (Grimaldi *et al.*, 2001). 4-aminopyridine has previously been shown to induce store mobilisation in microsomal vesicles and in human sperm briefly treated with EGTA buffered saline (chapter 5). In experiments designed to activate store-operated channels (SOCs) (treatment with bis-phenol or incubation in EGTA buffered saline) this report demonstrates that Ca^{2+} influx in the presence of 4-aminopyridine was enhanced. Furthermore pharmacological inhibition of SOC (with 2-APB and SFF-96365) had a significant effect on the 4-aminopyridine induced response. In the presence of inhibitors, 4-aminopyridine induced a transient $[Ca^{2+}]_i$ response, the SOC sustained influx was inhibited in a population of cells. Significantly this effect was absent when CatSper channel influx was activated by cytoplasmic alkalinisation (2-APB pretreatment did not significantly effect the NH_4Cl induced $[Ca^{2+}]_i$ response). These results indicate that the 4-aminopyridine induced $[Ca^{2+}]_i$ store mobilisation response may potentiate SOC within a population of human sperm.

6.2 Introduction

Mammalian sperm use Ca^{2+}-signalling to regulate or contribute to the regulation of many different functions including hyperactivation (Ho and Suarez, 2003; Jimenez-Gonzalez *et al*, 2006; Publicover *et al*, 2007).

Numerous studies have demonstrated that the CatSper channel proteins are required for normal hyperactivated motility in mouse sperm (Ren *et al*., 2001; Quill *et al.,* 2003; Carlson *et al.,* 2005; Qi *et al.,* 2007; Xia *et al*., 2007). In addition, studies on sperm from rodents, bulls and humans have provided strong evidence that Ca^{2+} stored in the region of the sperm neck contributes to regulation of flagellar activity (Ho and Suarez, 2001b, 2003; Naaby-Hansen *et al* 2001). Triggering mobilisation of this stored Ca^{2+} can induce hyperactivation in mouse sperm lacking CatSpers (Marquez *et al*., 2007). Recent data from this laboratory has demonstrated the presence of stromal interacting protein 1 (STIM1, a protein containing a calcium sensor located in the intracellular calcium store) and Orai proteins (a protein that forms a calcium channel in the plasma membrane) in human sperm, immunolocalised primarily to the neck/midpiece (Lefièvre *et al*., in prep), both these proteins are required for calcium release activated channel (CRAC) activity. Since capacitative calcium entry (CCE) has been observed in sperm (Blackmore 1993; Yoshida *et al*., 2003; Harper *et al*., 2003, 2005; Bedu-Addo *et al*., 2007; Costello *et al*., 2009), store mobilisation might even recruit this mechanism to supplement Ca^{2+} influx through CatSper channels (Olson *et al.,* 2010). Alternatively this pathway for induction of hyperactivation may play a separate role from that mediated by CatSpers. Observations of sperm, primarily in humans but also other mammals, suggest that the cells can 'switch' rapidly and probably repeatedly between activated and hyperactivated motility (Katz *et al*., 1989; Pacey *et al*., 1995a, 1995b; Mortimer and Swan 1995), a behaviour that may mediate interactions with cells of the female tract (Pacey *et al*., 1995a, 1995b). Mobilisation of stored Ca^{2+}, which is rapidly reversible and localised, may be better suited to such transient regulation of motility whereas pH regulated activation of CatSpers could support the prolonged

hyperactivation that permits 'permanent' detachment from the cells of the female tract (Ho *et al.,* 2009) and penetration of the zona.

Results have shown that 4-aminopyridine causes dramatic hyperactivation of human sperm, whereas cytoplasmic alkalinisation with 25mM NH_4Cl which hyperactivates bovine sperm (Marquez and Suarez, 2007) had a negligible effect on human sperm motility (chapter 3). Furthermore 4-aminopyridine can induce store mobilisation in microsomal vesicles although the exact mechanism involved remains unclear (chapter 5). Since 4-aminopyridine has been shown to potentiate capacitative calcium entry (CCE) in a number of cell types (Grimaldi *et al.*, 2001) store mobilisation was investigated and whether the 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response involves CCE.

The effects of a number of store operated Ca^{2+} channel (SOC) inhibitors (high dose 2-APB and SKF-96365) and agonist (low dose 2-APB) on 4-aminopyrdine induced $[Ca^{2+}]_i$ signals in OGB loaded spermatozoa was examined.

6.3 Materials and Methods

6.3.1 Materials

As described in chapter two. 1-[β-[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCL (SKF-96365 stock conc. 10mM was made up in H_2O) purchased from Calbiochem (UK). bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)methane (bis-phenol, stock conc. 20mM made up in DMSO) was purchased from Pfaltz and Bauer, Waterbury, CT. 2-aminoethoxydipenylborane (2-APB, stock conc. 200mM, made up in DMSO) was from Sigma Aldrich (Poole, Dorset, UK). All chemicals were cell-culture-tested grade where available.

Nominal Ca^{2+} free saline (no added Ca^{2+} , ($[Ca^{2+}]_o \leq 5\mu M$)) and EGTA buffered medium (5mM Ca^{2+}, 6mM EGTA, 25^oC ($[Ca^{2+}]_o$ 10^{-7} M)) were prepared as described in chapter 5. The calcium concentration calculator Maxchelator (V2.2) written by Chris Patton from Stanford University (http://www.stanford.edu/~cpatton/maxc.html) was used to calculate the free calcium concentration in saline containing EGTA.

6.3.2 Spermatozoa preparation and capacitation

As described in chapter two.

6.3.3 Evaluation of sperm hyperactivation

As described in chapter two.

6.3.4 Single cell Imaging

As described in chapter two.

6.3.5 Data Analysis

As described in chapter two.

6.4 Results

6.4.1 4-aminopyridine enhanced Ca^{2+} influx in Ca^{2+} store depleted sperm cells

To investigate whether mobilisation of stored Ca^{2+} (potentially activating store-operated Ca^{2+} influx mechanisms) might enhance the cells response to the 4-aminopyridine response, we used a protocol designed to evacuate the sperm's intracellular stores. Since high, non-specific doses of thapsigargin are required to mobilise stored Ca^{2+} in sperm, attempts to assess $[Ca^{2+}]_i$ may reflect effects on other Ca^{2+} pumps. Cells were exposed to EGTA-buffered medium ($<10^{-7}$M Ca^{2+}), conditions which cause a rapid fall in $[Ca^{2+}]_i$ and consequent depletion of Ca^{2+} from the sperm's intracellular stores (Harper *et al*, 2004; Bedu-Addo *et al*., 2007). A proportion of sperm cells briefly (~2 mins) treated with EGTA buffered saline ($[Ca^{2+}]_o$ at 10^{-7}M Ca^{2+}), respond to 2mM 4-aminopyridine with a small transient increase in $[Ca^{2+}]_i$ fluorescence (figure 5.2c), this response is lost when cells are continually superfused for longer periods (~10mins decreased $[Ca^{2+}]_i$ fluorescence by 36.3±4.1% below basal levels, figure 6.1a and b). Extracellular Ca^{2+} was re-introduced to the imaging chamber at a lower dose (10^{-5} M) to prevent saturation of the fluorescent signal (intracellular OGB saturated at 1-2μM Ca^{2+}) (Haugland, 2001). Restoration of extracellular Ca^{2+} resulted in a rapid recovery to baseline levels of $[Ca^{2+}]_i$ fluorescence (figure 6.1b, n=3 experiments, 128 cells). When the same experiments were performed in the presence of 4-aminopyridine $[Ca^{2+}]_i$ fluorescence increased 40-50% above baseline (figure 6.1a, n=5 experiments, 504 cells) a response maintained in the presence of 4-aminopyridine which promptly reversed upon washout.

