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IMAGING TECHNIQUES APPLIED FOR DETECTION OF SECRETION, TRANSGENE DELIVERY AND G PROTEIN-COUPLED RECEPTORS IN REPRODUCTIVE AND ENDOCRINE CELLS *IN VIVO* AND *IN VITRO*

by

Ramin Lindqvist

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From the Department of Physiology
Institute of Biomedicine
Faculty of Medicine
University of Turku
Finland

Supervised by

Professor Matti Poutanen, Ph.D.
Department of Physiology
Institute of Biomedicine
University of Turku
Turku, Finland

and

Professor Ilpo Huhtaniemi, MD., Ph.D.
Department of Physiology
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Dr. Ulla Petäjä-Repo, Ph.D.
Department of Anatomy and Cell Biology
Institute of Biomedicine
University of Oulu
Oulu, Finland

Professor Mika Scheinin, MD., Ph.D.
Department of Pharmacology, Drug Development and Therapeutics
Institute of Biomedicine
University of Turku
Turku, Finland

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To my Family

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ABSTRACT

Ramin Lindqvist

Imaging techniques applied for detection of secretion, transgene delivery and G protein-coupled receptors in reproductive and endocrine cells *in vivo* and *in vitro*.

Department of Physiology, Institute of Biomedicine, Faculty of Medicine, University of Turku, Finland
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Post-testicular sperm maturation occurs in the epididymis. The ion concentration and proteins secreted into the epididymal lumen, together with testicular factors, are believed to be responsible for the maturation of spermatozoa. Disruption of the maturation of spermatozoa in the epididymis provides a promising strategy for generating a male contraceptive. However, little is known about the proteins involved. For drug development, it is also essential to have tools to study the function of these proteins *in vitro*.

One approach for screening novel targets is to study the secretory products of the epididymis or the G protein-coupled receptors (GPCRs) that are involved in the maturation process of the spermatozoa. The modified Ca^{2+} imaging technique to monitor release from PC12 pheochromocytoma cells can also be applied to monitor secretory products involved in the maturational processes of spermatozoa. PC12 pheochromocytoma cells were chosen for evaluation of this technique as they release catecholamines from their cell body, thus behaving like endocrine secretory cells. The results of the study demonstrate that depolarisation of nerve growth factor -differentiated PC12 cells releases factors which activate nearby randomly distributed HEL erythroleukemia cells. Thus, during the release process, the ligands reach concentrations high enough to activate receptors even in cells some distance from the release site. This suggests that communication between randomly dispersed cells is possible even if the actual quantities of transmitter released are extremely small.

The development of a novel method to analyse GPCR-dependent Ca^{2+} signalling in living slices of mouse caput epididymis is an additional tool for screening for drug targets. By this technique it was possible to analyse functional GPCRs in the epithelial cells of the ductus epididymis. The results revealed that, both P2X- and P2Y-type purinergic receptors are responsible for the rapid and transient Ca^{2+} signal detected in the epithelial cells of caput epididymides. Immunohistochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses showed the expression of at least P2X1, P2X2, P2X4 and P2X7, and P2Y1 and P2Y2 receptors in the epididymis.

Searching for epididymis-specific promoters for transgene delivery into the epididymis is of key importance for the development of specific models for drug development. We used EGFP as the reporter gene to identify proper promoters to deliver transgenes into the epithelial cells of the mouse epididymis *in vivo*. Our results revealed that the 5.0 kb murine Glutathione peroxidase 5 (GPX5) promoter can be used to target transgene expression into the epididymis while the 3.8 kb Cysteine-rich secretory protein-1 (CRISP-1) promoter can be used to target transgene expression into the testis. Although the visualisation of EGFP in living cells in culture usually poses few problems, the detection of EGFP in tissue sections can be more difficult because soluble EGFP molecules can be lost if the cell membrane is damaged by freezing, sectioning, or permeabilisation. Furthermore, the fluorescence of EGFP is dependent on its conformation. Therefore, fixation protocols that immobilise EGFP may also destroy its usefulness as a fluorescent reporter. We therefore developed a novel tissue preparation and preservation techniques for EGFP. In addition, fluorescence spectrophotometry with epididymal epithelial cells in suspension revealed the expression of functional purinergic, adrenergic, cholinergic and bradykinin receptors in these cell lines (mE-Cap27 and mE-Cap28).