Figure 6.1

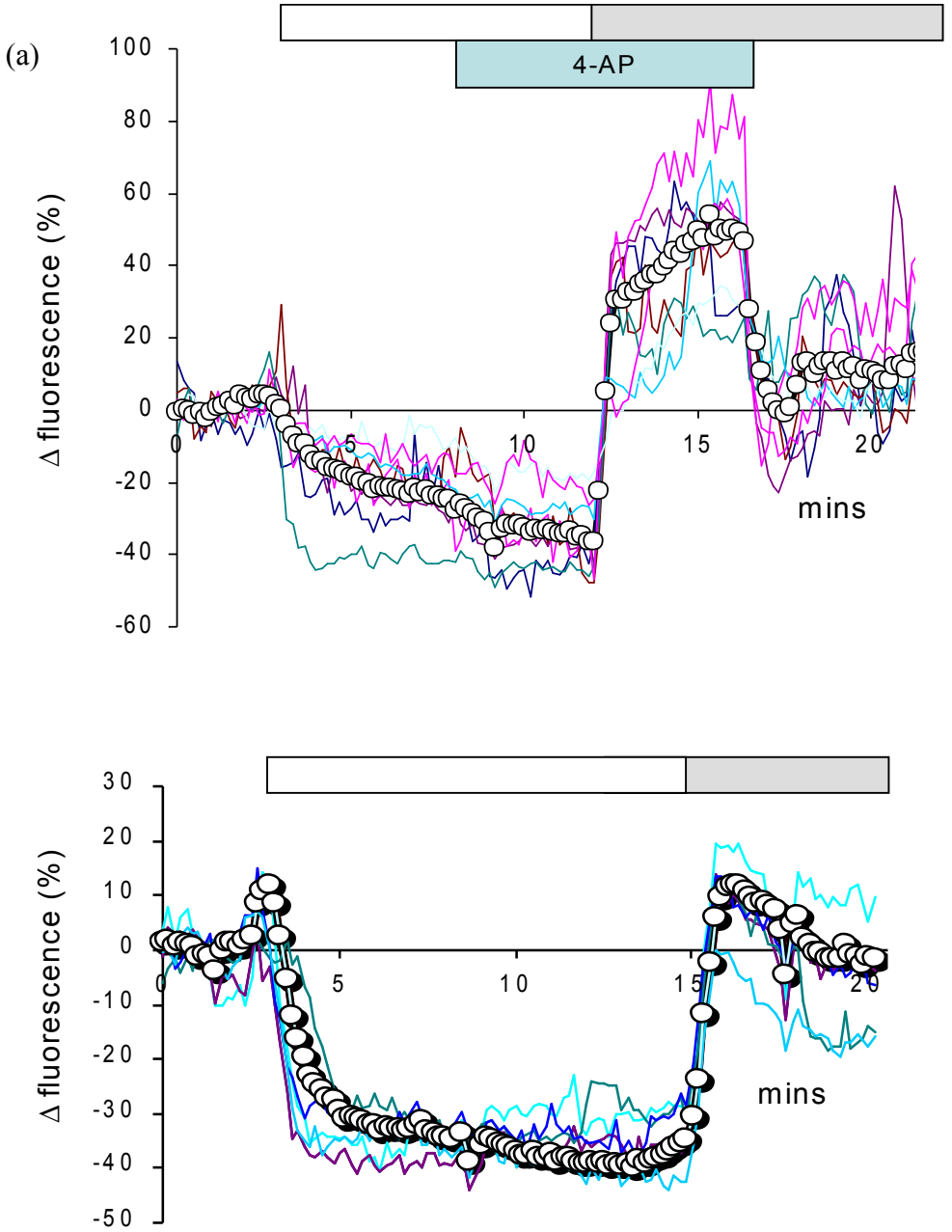

Figure 6.1. 4-aminopyridine enhanced Ca^{2+} influx in EGTA $[Ca^{2+}]_i$ depleted cells. **(a)** Plot showing cells pretreated with EGTA buffered saline ($<10^{-7}$M Ca^{2+}, white bar) to deplete stored calcium, decreased $[Ca^{2+}]_i$ fluorescent by 36.3% (n=5 experiments, 376 cells). Cells were exposed to 4-aminopyridine (blue bar) for ~5mins prior to the restoration of Ca^{2+} ($[Ca^{2+}]_o$ = 10^{-5} M, grey bar) which resulted in a ~50% increase in fluorescence above baseline. Responses of 7 representative cells are shown and also mean response (○-○) of all the 77 cells in the experiment. Upon 4-aminopyridine washout $[Ca^{2+}]_i$ fluorescence decreased rapidly. **(b)** Superfusion of cells with EGTA buffered saline to deplete stored calcium decreased $[Ca^{2+}]_i$ fluorescent by ~40% (n=3 experiments,

128 cells). Restoration of ($[Ca^{2+}]_o = 10^{-5}$ M, grey bar) resulted in a $[Ca^{2+}]_i$ fluorescence recovering to ~10% above baseline which promptly returned to basal levels. Responses of 5 representative cells are shown and also mean response (○-○) of all the 66 cells.

In sperm, high non-specific doses of thapsigargin (to inhibit SERCA) does not induce a rapid elevation of $[Ca^{2+}]_i$ but a gradual release, the effect is irreversible resulting in a loss of motility (Williams and Ford, 2003). In human cells data from this laboratory has shown that the neck/midpiece store is not mobilised by thapsigargin, but is sensitive to bis-phenol, an inhibitor of Ca^{2+} ATPases (both SERCA and SPCA) (Brown *et al*., 1994; Harper *et al*., 2001, 2005; Bedu-Addo *et al*., 2007). Bis-phenol mobilises stored Ca^{2+} in human sperm and prevents generation of store-mediated Ca^{2+}-oscillations (Harper *et al.*, 2005; Bedu-Addo *et al.*, 2007).

When cells were incubated in the presence of bis-phenol (20 μM) a sustained increase in $[Ca^{2+}]_i$, was observed due to impairment of Ca^{2+} clearance mechanisms (Brown *et al.,* 1994; Bedu-Addo *et al.*, 2007). An increase in fluorescence (calculated with respect to steady-state level in the presence of bis-phenol) upon application of 2mM 4-aminopyridine was not only still evident, but was enhanced, (mean = 52.9±0.9%; 597 cells from 5 experiments; figure 6.2a), significantly greater than the response in cells not pretreated with bis-phenol ($P<10^{-5}$). When these experiments were repeated in medium with no added Ca^{2+} ($[Ca^{2+}]_o \leq 5$ μM) (Harper *et al.*, 2004), to reduce Ca^{2+} influx at the plasma membrane, bis-phenol again caused a sustained (though smaller) rise in $[Ca^{2+}]_i$ (Bedu-Addo *et al.*, 2007), the effect of 4-aminopyridine was greatly reduced. The increase in fluorescence 5 min after application of 4-aminopyridine was just 12.2±0.5%, significantly smaller than in non-pretreated preparations (paired *t*-test, $P<0.0005$) and in some experiments was barely detectable (figure 6.2b).

Treatment with bis-phenol in the presence or absence of extracellular Ca^{2+} does not significantly enhance hyperactivation in human spermatozoa. Application of 4-aminopyridine

was required to significantly enhance hyperactivation (figure 6.2c). Examination of VCL (the total distance the sperm covered in the observation period) highlighted the potent effect of 4-aminopyridine on motility (increased from 85.0± 4.9 to 129.7±7.6 μm/sec in NCFsEBSS) an effect not mirrored in cells treated with bis-phenol. However, treatment with bis-phenol decreased % motility in both normal and nominal free Ca^{2+} saline (table 6.1) and with addition of 4-aminopyridine many cells were observed to 'freeze' with tightly bent flagella (arrested with angulated flagellum), which is typical cytoplasmic Ca^{2+} overload (apparently due to impaired Ca^{2+} clearance and Ca^{2+} influx).

Figure 6.2

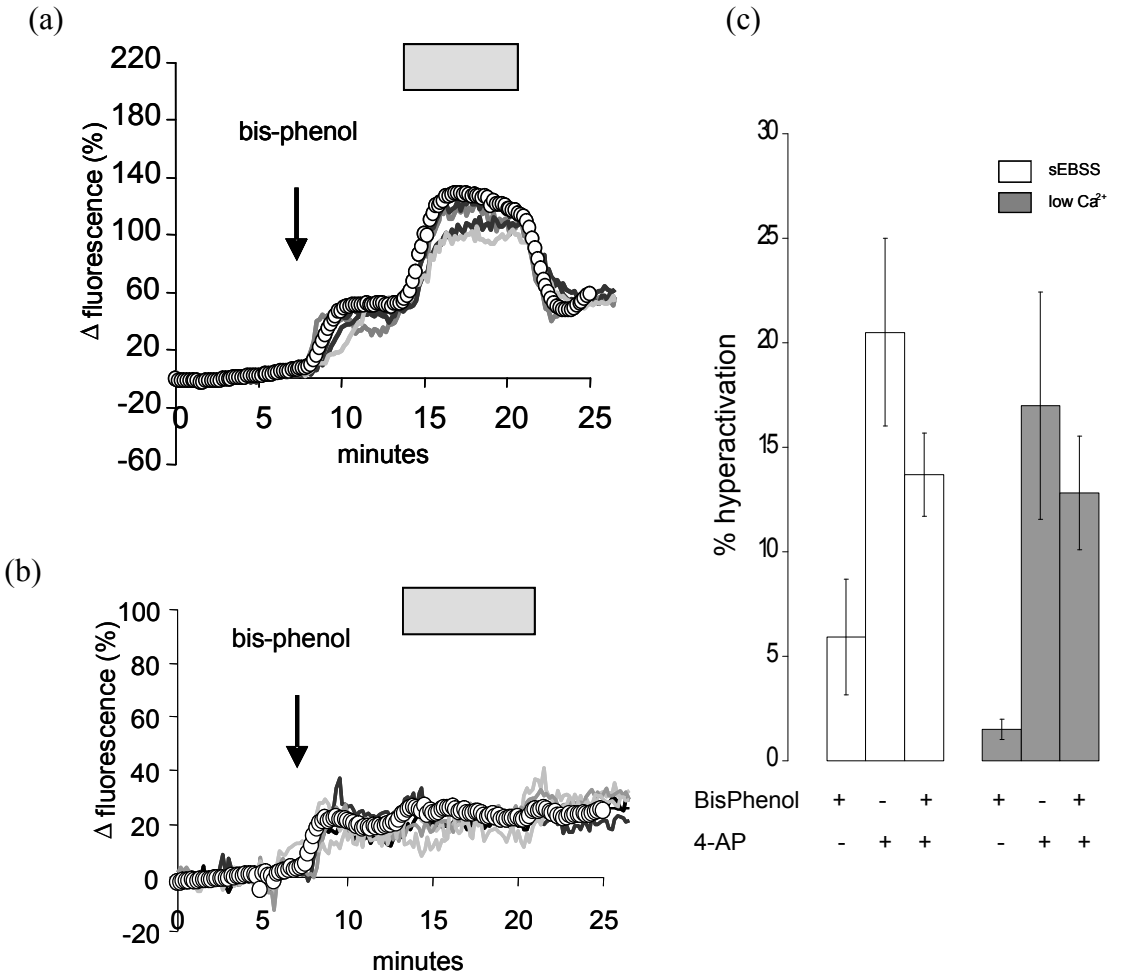

Table 6.1. Effect of bis-phenol (20µM) ± 4-aminopyridine (2mM) on sperm motility, VCL, ALH and LIN (mean±SEM; n=6donors) in normal saline and saline with no added Ca2+ ($[Ca^{2+}]_o$ ⩽5 mM)