In conclusion, we developed new tools for studying the role of the epididymis in sperm maturation. We developed a new technique to analyse GPCR dependent Ca^{2+} signalling in living slices of mouse caput epididymis. In addition, we improved the method of detecting reporter gene expression. Furthermore, we characterised two epididymis-specific gene promoters, analysed the expression of GPCRs in epididymal epithelial cells and developed a novel technique for measurement of secretion from cells.

Key words: Epididymis, GPCRs, Ca^{2+} imaging, cell to cell communication, vibratome slices, ATP, EGFP, epididymal epithelial cell line

TIIVISTELMÄ

Kuvantamistekniikoiden käyttö tutkittaessa hormonaalisesti säädeltyjen solujen eritystoimintaa sekä G-proteiinien ilmentymistä
Fysiologian osasto, Biolääketieteen laitos, Lääketieteellinen tiedekunta, Turun Yliopisto
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G-proteiinivälitteiset reseptorit muodostavat yhden keskeisimmän lääkekehityksen kohteen ja hypoteesimme mukaan niiden toiminnan esto lisäkiveksessä on yksi mahdollinen tapa estää siittiöiden kypsyminen hedelmöityskykyisiksi. Useat G-proteiinivälitteiset reseptorit välittävät signaalinsa muuntamalla solun sisäistä Ca^{2+}-pitoisuutta. Työssä kehitettiin uusi kuvantamistekniikka solun sisäisen Ca^{2+}-pitoisuuden mittaamiseksi viljellyillä soluilla ja elävillä kudosleikkeillä. Menetelmä kehitettiin käyttäen PC12 feokromosytoma soluja. Menetelmän avulla osoitimme, että PC12 solujen erittämät erittäin pienet pitoisuudet katekoliamiineja saavat aikaan solun sisäisen Ca^{2+}-pitoisuuden muutoksia niiden kanssa viljellyissä soluissa. Käytetyllä menetelmällä osoitettiin solujen kykenevän kommunikoimaan toistensa kanssa erittämällä erittäin pieniä pitoisuuksia välittäjäaineita ja kehitettyä menetelmää voidaan soveltaa solujenvälisen kommunikaation tutkimisessa. Menetelmää sovellettiin myös G-proteiinivälitteisten reseptoreiden ilmenemisen tunnistamiseen lisäkiveksen epiteelisoluissa. Tulokset osoittivat lisäkiveksen epiteelisolujen ilmentävän P2X- ja P2Y-tyypin purinergisiä reseptoreita. Immunohistokemian ja reseptoreiden lähetti-RNA-analyysien avulla osoitimme, että ainakin P2X1, P2X2, P2X4, P2X7, P2Y1 ja P2Y2 reseptorit ilmenevät lisäkiveksessä. Lisäksi fluoresenssispektrofotometriaa käyttäen osoitimme hiiren lisäkiveksestä tuotettujen solulinjojen ilmentävän purinergisiä, adrenergisiä, kolinergisiä reseptoreita sekä bradykiniini reseptoria.

Solun sisäisen Ca^{2+}-pitoisuuden mittaamisen lisäksi työssä kehitettiin aiempaa herkempi menetelmä fluoresoivan EGFP-proteiinin tunnistamiseksi kudosleikkeillä. Menetelmän avulla tutkimme eräiden lisäkiveksessä ilmentyvien geenien (Glutathione peroxidase 5, GPX5; Cysteine-rich secretory protein-1, CRISP-1) säätelyalueita siirtogeenisissä hiirissä. Ennustetut GPX5 ja CRISP-1 geenien säätelyalueet liitettiin muuntogeeniin siten, että ne ohjasivat EGFP-proteiinin ilmentymistä. Tulokset osoittivat, että 5000 emäsparia pitkä GPX5 geenin säätelyalue ilmentää siirtogeenin spesifisesti lisäkiveksessä, kun taas käyttämämme 3800 emäsparin pituinen CRISP-1- geenin säätelyalue ohjaa siirtogeenin ilmentymistä kiveksessä. Tunnistetulla lisäkivesspesifisellä GPX5-geenin säätelyelementillä on käyttöä esim. muuntogeenisiä eläinmalleja tuotettaessa tutkimuskäyttöön ja lääkekehitykseen.

Yhteenvetona voidaan todeta, että työssä kehitettiin uusia kuvantamismenetelmiä, joilla voidaan selvittää lisäkiveksen epiteelisolujen toimintaa sekä niissä tapahtuvaa geenisäätelyä. Siittiöiden kypsymisen estäminen lisäkiveksessä on yksi mahdollisuus tuottaa uusi ehkäisymenetelmä miehille. Siten kehitetyillä menetelmillä on käyttöä lääkekehitystyössä, pyrittäessä kehittämään miehen ehkäisymenetelmiä.

Avainsanat: ATP, Ca^{2+}, G-proteiini, EGFP, kuvantaminen, lisäkives, solujen kommunikaatio, reseptori

LIST OF ORIGINAL COMMUNICATIONS

This study is based on the following original publications, which are referred to in the text by the Roman numerals I-IV:

I Shariatmadari R., Lund P.E., Krijukova E., Sperberg G.O., Kukkonen J.P. and Åkerman K.E.O. (2001) Reconstitution of neurotransmission by determining communication between differentiated PC-12 pheochromocytoma and HEL 92.1.7 erythroleukemia cells. Pflügers Arch - Eur. J. Physiology 442, 312-320

II Shariatmadari R., Sipilä P., Vierula M., Törnquist K., Huhtaniemi I.T. and Poutanen M. (2003) Adenosine triphosphate induces Ca^{2+} signal in epithelial cells of the mouse caput epididymis through activation of P2X and P2Y purinergic receptors. Biol. Reprod., 68, 1185-1192

III Lahti P., Shariatmadari R., Penttinen J., Drevet J., Haendler B., Vierula M., Parvinen M., Huhtaniemi I. and Poutanen M. (2001) Evaluation of the 5'-flanking regions of glutathione peroxidase 5 (GPX5) and Cysteine rich protein-1 (CRISP-1) genes for targeting epididymis in transgenic mice. Biol. Reprod., 64, 1115-1121

IV Shariatmadari R., Sipilä P., Huhtaniemi I. and Poutanen M. (2001) Improved technique for detection of enhanced Green Fluorescent Protein (EGFP) in transgenic mice. BioTechniques 30, 1282-1285