	Motile(%)	VCL (µm/sec)	ALH (µm)	LIN (%)
Cntrl ($[Ca^{2+}]_o$ 1.8mM)	83.0±3.5	113.4±12.1	4.5±0.6	59.6±1.4
+ 4AP	81.2±3.6	149.5±8.2	6.1±0.3	49.6±1.4
+ bis-phenol	74.6±4.7	117.8±6.8	4.8±0.1	53.6±1.2
+ bis-phenol and 4AP	59.0±9.0	123.6±4.8	5.1±0.1	16.5±2.9
Cntrl ($[Ca^{2+}]_o$ ⩽5 µM)	75.8±4.6	85.0±4.9	3.4±0.2	64.2±1.4
+ 4AP	75.5±4.1	129.70±7.7	5.5±0.3	52.0±1.0
+ bis-phenol	62.0±2.6	87.6±3.1	4.0±0.2	55.0±0.9
+ bis-phenol and 4AP	53.2±6.1	115.2±9.1	5.4±0.5	46.3±1.4

Figure 6.2: Effect of prior mobilisation of stored Ca^{2+} (by inhibition intracellular Ca^{2+} store uptake with bis-phenol) on the response to 4-aminopyridine in normal and nominal Ca^{2+} free saline. **(a)** Pre-treatment of cells with bis-phenol in standard saline. Responses of 7 representative cells are shown and also mean response (○-○) of all the 150 cells in the experiment. Cells were exposed to 20 μM bis-phenol (arrow) for ≈5 min, causing a sustained elevation of $[Ca^{2+}]_i$ Application of 2mM 4-aminopyridine (grey bar) caused a further large rise in $[Ca^{2+}]_i$ which exceeded that seen in non-pretreated cells. This effect reversed upon washout of 4-aminopyridine. **(b)** Response to 4-aminopyridine in bis-phenol pretreated cells is dependent upon Ca^{2+} influx. Cells superfused with nominally Ca^{2+} free sEBSS (no added Ca^{2+}; $[Ca^{2+}] \leq 5$ μM) were exposed to 20 μM bis-phenol (arrow) for ≈5 min, causing a sustained elevation of $[Ca^{2+}]_i$ that was <50% of that seen in standard sEBSS (Harper *et al*., 2005). Application of 2mM 4-aminopyridine (grey bar) caused only a small further rise in $[Ca^{2+}]_i$, the effect being negligible in many cells. Responses of 6 representative cells are shown and also mean response (○-○) of all the 103 cells in the experiment. **(c)** Proportion of hyperactivated cells (mean ± SEM) in response of bis-phenol ± 4-aminopyridine in normal saline (white bars) and nominal Ca^{2+} free saline (grey bars). **Table 6.1** Effects of bis-phenol ± 4-AP on human sperm motility, VCL, ALH and LIN in standard or nominal Ca^{2+} free saline ($[Ca^{2+}]_o \leq 5$ μM) (mean ± SEM, n=6 donors).

6.4.2 Pharmalogical inhibition of store operated Ca^{2+} channels modified the 4-aminopyridine induced $[Ca^{2+}]_i$ response

Previous reports have shown that treatment of sperm with store-operated channel blockers (high dose 2-APB and SKF-96365) inhibits asymmetrical flagellar beating patterns and chemotaxis in ascidian and human sperm (Yoshida *et al.,* 2003; Teves *et al.,* 2009).

2-APB has a reported bimodal effect on CRAC channel activity, low doses (<10μM) enhance Ca^{2+} entry whereas high doses (<50μM) are inhibitory (Peinelt *et al*., 2008). Pre-treatment of sperm cells with 2-APB (10μM and 50μM) resulted in a slow tonic increase in $[Ca^{2+}]_i$ fluorescence of ~10% in ~50-60% of cells. Application of 4-aminopyridine induced a transient increase of $[Ca^{2+}]_i$ (amplitude 20-30%) in 50-60% of sperm (n=6 experiments, 596 cells, figure 6.3a) while the remaining cells (~30%) responded with a sustained increase in $[Ca^{2+}]_i$ (amplitude ~10%). The 4-aminopyridine induced transient response was absent from parallel control experiments, where the majority of cells (~80%) responded to with a sustained increase of 24.0±1.7% (n=3 experiments, 269 cells). The effect of 2-APB on the 4-aminopyridine induced response was independent of cytoplasmic alkalinisation. In parallel experiments where 4-aminopyridine was replaced with NH_4Cl (25mM), pre-treatment with 2-APB (10μM and 50μM) did not significantly alter the kinetics or amplitude of the NH_4Cl induced $[Ca^{2+}]_i$ response (figure 6.3b, n=7 experiments, 450 cells, paired *t*-test, $P>0.05$).

The compound SKF-96365 is widely used to block Ca^{2+} channels including store operated channels (Merritt *et al.*, 1990; Hirohashi and Vacquier, 2003). Treatment of sperm cells with SKF-96365 (10μM for 10 minutes, figure 6.3c, white bar) resulted in a increase in $[Ca^{2+}]_i$ of ~10±2.7% in the majority (~80%) of cells. Application of 4-aminopyridine (black arrow) induced a transient $[Ca^{2+}]_i$ fluorescence response in ~64% of cells of 26.8±5.8% (n=4experiments, 353 cells) (calculated with respect to steady-state level in the presence of

SKF-96365). In parallel control experiments (non-SKF-96365 treated) ~80% responded to 4-aminopyridine with a sustained response of 27.0±1.7% (n=4 experiments, 511 cells).

There was no significant correlation between the gradual tonic increase in $[Ca^{2+}]_i$ fluorescence (induced by 2-APB or SKF96395) with the 4-aminopyridine induced transient $[Ca^{2+}]_i$ response ($P>0.05$;. chi square test).

Figure 6.3

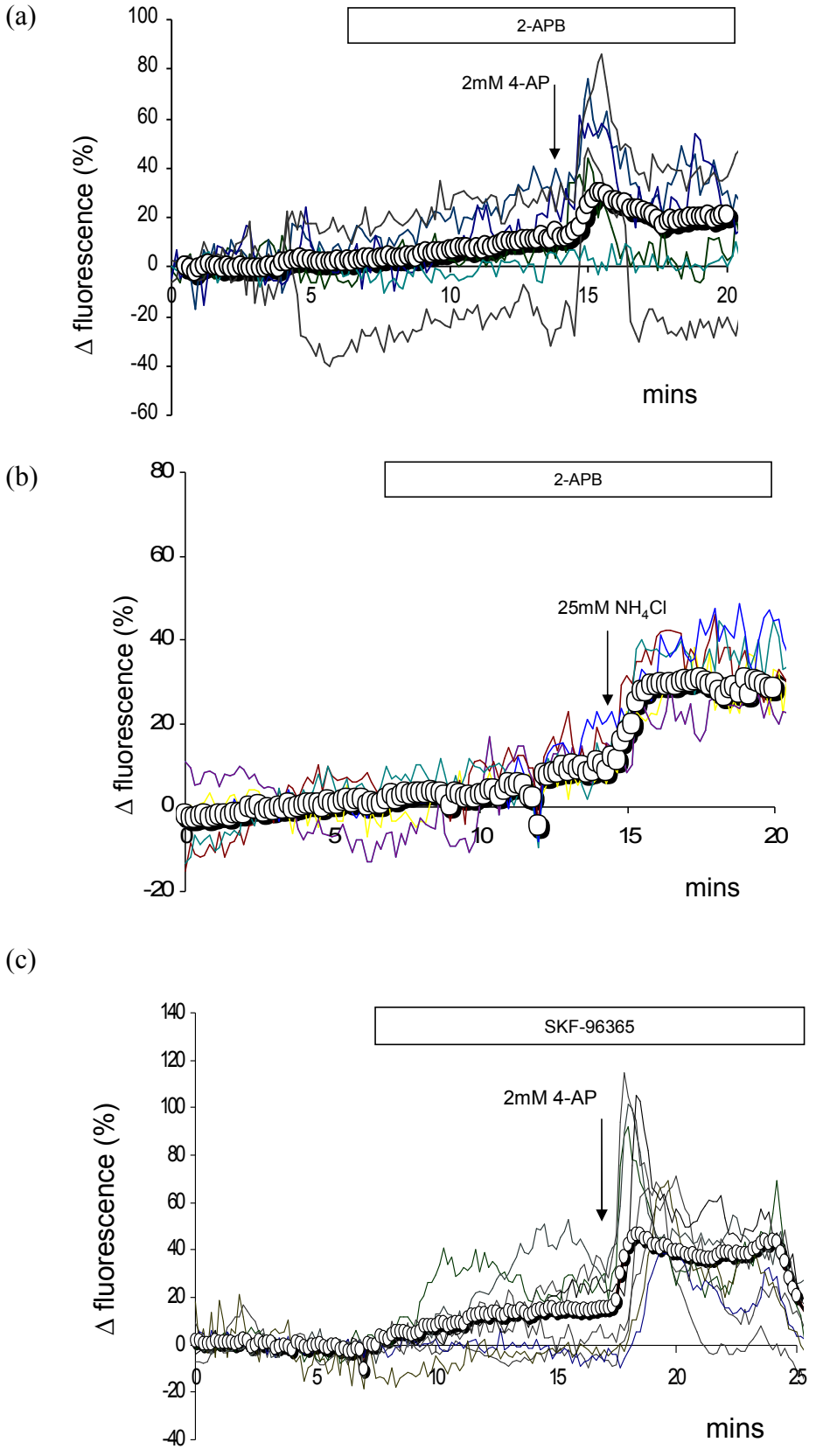
(a)
2-APB
2mM 4-AP
Δ fluorescence (%)
100
80
60
40
20
0
-20
-40
-60
0
5
10
15
20
mins
(b)
2-APB
25mM NH4Cl
Δ fluorescence (%)
80
60
40
20
0
-20
0
5
10
15
20
mins
(c)
SKF-96365
2mM 4-AP
Δ fluorescence (%)
140
120
100
80
60
40
20
0
-20
-40
0
5
10
15
20
25
mins