ABBREVATIONS

nAChR	nicotinic acetylcholine receptor
mAChR	muscarinic acetylcholine receptor
AC	adenylyl cyclase
AR	androgen receptor
ATP	adenosine triphosphate
CA II	carbonic anhydrase II
CaMK	calmodulin kinase
cAMP	cyclic adenosine monophosphate
CCH	carbamyl choline chloride
CRISP-1	cysteine rich secretory protein-1
Dop	dopamine
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FMM	fluorescent mounting medium
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GPX5	glutathione peroxidase 5
H^+V-ATPase	vacuolar proton adenosine-triphosphatase pump
HBM	Hepes buffered medium
hCG	human chorionic gonadotropin
HEL	human erythroleukemia cell
IP_3	inositol-1,4,5-triphosphate
IP_3R	inositol-1,4,5-triphosphate receptor
K^+70	potassium chloride 70 mM
NCX	Na^+/Ca^{2+} exchanger
NGF	nerve growth factor
Nic	nicotine
Nif	nifedipine
Nor	noradrenaline
OR	odorant receptor
P2X	ligand gated purinergic ion channel
P2Y	G-protein coupled purinergic receptor
PBS	phosphate buffered saline
PC12	rat pheochromocytoma cell line
PFA	paraformaldehyde
PIP2	phosphoinositol-phospholipid biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMCA	plasma-membrane Ca^{2+}-ATPase
RT-PCR	reverse transcriptase polymerase chain reaction
SOC	store-operated channel
SOCC	store-operated calcium channel
SR	sarcoplasmic reticulum
T	testosterone
TBM	Tes buffered medium
UK14,304	5-bromo-N-(4,5-dihydro-1H- imidazol-2-yl) -6-quinoxalinamine
VOCC	voltage operated Ca^{2+}-channel
VSCC	voltage sensitive calcium channel
ω-aga-IVA	ω-agatoxin IVA
ω-ctxGVIA	ω-conotoxin GVIA
ω-ctxMVIIC	ω-conotoxin MVIIC

1 INTRODUCTION

The male sex organs in mammals include the testicles, the efferent duct system, including the epididymis and vas deferens, accessory glands, and the penis (Setchell *et al.,* 1988). The two testicles produce testosterone, which stimulates the production of sperm. The vas deferens is a muscular tube that passes upward alongside the testis and transports the sperm-containing fluid called semen. Each epididymis is a set of coiled tubes (~ 6-7 meters, when uncoiled) that lies against the testis, connecting the testis with the vas deferens (Setchell *et al.,* 1988, Robaire and Hermo, 1988). They are located in a pouch-like structure behind the penis called the scrotum. The accessory glands, including the seminal vesicles and the prostate gland, provide fluids that lubricate the duct system and nourish the sperm. The seminal vesicles are saclike structures attached to the vas deferens. The prostate gland, which produces some of the components of semen, surrounds the ejaculatory ducts at the base of the urethra, just below the urinary bladder. The urethra carries the semen through the penis to the outside.

An adult male produces several million sperm cells every day. Sperm develop in the testicles within a system of tubes called seminiferous tubules. Spermatozoa in the testis are not motile and are incapable of fertilizing ova. Spermatozoa become functional gametes only after they migrate through the epididymis and undergo an additional maturation process, thereby acquiring the capacitis for both progressive motility and fertility (Gong *et al.,* 2000). The main functions of the epididymis are the storage and maturation of spermatozoa until they attain their mobility in the proximal parts of this organ. These maturation events are believed to be dependent on the local environment provided by the epididymal fluid (Gong *et al.,* 2000, Jervis and Robaire 2001). Anatomically, the epididymis can be divided into four regions: the initial segment, caput, the corpus, and the cauda epididymis (Abe *et al.,* 1983, Abou-Haila and Fain Maurel 1984). However, these anatomical divisions have been defined based on findings in animals, not in humans. The human epididymal epithelium is relatively homogeneous as viewed under the microscope, and the epididymis does not have the same distinct gross anatomical subdivisions that are easily seen in the rat, mouse and many other animals. Unfortunately, compared to mice there is less information available regarding the functional diversity of these three regions of the human epididymis.

In recent years, the epididymis has gained increased attention as a possible target for the action of non-hormonal male contraceptives due to its crucial role in the final steps of sperm maturation. An ideal male post-testicular contraceptive should allow normal sperm production in the testis, but block maturation of spermatozoa in the epididymis. Hence, disruption of the maturation of spermatozoa in the epididymis provides a promising strategy for generating a male contraceptive. G-protein coupled receptors (GPCRs) are possible targets for such pharmacological approaches. Recently, this view has been supported by targeted disruption of the epididymis-specific HE6/Gpr64 gene in mice (Gottwald *et al.,* 2006, Davies *et al.,* 2007). These knockout