Figure 6.3. Pre-treatment with store operated channel (SOC) inhibitors (50µM 2-APB or 10µM SKF-96365) (white bar) altered the 4-aminopyridine induced $[Ca^{2+}]_i$ fluorescence response in a proportion of sperm cells. **(a)** 2-APB (50µM) pretreatment (10 mins, white bar) causes a tonic increase of $[Ca^{2+}]_i$ fluorescence in cells superfused with sEBSS. Upon application of 4-aminopyridine (arrow) many cells respond with a transient increase in $[Ca^{2+}]_i$ fluorescence cells. Responses of 5 representative cells are shown and also mean response (○-○) of all the 95 cells in the experiment. **(b)** Pre-treatment with 2-APB (50µM, for 10 mins, white bar) did not significantly alter the NH_4Cl (25mM, arrow) induced $[Ca^{2+}]_i$ fluorescence response (paired *t*-test, $P>0.05$). Responses of 6 representative cells are shown and also mean response (○-○) of all the 79 cells in the experiment. **(c)** SKF-96365 (10µM) pretreatment (10mins, white bar) causes a tonic increase of $[Ca^{2+}]_i$ fluorescence in cells superfused with standard saline. Upon application of 4-aminopyridine ~60% of cells respond with a transient increase in $[Ca^{2+}]_i$ fluorescence cells. Responses of 10 representative cells are shown and also mean response (○-○) of all the 94 cells in the experiment.

6.5 Discussion

The role of hyperactivated motility is not restricted to penetration of the zona pellucida. It facilitates mucus penetration (Suarez and Ho, 2003a, 2003b) and aids detachment of cells bound to the walls of the female tract (Suarez and Pacey, 2006). The ability to control and rapidly 'switch' hyperactivation is likely to be important in interaction of human and animal sperm with the fluids and cells of the female tract (Pacey *et al.*, 1995a, 1995b; Suarez and Pacey, 2006). Increased pH_i, leading to activation of CatSper channels (Navarro *et al.,* 2007), could provide prolonged hyperactivation in capacitated cells at the site of fertilisation and may even cause hyperactivation of cells upon binding to zona proteins (Xia and Ren, 2009b). In contrast, activation by Ca^{2+} store mobilisation and capacitative calcium entry (CCE) may be better suited to generation of bursts of hyperactivation 'on demand', regulated by a range of factors encountered at various points within the female tract such as progesterone and nitric oxide (Harper *et al*., 2004, 2005; Machado-Oliveira *et al.*, 2008). Marquez *et al.,* (2007) demonstrated that store mobilisation induced hyperactivation in CatSper null sperm providing strong evidence that store mobilisation and CCE play a role in regulating flagellar activity.

CCE is carried by store-operated channels and the calcium release-activated Ca^{2+} current (I_{CRAC}) is the best characterised. The molecular components of I_{crac} have been identified, depletion of stored calcium is sensed by the ER membrane protein STIM which reorganize into distinct puncta to interact with Orai subunits to form a functional Ca^{2+} channel (Frischauf *et al.,* 2008). The ubiquitously expressed STIM and Orai proteins have recently been identified in human sperm, immunolocalised primarily to the posterior neck/midpiece region (Lefièvre *et al.*, in prep). Due to the small size of sperm there seems little scope for STIM reorganisation indeed Ca^{2+} mobilisation from the posterior head/midpiece store would be negligible, it is likely that STIM and Orai proteins are already primed and in place. The transient receptor potential (TRP) channels activated by STIM1 can act as store operated Ca^{2+} channels, several TRP channels have been identified in human sperm (TPRC and TRPM) are linked with

motility regulation and the acrosome reaction (Castellano *et al.,* 2003; De Blas *et al.,* 2009).

4-aminopyridine potentiates CCE in store depleted human sperm

4-aminopyridine, at concentrations comparable with those used here, strongly potentiated CCE in astrocytes and skeletal muscle cells (Grimaldi *et al*., 2001). To investigate the effect of 4-aminopyridine on CCE spermatozoa were exposed to EGTA-buffered medium ($<10^{-7}$M Ca^{2+}), conditions which cause a rapid fall in $[Ca^{2+}]_i$ and concomitant depletion of Ca^{2+} from the sperm's intracellular stores (Harper *et al,* 2004; Bedu-Addo *et al*., 2007) before application of 2mM 4-aminopyridine. Restoration of extracellular calcium (10^{-5}M) caused a significant sustained increase in $[Ca^{2+}]_i$ fluorescence which was maintained in the presence of 4-aminopyridine. CRAC channels have high Ca^{2+} selectivity but in calcium free conditions readily allow Na^+ permeation until extracellular Ca^{2+} is restored (Parekh and Putney, 2005). Torres-Flores and colleagues (2008) recently demonstrated Na^+ dependent depolarisation of human spermatozoa when $[Ca^{2+}]_o$ $<10^{-8}$ M due to Na^+ permeation presumably through a Ca^{2+} channel. Calcium restoration resulted in E_m hyperpolarisation and a large Ca^{2+} influx although the identity of the Ca^{2+} influx channel is unclear (Gonzalez-Martinez *et al*., 2002; Gonzalez-Martinez, 2003; Trevino *et al.,* 2004; Torres-Flores *et al.,* 2008). Since $[Ca^{2+}]_o$ was maintained at 10^{-7}M in these experiments it is probable that the 4-aminoyridine enhanced Ca^{2+} influx was SOC dependent however depolarisation induced Ca^{2+} influx may occur through other channels including the CatSper channels or voltage-gateed Ca^{2+} channels (Darszon *et al.,* 2007).

The site within human sperm at which the $[Ca^{2+}]_i$ response to 4-aminopyridine initiates, the sperm neck/midpiece (figure 5.1a), is consistent with previous reports of a store in the neck region of mammalian sperm (Ho and Suarez, 2001b, 2003; Naaby-Hansen *et al*., 2001; Harper *et al*., 2004). In human cells we have shown that this store is not mobilised by SERCA inhibition, but is sensitive to bis-phenol (Harper *et al.*, 2001, 2005). In cells superfused with

sEBSS the elevation of $[Ca^{2+}]_i$ induced by 4-aminopyridine was strongly enhanced by pre-treatment with 20 μM bis-phenol (figure 6.2a), probably reflecting induction of Ca^{2+} influx by 4-aminopyridine against a background of impaired Ca^{2+} clearance due to the effect of bis-phenol on the plasma membrane Ca^{2+}-ATPase (Brown *et al*., 1994; Bedu-Addo *et al*., 2007). In contrast, when these experiments were repeated in medium with no added Ca^{2+} ($[Ca^{2+}]_o \leq 5$ μM) the action of 4-aminopyridine in bis-phenol pretreated cells was greatly reduced compared to parallel experiments without pre-treatment, sometimes being barely detectable (compare responses in figures 6.2a and 6.2b). The proportion of 4-aminopyridine induced hyperactivated cells was reduced in the presence of bis-phenol, although many cells arrested with angulated flagellum (Gibbons and Gibbons, 1980; Sale, 1986).

Regardless of the method used to activate CCE (store mobilisation with bis-phenol or EGTA induced $[Ca^{2+}]_i$ depletion) the presence of 4-aminopyridine maintained a considerably enhanced influx of calcium which reversed upon washout.

Inhibition of store operated channels altered the 4-aminopyridine induced $[Ca^{2+}]_i$ response

Despite the physiological importance of store operated channels only a few pharmacological tools are available including 2-APB and SKF-96365 (Parekh and Putney, 2005). The reported bimodal effect of 2-APB on CRAC current are complex with potentiation at low doses (5-10μM) whereas higher dose (>10μM) are inhibitory (Prakriya and Lewis, 2001; Parekh and Putney, 2005; Peinelt *et al*., 2008). The reported dual effect of 2-APB was not evident in these experiments as both low and high dose 2-APB (10μM and 50μM) altered the 4-aminopyridine response in ~50-60% of cells to a transient which indicated store mobilisation. Human sperm may be particularity sensitive to the effects with 10μM 2-APB having an inhibitory effect on Ca^{2+} influx. Recent data by Lefièvre *et a*l., (in prep) demonstrates that 2-APB (5μM) enhanced CCE in EGTA $[Ca^{2+}]_i$ depleted human sperm and significantly enhanced the progesterone

induced transient response. The effects of high dose 2-APB (50μM) are complex and has different effects depending on the molecular components of I_{CRAC} (Peinelt *et al.* 2008).

Previously results have shown that increased pH_i induced by NH_4Cl (25mM) enhanced $[Ca^{2+}]_i$ fluorescence in human sperm presumably apparently due to CatSper channels activation (chapter 3). Pre-treatment with 2-APB (10 or 50μM) had little effect on NH_4Cl induced $[Ca^{2+}]_i$ fluorescence response indicating that CatSper dependent Ca^{2+} influx was unaffected. These results signify that the sustained part of the 4-aminopyridine induced Ca^{2+} response is carried by SOC which is absent in the presence of 2-APB.

Similarly pre-treatment with SKF-96365 altered the 4-aminopyridine induced $[Ca^{2+}]_i$ response in many cells. These results indicate that the sustained part of the 4-aminopyridine induced response is dependent on SOC. Both 2-APB and SKF-96365 have non-specific responses which may account for the increase in $[Ca^{2+}]_i$ fluorescence prior to the application of 4-aminopyridine.