mice were infertile, and no histologically epididymis-specific phenotype was observed. Instead, the reabsorption of the testicular fluid along the efferent duct was strongly affected. At molecular level, many genes that belong to the cystatin, lipocalin and β-defensin families were down-regulated. The functions of these genes are still unknown, but there are many studies that suggest that these genes are important for sperm maturation and/or protection (Kim *et al.,* 2006, Nishimura *et al.,* 2007). The importance of membrane receptors for fertility was previously indicated e.g. by the finding that P2X1 purinergic receptor knock-out mice were infertile (Mulryan *et al.,* 2000). However, their infertility was not caused by maturational defects of the spermatozoa in the epididymis, but rather by reduction of contraction of the vas deferens, thus reducing the amount of ejaculated spermatozoa. It has previously been shown that extracellular purine nucleotides acting on purinergic receptors stimulate Cl^- secretion in a primary culture of epithelial cells in both rat and mouse epididymis (Wong 1988, Leung *et al.,* 1996). However, nothing is known about the purinergic receptor subtypes responsible for this action.

The present studies were focused on developing tools for studying the role of the epididymis in sperm maturation. We developed a new technique to analyze GPCR dependent Ca^{2+} signalling in living slices of mouse caput epididymis. In addition, we improved the method for detecting reporter gene expression in the epididymis. Furthermore, we characterized two epididymis-specific promoters and developed a novel technique for detection of secretion from cells.

2 REVIEW OF THE LITERATURE

2.1 Epididymal structure

2.1.1 Development and segmentation

Spermatozoa in the testis are not motile and are incapable of fertilizing ova. Spermatozoa become functional gametes only after they migrate through the epididymis and undergo an additional maturation process, thereby acquiring the capacities for both progressive motility and fertility. The main functions of the epididymis are the storage and maturation of spermatozoa until they attain their mobility in the proximal parts of this organ. These maturation events are believed to be dependent on the local environment provided by the epididymal fluid (Gong *et al.,* 2000, Jervis et *al.,* 2001). The principal components of the epididymal fluid environment are inorganic ions, small organic molecules and specific proteins synthesized and secreted by the epididymal epithelium in a highly regionalized manner. Histologically, the epididymis can be divided into four different main regions; the initial segment, the caput, the corpus and the cauda (Figure 1A). These regions are