Conclusion

This report provides further evidence that SOC play a role and contribute to the 4-aminopyridine induced $[Ca^{2+}]_i$ response in human speramtozoa. Whether mobilisation of stored Ca^{2+} may affect CatSper channels is not known. It is possible that the different sources of Ca^{2+} have different functions in human sperm, transient bursts of hyperactivation facilitated by Ca^{2+} mobilisations and concomitant CCE whereas prolonged hyperactivation is facilitated by CatSper activation.

CHAPTER SEVEN

GENERAL DISCUSSION

It has been 40 years since Ryuzo Yanagimachi observed that hamster sperm swimming in the oviduct exhibited a vigorous motility pattern which he termed hyperactivation (Yanagimachi, 1970). Since then hyperactivation has been studied extensively however the physiological factor(s) that activate hyperactivation *in vivo* remains elusive. Due to ethical constraints knowledge of *in vivo* human sperm hyperactivation is particularly limited.

Previous data from this laboratory demonstrated that 4-aminopyridine is a potent inducer of hyperactivation in human spermatozoa (Gu *et al*., 2004). The aim of this thesis was to elucidate the effects of 4-aminopyridine on $[Ca^{2+}]_i$, examine its target and mechanism of action and to assess the importance of store mobilisation on $[Ca^{2+}]_i$ and its potential to regulate human sperm hyperactivation.

Key findings

This report examined in detail the effects of 4-aminopyridine on $[Ca^{2+}]_i$ fluorescence and hyperactivation on human spermatozoa. Ca^{2+} imaging showed that treatment with 4-aminopyridine induced a parallel elevation of $[Ca^{2+}]_i$, (chapter 2) which initiated at the sperm neck/midpiece and was associated with asymmetric flagellar bending in this region (section 5.4.1). 'Clamping' of E_m at E_K (1 μM valinomycin) had no effect on these actions of 4-aminopyridine (section 2.4.5).

Elevation of $[Ca^{2+}]_i$ and hyperactivation showed similar dose-dependence and both were enhanced at elevated pH_o, which facilitates entry of 4-aminopyridine across the plasmalemma (chapter 3). Since various studies have shown that a pH-stimulated Ca^{2+} increase in sperm, triggers hyperactivation (Mújica *et al*., 1994; Marquez and Suarez, 2004, 2007) the effects of 4-aminopyridine on cytoplasmic alkalinisation was examined (chapter 3). Assessment using BCECF confirmed that 2mM 4-aminopyridine caused an immediate increase in pH_i of ≈0.2 units in human sperm. To investigate whether cytoplasmic alkalinisation of human sperm might underlie the effect of 4-aminopyridine on $[Ca^{2+}]_i$ and hyperactivation NH_4Cl was used directly to increase pH_i. Exposure of sperm to NH_4Cl had an almost identical effect on pH_i and also caused a sustained increase in $[Ca^{2+}]_i$ only slightly less than that seen with 2mM 4-aminopyridine. However, there was negligible induction of hyperactivation compared to cells treated with 4-aminopyridine. Treatment of sperm with propionate (to decrease pH_i and inhibit CatSper channel) did not significantly effect 4-aminopyridine induced hyperactivation but enhanced the effect of 4-aminopyridine on $[Ca^{2+}]_i$. In summary the 4-aminopyridine induced hyperactivation response in human sperm is not solely pH dependent.

The sperm specific pH-sensitive flagellar CatSper channels have been shown to be an important contributor to hyperactivation in mouse spermatozoa (Ren *et al*., 2001; Quill *et al*., 2001). The effect of calcium channel inhibition (with Ni^{2+}, Cd^{2+} and ruthenium red) on 4-aminopyridine induced hyperactivation was therefore investigated (chapter 4). Inhibition of CatSper channels did not occlude the 4-aminopyridine induced hyperactivation in a population of human spermatozoa. The application of 4-aminopyridine to cells briefly pre-treated with Ni^{2+} triggered a transient increase in $[Ca^{2+}]_i$ fluorescence indicative of stored Ca^{2+} mobilisation in a small proportion of cells. These results provide further evidence that 4-aminopyridine induced hyperactivation is not solely dependent on CatSper dependent calcium influx. The presence of a population of 4-aminopyridine responsive cells suggest that store mobilisation

and channels resistant to the divalent metals (Ni^{2+} and Cd^{2+}) play a significant role in human sperm hyperactivation.

Evidence suggests that Ca^{2+} store mobilisation plays a key role in hyperactivation (Mareqez *et al*., 2007). In low Ca^{2+} saline the ability of 4-aminopyridine to cause an elevation of $[Ca^{2+}]_i$ was similar to that seen in standard saline (chapter 5). Application of 4-aminopyridine to cells briefly (4-5min) exposed to EGTA-buffered saline ($<10^{-7}$ M Ca^{2+}) induced a $[Ca^{2+}]_i$ transient. Hyperactivation induced by 4-aminopyridine was similarly transient (10-15min) in EGTA-buffered saline, though motility persisted for 20-30 min. 4-aminopyridine acted as a non-specific intra-calcium store agonist however it remains unclear how this compound triggers store mobilisation in microsomal vesicles. In conclusion intracellular Ca^{2+} store mobilisation contributes to 4-aminopyridine induced hyperactivation in human sperm.

4-aminopyridine, at concentrations comparable with those used here, strongly potentates capacitative Ca^{2+} entry (CCE) in astrocytes and skeletal muscle cells (Grimaldi *et al.*, 2001). In experiments designed to activate store-operated channels (SOCs) (treatment with bis-phenol or incubation in EGTA buffered saline) this report demonstrates that Ca^{2+} influx in the presence of 4-aminopyridine was enhanced (chapter 6). Furthermore pharmacological inhibition of SOC (with 2-APB and SFF-96365) had a significant effect on the 4-aminopyridine induced response. In the presence of inhibitors, 4-aminopyridine induced a transient $[Ca^{2+}]_i$ response, the SOC sustained influx was inhibited in a population of cells. Significantly this effect was absent when CatSper channel influx was activated by cytoplasmic alkalinisation (2-APB pretreatment did not significantly effect the NH_4Cl induced $[Ca^{2+}]_i$ response). These results indicate that the 4-aminopyridine induced $[Ca^{2+}]_i$ store mobilisation response may potentiate SOC within a population of human sperm.

In summary, this report demonstrates that the elevation of $[Ca^{2+}]_i$ that occurs upon mobilisation from a store in the sperm neck/mid-piece region regulates hyperactivation at least in a population of cells.

Why would human sperm cells require two mechanisms for hyperactivation? Could the different sources of Ca^{2+} have different functions?

The female tract represents a formidable barrier for sperm where numerous poorly understood interactions occur to ensure that only sperm of good quality, characterised by normal morphology and vigorous motility, will meet the oocyte (figure 7.1a, Suarez and Pacey, 2006). Human sperm are deposited in the vagina but only ~1% of sperm are retained by the female reproductive tract (~95% is lost following coitus due to 'flowback' and ~4% trapped by cervical mucus). This suggests that the female is exercising sperm selection favouring particular sperm from the ejaculate (Suarez and Pacey, 2006). In other species such as the pig, boar sperm are ejaculated directly into the uterine cavity hence female sperm selection is likely to occur elsewhere. The role of hyperactivated motility is not restricted to penetration of the zona pellucida. It also facilitates mucus penetration (Suarez and Dai,1992; Suarez and Ho, 2003a; 2003b) and aids detachment of cells bound to the walls of the female tract (Suarez and Pacey, 2006; Suarez, 2007; Ho *et al*., 2009). The ability to control and rapidly 'switch' hyperactivation is likely to be important for interaction of human and animal sperm with the fluids and cells of the female tract (Pacey *et al*., 1995a, 1995b; Suarez and Pacey, 2006). The utero-tubal junction presents another major anatomical challenge. In some mammals (rabbits, pigs, cows) the narrow mucus filled lumen is complicated by mucosal folds, in mouse/rat the junction is patent for only a short time after coitus, whereas in humans the entrance is fairly simple. As sperm pass through the utero-tubal junction they enter the tubal isthmus which serves as a sperm storage reservoir by binding sperm tightly to the endosalpingeal (oviductal) epithelium (De Mott and Suarez, 1992; Suarez, 2002a, 2007, 2008b). The storage reservoir

preserves sperm viability/fertility until ovulation and may serve to prevent polyspermic fertilisation (Hunter and Leglise, 1971; Suarez *et al*., 1991; Pollard *et al*., 1991). Capacitation associated changes within the sperm plasma membrane reduce binding affinity and hyperactivation is required to provide the force to gradually free sperm cells from endosalpingeal epithelium (De Mott and Suarez, 1992; Ho *et al*., 2009). The human oviduct differs from other mammals in that no distinct anatomical tubal reservoir has been located. Although human sperm do associate intermittently with oviduct epithelium, *in vitro* they do not bind as quickly or tightly as those of other non-primate mammals (De Mott *et al*., 1995; Pacey *et al*., 1995a, 1995b; De Jonge, 2005; Suarez and Pacey, 2006). It is possible that activation of Ca^{2+} store mobilisation and capacitative Ca^{2+} entry may be better suited in human sperm to generate bursts of hyperactivation 'on demand' regulated by mediators of Ca^{2+} influx (such as progesterone) or direct inducers of store mobilisation (such as NO•) (Machado-Oliveira *et al*., 2008). Finally, only hyperactivated sperm can penetrate the outer vestments of the mouse oocyte in order to reach and fuse with the oocyte plasma membrane (figure 7.1b) (Overstreet and Cooper, 1979; Ren *et al*., 2001; Quill *et al*., 2003). Increased pH_i, leading to activation of CatSper channels, could provide prolonged hyperactivation in capacitated cells at the site of fertilisation and may even cause hyperactivation of cells upon binding to zona proteins (Xia and Ren, 2009b).

How important are CatSper channels in human sperm and are they absolutely necessary for hyperactivation?

Evidence suggests that sperm competition is a potent driving force in the evolution of sperm traits and likely to influence the diversity of design, total sperm size and midpiece volume associated with sperm competition (Immler *et al*., 2007). It is physiologically 'costly' for male mammals to engage in sperm competition but compared with rodents and other mammals, humans are unlikely to face sperm competition or sexual conflict. Of the millions of human

sperm ejaculated, a small proportion of sperm actually reach the site of fertilisation and of these only a fraction may be competent to fertilise (De Jonge, 2005; Suarez and Pacey, 2006; Lefièvre *et al.*, 2009). Furthermore, reproduction in humans is relatively inefficient, the probability of conceiving during a menstrual cycle is about 40% and approximately 30% of conceptions will not result in a live birth (Dey, 2010).

Although basic biological processes are usually conserved in mammals, reproductive system proteins are subjected to selective evolutionary forces and often undergo accelerated evolution and functional divergence so that a degree of redundancy is often observed. Since fertilisation occurs deep within the female tract, sperm have evolved the ability to alter motility pattern, indeed the CatSper channels are a recent evolutionary invention, since CatSper-like channels have not been detected in species lower than mouse (Lobley *et al*., 2003; Cai and Clapham, 2008). The intriguing observation that, whereas 4-aminopyridine causes dramatic hyperactivation of human sperm, cytoplasmic alkalinisation with 25mM NH_4Cl (which hyperactivates bovine sperm (Marquez and Suarez, 2007) had a negligible effect on motility, may reflect species variation in the mechanisms of control of hyperactivation (and thus in sensitivity to hyperactivating stimuli) due to differences in the behavioural requirements for sperm.

Approximately 1:15 human male are sub-fertile or infertile (HFEA, 2005, www.hfea.gov.uk) and of these approximately 15% are linked with genetic abnormalities (Lefièvre *et al*., 2007). Since large proportions of infertile males are diagnosed as idiopathic, genetic causes of male infertility may be underestimated. However, it is almost 10 years since Ren *et al*., (2001) first identified the CatSper channels in mouse sperm but to date there are very little data on CatSper related infertility in humans. Avenarius *et al*., (2009) identified two males in an Iranian family with nonsyndromic male fertility resulting from insertion mutations in the CatSper1 gene (Avenarius *et al*., 2009). Nikpoor *et al*., (2004) report a positive correlation between lowered

CatSper gene expression and immotile sperm in humans, but the study was very small (18 patients in total). Only one group have examined human sperm motility in relation to CatSper channels. Li *et al*., (2007) demonstrated that CatSper1 immunolocalised to the principal piece of human sperm and incubation with an antibody reduced % progressive motility by ~25% after a 6 hour incubation. The same group report that human sperm hyperactivation (12/173 cells) was inhibited in the presence of CatSper antibody (Li *et al*., 2009). Without more evidence on gene expression/mutations and availability of specific inhibitors of CatSper channel activation, it is impossible to determine their importance in human sperm motility and hyperactivation.

We can speculate that due to a lack of sperm competition and the physiology/anatomy of the female reproductive tract, human sperm motility may require periods of quiescence followed by 'bursts' of hyperactivation to respond rapidly upon receiving the signals that ovulation has occurred. In other mammals where insemination is synchronised with ovulation, spermatozoa may require more prolonged hyperactivation (CatSper dependent). Prolonged and sustained hyperactivation may confer an advantage (over sperm from rival males) enabling spermatozoa to quickly reach the oviduct, pull free from the sperm reservoir and fertilise the egg(s) (Dixson and Anderson, 2004; Pennisi, 2010).

The data presented here demonstrates that 4-aminopyridine induced hyperactivation in human sperm is not solely pH/CatSper channel dependent and that mobilisation of stored Ca^{2+}, possibly causing activation of store-operated Ca^{2+} influx, is essential for hyperactivation in a population of human sperm (figure 7.1d). Of course there are many aspects of the activation and control of store mobilisation of which we are still ignorant and further characterisation of the store is required. Although basic semen analysis is rudimentary, 4-aminopyridine could provide a simple, non-invasive pharmacological tool to identify patients with motility related

sub-fertility. In addition, these data have important implications for the development of the elusive male contraceptive. Pharmaceutical targeting of native CatSper channel in mouse sperm produced a CatSper null phenocopy (Carlson *et al*., 2009) but may be insufficient to fully inhibit hyperactivation in human sperm.

Figure 7.1

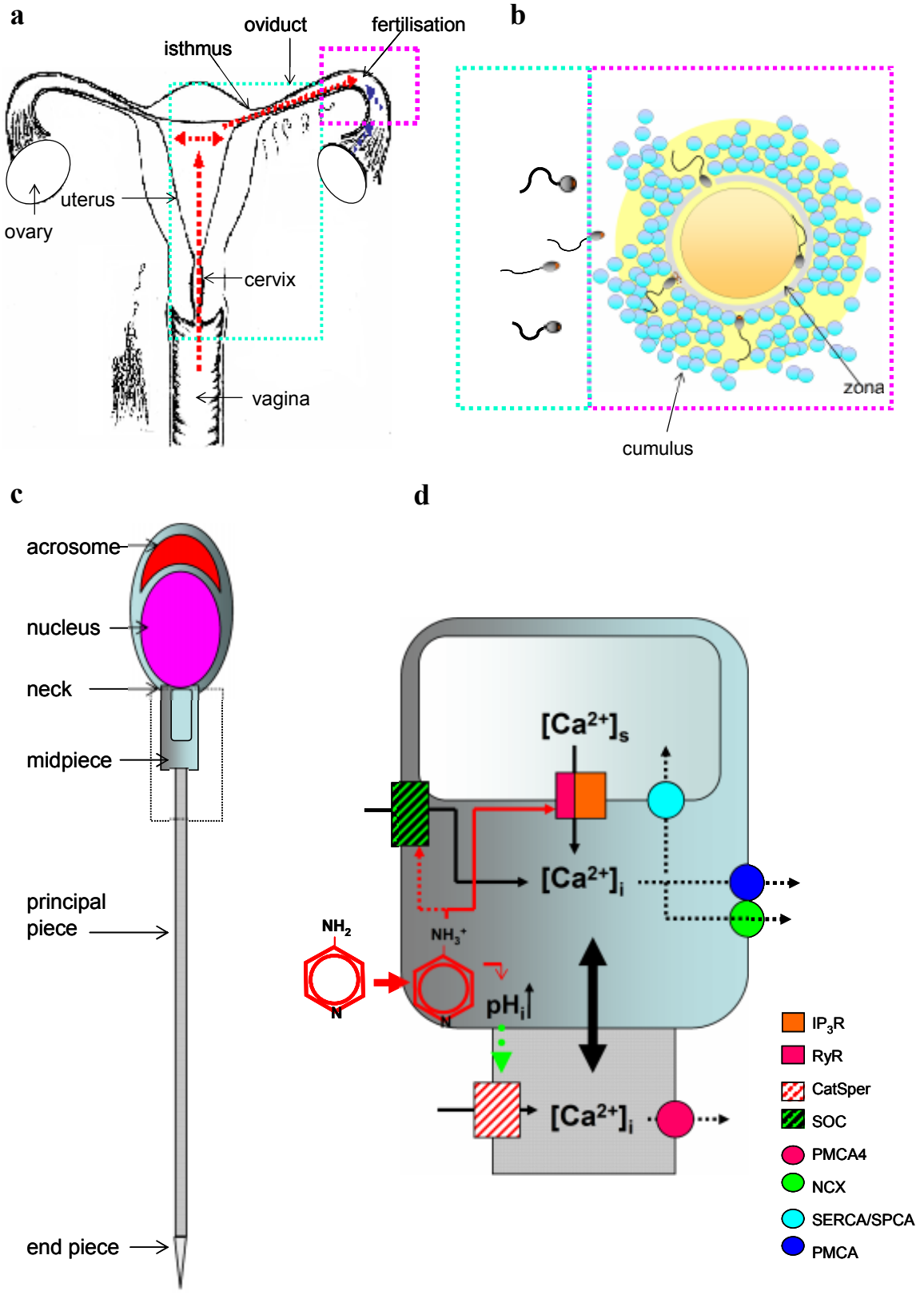
a
oviduct
fertilisation
isthmus
uterus
ovary
cervix
vagina
b
zona
cumulus
c
acrosome
nucleus
neck
midpiece
principal
piece
end piece
d
[Ca2+]s
[Ca2+]i
NH2
NH3+
N
pHi
[Ca2+]i
IP3R
RyR
CatSper
SOC
PMCA4
NCX
SERCA/SPCA
PMCA

Figure 7.1**:** **(a)** Schematic representation of the female reproductive tract illustrating the transport of gametes to the site of fertilisation. Sperm (red arrow) are deposited in the vagina, from where a limited number quickly swim through the cervix/cervical mucus. Transport through the uterus is aided by uterine contractions. Sperm must then swim through the isthmus and oviduct to the ampulla where fertilisation can occur. The oocyte (blue arrow) is released from the ovary and is moved along to the site of fertilisation. **(b)** In order to successfully complete the journey through the female reproductive tract human sperm may switch between periods of quiescence followed by 'bursts' of hyperactivation on demand. However prolonged (possibly CatSper dependent) hyperactivation may be required upon receiving ovulation signals and to enable penetration of the cumulus oocyte complex. **(c)** Schematic structure of human sperm, boxed area shows the position of the Ca^{2+} store at the neck/midpiece and a short segment of the principal piece. **(d)** Model for the effects of 4-aminopyridine on $[Ca^{2+}]_i$ in human sperm. 4-aminopyridine crosses the plasma membrane in an uncharged form however it is the charged form which is biologically active. 4-aminopyidine can activate store mobilisation (via RyR or IP_3R in microsomal vesicles) in sperm in the absence of extracellular Ca^{2+} (buffered with EGTA) (chaper 5). 4-aminopyridine induced store mobilisation is likely to activate store operated Ca^{2+} channels and enhance Ca^{2+} influx. 4-aminopyridine has been shown to potentiate CCE following store mobilisation (bis-phenol or depletion with EGTA buffered saline) (chapter 6). Store mobilisation and concomitant SOC activation may provide a localised signal to facilitate short bursts of hyperactivation on demand. Furthermore 4-aminopyridine acting as weak base could induce cytoplasmic alkalinisation and CatSper channels activation. Surprisingly cytoplasmic alkalinisation with NH_4Cl did not induce hyperactivation in human sperm. In conclusion the actions of 4-aminopyridine are not solely pH/CatSper dependent in a population of human sperm. However it is likely that combined activation of CatSper channels and store mobilisation/SOC activation is required to maintain high $[Ca^{2+}]_i$ and sustain hyperactivation enabling penetration of the outer vestments of the oocyte.

7.1 Future research

Strict regulation of sperm functions (including motility) is essential to achieve successful fertilisation. Although *in vitro* studies have been carried out to understand the complex events that precede human fertilisation, translation to what happens *in vivo* is far from being simple. Evidence from this study indicates a central role for Ca^{2+} stores in hyperactivation of human sperm. This report focused on 4-aminopyridine which is a remarkablely effective inducer of hyperactivation in human sperm. Although 4-aminopyridine can induce cytoplasmic alkalinisation and CatSper channel activation these results demonstrate a key role for mobilisation of stored Ca^{2+} in the sperm neck and human sperm hyperactivation. Although this compound is a useful tool in studying store mobilisation and hyperactivation in human sperm it is difficult to isolate these responses from CatSper channel activation. A specific CatSper channel inhibitor is currently not available commercially however the compound HC-056456 has been shown to cause a rapid reversible loss of CatSper dependent hyperactivation in the mouse (Carlson *et al*., 2009). The effect of this compound on human sperm remains unknown. Further characterisation of the neck/midpiece store is essential to understanding the signalling pathways involved in the regulation of motility and hyperactivation. 4-aminopyridine presents a useful non-invasive tool for examination of sperm hyperactivation response. Investigation of 4-aminopyridine induced hyperactivation in assisted conception patients would provide an opportunity to determine the biological role of defective, Ca^{2+} stores and hyperactivation. We would hypothesise that failure of Ca^{2+} store mobilisation/CCE would result in sperm dysfunction and infertility.

APPENDIX I: Supplement calcium channel information

Voltage gated calcium channel subunits and currents

The voltage-gated Ca^{2+} channels (VGCC) (Table 7.0, figure 8.0) that have been characterised bio-chemically are complex proteins composed of four or five distinct subunits that are encoded by multiple genes (Catterall *et al*., 2005). The α_1 subunit of 190 to 250 kDa is the largest subunit and it incorporates the conductance pore, the voltage sensor and gating apparatus and most of the known sites of channel regulation by second messengers, drugs and toxins. Like the α subunits of sodium channels, the α_1 subunit of voltage-gated Ca^{2+} channels (VOCC) is organised into four homologous domains (I-IV), with six trans-membrane segments (6TM) (S1-S6) in each. The S4 segment serves as the voltage sensor. The pore loop between tansmembrane segments S5 and S6 in each domain determines ion conductance and selectivity. An intracellular β subunit and a tansmembrane disulphide-linked $\alpha_2\delta$ subunit complex are components of most Ca^{2+} channels. A γ subunit has been found in skeletal muscle Ca^{2+} channels. Although the auxiliary subunits modulate the properties of the channel complex, the pharmacological and electrophysiological diversity of calcium channels arise primarily from the existence of multiple α1 subunits (Catterall *et al*., 2005).

Figure 8.0.

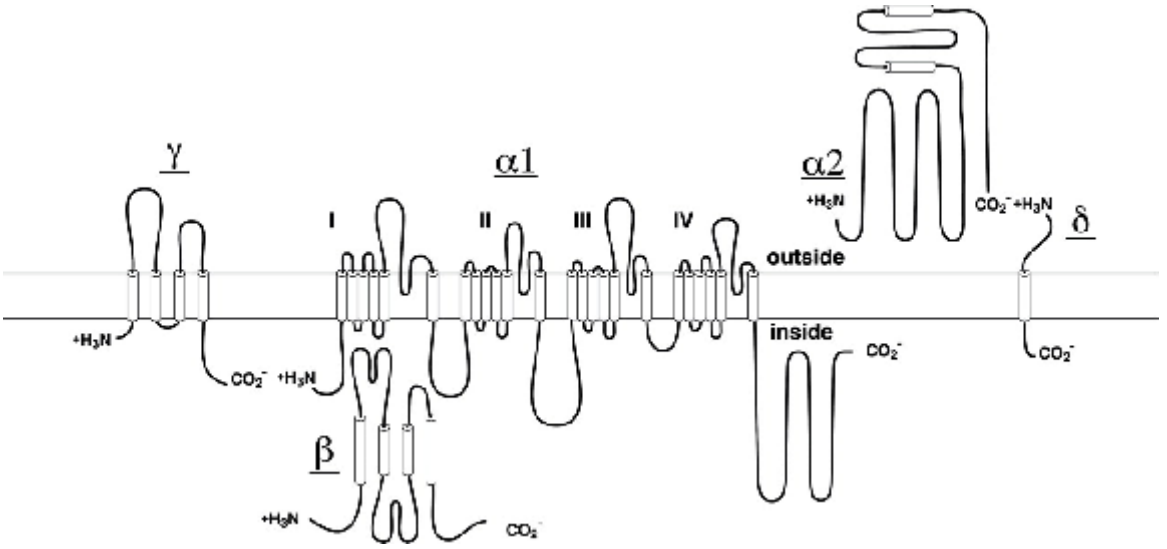

Figure 8.0 Subunit structure of Ca_v1 channels. The subunit composition and structure of calcium channels purified from skeletal muscle are illustrated. Predicted α helices are depicted as cylinders (Catterall *et al.*, 2005).

Calcium currents recorded in different cell types have diverse physiological and pharmacological properties. L-type Ca^{2+} currents typically require strong depolarisation for activation, are long lasting and are blocked by organic L-type channel antagonists (including dihydropyridines, phenyalkylamines and benzothiazepines). They are the main Ca^{2+} currents recorded in muscle and endocrine cells where they initiate contraction and secretion. N-type, P/Q-type and R-type Ca^{2+} currents also require strong depolarisation of activation. They are relatively unaffected by L-type Ca^{2+} channel antagonist but are blocked by specific polypeptides toxins from snail and spider venom. They are expressed primarily in neurons where they initiate neurotransmission. T-type Ca^{2+} currents are activated by weak depolarisation and are transient. They are resistant to both organic antagonists and to the snail and snake toxins used to define N- and P/Q-type Ca^{2+} currents. They are expressed in a wide variety of cells.

Application of electrophysiological techniques is technically difficult but patch clamping of immature male germ cells (both rodent and human) has shown consistently that that these cells display both L-type and T-type currents. Immunostaining experiments using specific antibodies

detected the presence and distribution of T-type, L-, R- and P/Q-type and N-type channels in rodent sperm (Wennemuth *et al*., 2000).

CatSper channels

CatSpers (Table 8.0) are putative 6TM voltage-gated Ca^{2+}-permeant channels that are specific to sperm (Clapham and Garbers, 2005). CatSpers are structurally related to the 6TM voltage-gated Na^{+} channel classes. Patch-clamp experiments on mouse spermatozoa demonstrated an alkaline-potentiated, voltage-activated, calcium selective channel (Kirichok *et al*., 2006). The two-pore channels TPC1 and TPC2 are putative cation-selective ion channels related to CatSper and transient receptor potential channels.

Transient receptor potential (TRP) channels

The TRP ion channels (Table 9.0) are named after the *Drosophila* phototransduction channels. There are six mammalian protein families including the classical TRPs (TRPC). TRPs are generally assumed to have 6TM domains, one (S1-S4) containing the S4 voltage sensor and a second (S5-S6) containing the 2TM pore and gate. A high resolution structure of a TRP channel has not yet been solved. However, the 2TM structure of a bacterial K^{+} channel is analogous to the S5 and S6 domain (Clapham *et al*., 2005).

TRP channels are relatively non-selective. Upon opening they depolarise cells from resting E_m to around 0mV (raise intracellular N^{+} and usually Ca^{2+}) (Clapham *et al*., 2005).

Cyclic-nucleotide-regulated channels

The family of cyclic nucleotide-regulated channels comprises two groups: the cyclic nucleotide gated (CNG) and the hyperpolarisation-activated nucleotide-gated (HNG) channels (Hofmann *et al*., 2005). CNG cation channels activation is mediated by the direct binding of cGMP or cAMP to the channel protein. CNG channels are expressed in the cilia of olfactory neurons where they

play a key role in sensory transduction. CNG channels are heterotetramers composed of an A subunit and a B subunit. Both types of subunits are members of 6TM segment channel superfamily. In the cytosolic C terminus, CNG channel subunits carry a cyclic nucleotide binding domain that serves as an activation domain. CNG channels pass monovalent cations (Na^+ and K^+), Ca^{2+} is also permeable and provides feedback when bound to calmodulin. A CNG, Ca^{2+} permeable channel has been described in human spermatozoa.

Table 7.0: Supplement Ca_v channel information (including channel current, specific antagonists, male germ cell tissue (human or rodent), localisation and method used) (adapted from Catterall *et al*., 2005; Jimenez-Gonzalez *et al*., 2006).

Channel	Current	Specific Antagonists		Tissue Present	Method	Knock-out mouse
$Ca_v1.1$	L	Dihydropyridines, phenyalkylamines, benzothiazepines		Rat testis sections (Goodwin *et al*., 1997)	Immunostaining	
Ca_v 1.2	L	Dihydropyridines, phenyalkylamines, benzothiazepines		Rat Testis sections (Goodwin *et al*., 2000) Human spermatozoa (Goodwin *et al*., 2000; Park *et al*., 2003)	*In situ* RT-PCR *In situ* RT-PCR, Immunogold transmission EM	
Ca_v 1.3	L	Dihydropyridines, phenyalkylamines, benzothiazepines		Not detected		
Ca_v 1.4	L	Dihydropyridines, phenyalkylamines, benzothiazepines		Not detected		
Ca_v 2.1	P/Q	ω-Agatoxin IVA (funnel web spider)		Mouse spermatozoa (Wennemuth *et al*., 2000).	Immunostaining/immunoblotting	
$Ca_v2.2$	N	ω-Conotoxin-GVIA (cone snail toxin)		Mouse spermatozoa (Wennemuth *et al*., 2000)	Immunostaining/immunoblotting	KO mouse fertile (Sakata *et al*., 2001)
$Ca_v2.3$	R	SNX-482 (tarantula venom)		Mouse spermatozoa (Wennemuth *et al*., 2000). Human spermatozoa (Trevino *et al*., 2004).	Immunostaining/immunoblotting Immunostaining	KO mouse remain fertile (Sakata *et al*., 2001).
$Ca_v3.1$	T	None		Human sperm head regions (Trevino *et al*., 2004)	Immunostaining	KO mouse fertile (Escoffier *et al*., 2007
$Ca_v3.2$	T	None		Human sperm head region (Trevino *et al*., 2004)	Immunostaining	KO mouse remain fertile (Escoffier *et al*., 2007)
$Ca_v3.3$	T	None		Human sperm midpiece region (Trevino *et al*., 2004)	Immunostaining	

Table 8.0: Supplement CatSper channel information (including channel current, specific antagonists, male germ cell tissue (human), localisation and method used) (adapted from Clapham and Garbers, 2005).

Channel name	Current	Specific Antagonists	Tissue Present	Method
CatSper 1	Ca^{2+} selective, voltage dependent, potentiated at pH8	Not established HC-056456 (Carlson *et al.*, 2009)	Mouse spermatozoa	Ca^{2+} imaging, patch clamp Ren *et al.*, 2001)
CatSper 2	Putative Ca^{2+} current	Not established	Mouse spermatozoa	Ca^{2+} imaging (Quill *et al.*, 2001)
CatSper 3	Putative Ca^{2+} current	Not established	Mouse Testis	Lobley *et al.*, 2003
CatSper 4	Putative Ca^{2+} current	Not established	Mouse Testis	Lobley *et al.*, 2003

Table 9.0: Supplement TRPC channel information (including channel current, specific antagonists, male germ cell tissue (human or rodent), localisation and method used) (adapted from Clapham *et al.*, 2005).

Channel name	Current	Specific Antagonists	Tissue Present	Method
TRPC1	Na^{+}/Ca^{2+}	2-APB, SKF96365 and Gd^{3+}	Human spermatozoa	Immunolocalised flagellum (Castellano *et al.*, 2003)
TRPC2	Mouse pheromone channel	None	Mouse sperm	Immunolocalised acrosome region (Jagannathan *et al.*, 2002) Pseudogene in human and bovine (Vannier *et al.*, 1999).
TRPC3	Na^{+}/Ca^{2+}	2-APB, 1-25µM Gd^{3+}, La^{3+}, RR	Human spermatozoa	Immunolocalised flagellum (Castellano *et al.*, 2003)
TRPC4	Na^{+}/Ca^{2+}	2-APB, mM La^{3+}	Human spermatozoa	Immunolocalised flagellum (Castellano *et al.*, 2003)
TRPC6	Na^{+}/Ca^{2+}	100µM 2-APB, 2µM Gd^{3+}, 4µM La^{3+}, 4µM SKF96365	Human spermatozoa	Immunolocalised flagellum (Castellano *et al.*, 2003)

APPENDIX II: Media

Supplemented Earle's Balanced Salt Solution (sEBSS)

Sodium Dihyd. Phosphate (1.0167 mM)

Potassium Chloride (5.4 mM)

Magnesium Sulphate.7H_2O (0.811 mM)

Dextrose Anhydrous (5.5 mM)

Sodium Pyruvate (2.5 mM)

DL-Lactic Acid, Sodium (19.0 mM)

Calcium Chloride.2H_2O (1.8 mM) Sodium

Bicarbonatel (25.0 mM)

Sodium Chloride (≈116.4 mM)

HEPES (15.0mM)

Sodium chloride was added in increments until osmolarity was 285-295 mOsm (Advanced Micro Osmometer, Vitech Scientific Ltd, West Sussex, UK, which has been pre-calibrated using a 50 mOsm/Kg H_2O and a 850 mOsm/Kg H_2O calibration standards). sEBSS pH was adjusted to 7.3-7.4 with 1M HCl and 1M NaOH and subsequently stored as 100 ml volumes in glass bottles at 4°C until use. 0.3% Bovine serum albumin (BSA) (0.3%) was added on experimental day.

Nominal Ca^{2+}-free sEBSS (NCFsEBSS)

Sodium Dihyd. Phosphate (1.0167 mM)

Potassium Chloride (5.4 mM)

Magnesium Sulphate.7H_2O (0.811 mM)

Dextrose Anhydrous (5.5 mM)

Sodium Pyruvate (2.5 mM)

DL-Lactic Acid, Sodium (19.0 mM)

Sodium Bicarbonate (25.0 mM)

Sodium Chloride (≈ 118.4 mM)

Prepared as for standard sEBSS but stored as 100 ml volumes in polystyrene bottles at 4°C, pH was adjusted to 7.3-7.4, 0.3% BSA was added on experimental day.

Non-capacitating sEBSS (NCsEBSS)

Sodium Dihyd. Phosphate (1.0167 mM)

Potassium Chloride (5.4 mM)

Magnesium Sulphate.7H_2O (0.811 mM)

Dextrose Anhydrous (5.5 mM)

Sodium Pyruvate (2.5 mM)

DL-Lactic Acid, Sodium (19.0 mM)

Calcium Chloride.2H_2O (1.8 mM)

Sodium Chloride (≈ 116.4 mM)

NCsEBSS recipe was based upon standard sEBSS receipe (osmolarity was 285-295 mOsm as before), pH was adjusted to 7.3-7.4. NCsEBSS was stored as 100 ml volumes in glass bottles at 4°C until use, 0.3% BSA added on day of use.

sEBSS-Ethylene glycol-bis (β-amino-ethylether)-N,N,N'N'-tetraacetic acid (sEBSS-EGTA)

Sodium Phosphate Monobasic (1.018 mM)

Potassium Chloride (5.366 mM)

Magnesium Sulphate.7H_2O (0.811 mM)

Dextrose Anhydrous (5.551 mM)

Sodium Pyruvate (2.724 mM)

DL-Lactic Acid, Sodium (41.763 mM)

Calcium Chloride.2H_2O (4.966 mM)

Sodium Bicarbonate (26.187 mM)

Sodium Chloride(≈85.0 mM)

EGTA (5.994 mM)

Prepared as for standard sEBSS. 0.3% BSA was added on experimental day.

The calcium concentration calculator Maxchelator (V2.2) written by Chris Patton from Stanford University (http://www.stanford.edu/~cpatton/maxc.html) was used to calculate the free calcium concentration in saline containing EGTA: 25°C ($[Ca^{2+}]_o$ 10^{-7} M)

Simplified Saline

Potassium Chloride (5.4 mM)

Magnesium Chloride 6H_2O (1.0 mM)

Dextrose Anhydrous (9.0 mM)

Calcium Chloride.2H_2O (1.8 mM)

Sodium Chloride (approx 132.0 mM)

Osmolarity was 285-295 mOsm as before, pH adjusted to 7.2-7.4, saline stored as 100 ml volumes in glass bottles at 4°C until use, 0.3% BSA added on day of use.

APPENDIX III: Research Publications

In prep

Costello, S., Barratt, C.L.R., Kirkman-Brown, J. and Publicover, S. Hyperactivation of human sperm by 4-aminopyridine: key role for mobilisation of stored Ca^{2+} in the sperm neck (in prep)

Reviews

Costello, S., Michelangeli, F., Nash, K., Lefièvre, L., Morris, J., Machado-Oliveira, G., Barratt, C., Kirkman-Brown, J.K., and Publicover, S. (2009). Ca^{2+}-stores in sperm: their identities and functions. *Reproduction* **138**, 425-437.

Bedu-Addo, K., **Costello, S**., Harper, C., Machado-Oliveira, G., Lefièvre, L., Ford, C., Barratt, C. and Publicover, S. (2008). Mobilisation of stored calcium in the neck region of human sperm – a mechanism for regulation of flagellar activity. *International Journal of Developmental Biology* **52**, 615-26.

Abstracts

4-aminopyridine mobilises calcium in human sperm
Young Physiologist Symposium (Cations 2008) at University of Manchester 2008

4-aminopyridine mobilises calcium in human sperm
Society for Reproduction and Fertility Meeting (2008)

4-aminopyridine mobilises calcium in human sperm
Proceedings of the Physiological Society (2008)

The modulation of $[Ca^{2+}]_i$ homeostasis by 4-aminopyridine in human spermatozoa
Fertilization & Activation of Development Gordon Research Conference (July 2007), U.S.

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