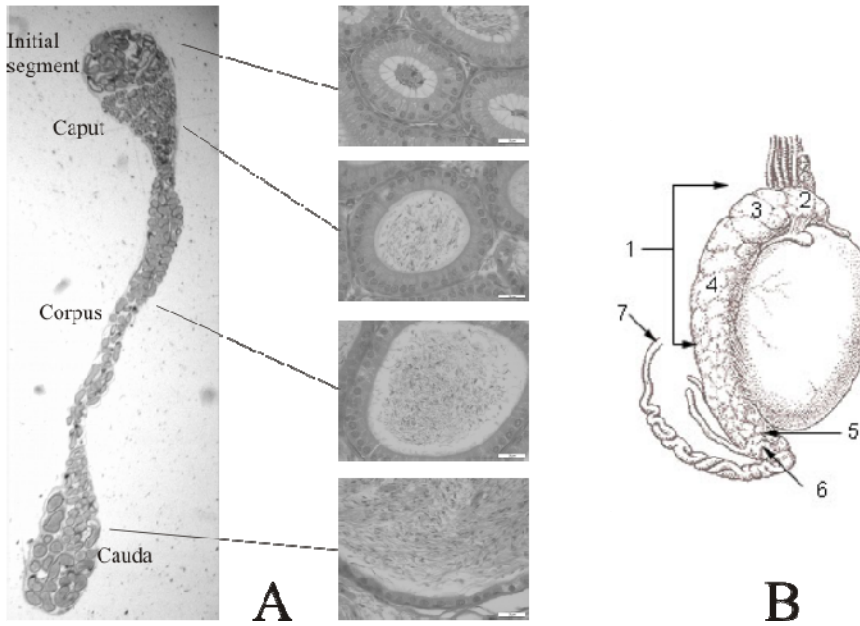

Figure 1. A. Micrograph of the mouse epididymis, showing the different regions: initial segment, caput, corpus and cauda. Cross-sectional images of the epididymal duct from each region are shown to the right. Note that the luminal diameter increases and the cell height decreases from the initial segment to the cauda (scale bar, 25 μm). B. Schematic representation of the human testis and epididymis showing the different regions of the epididymis. 1-Epididymis, 2-Head of the epididymis, 3-Lobules of the epididymis, 4-Body of the epididymis, 5-Tail of the epididymis, 6-Duct of the epididymis and 7-Vas deferens. Image A captured by Ramin Lindqvist, Image B from Wikipedia, the free encyclopedia.

further subdivided into several segments by connective tissue septa (Figure 2; Abou-Haila and Fain-Maurel, 1984). Epididymal gene expression is characterized by tissue-, segment- and cell-type specifity leading to a checkerboard -type expression pattern. This regional expression of epididymal proteins and changing ion concentrations results in a unique luminal fluid environment in each part of the epididymis. Progress through this unique environment ultimately results in sperm maturation (Hinton and Palladino, 1995). However, these anatomical divisions have been defined based on findings in animals, not in humans. The human epididymal epithelium is relatively homogeneous as viewed under the microscope, and the epididymis does not have the same distinct gross anatomical subdivisions that are easily seen in mice, rats, rabbits, dogs and other animals (Figure 1B).

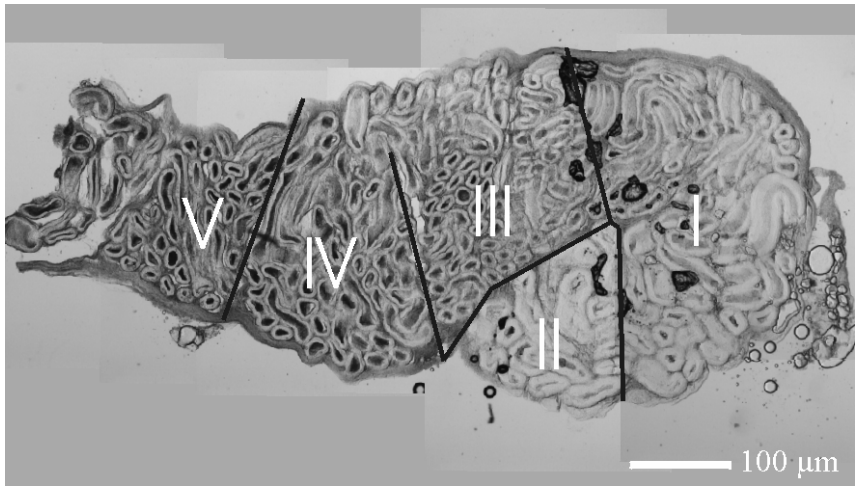

Figure 2. Light microscopic image of the mouse caput epididymis representing the different regions (defined as segments I-V) according to Abou-Haila & Fain Maurel (1984). (Image captured by Ramin Lindqvist)

2.1.2 Epididymal function

After leaving the testis, mammalian spermatozoa are not yet able to fertilize an oocyte *in vivo*. Sperm maturation involves, in addition to motility of spermatozoa, their ability to recognize and bind to the zona pellucida, and to fuse with the oocyte. These properties are only gained after their transit through the epididymis. The epididymal epithelium reabsorbs water in the efferent ducts and the initial segment, where 90 % of the testicular luminal fluid is removed (Ilio and Hess, 1994, Robaire and Hermo, 1998). Secretion of electrolytes and water by the epididymal epithelium is important in the formation of an optimal fluid environment for sperm maturation and transport. This process is disrupted in cystic fibrosis, a heritable disorder caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Wong, 1998). Contraction of smooth muscle cells surrounding the epididymal duct is responsible for the transport of sperm from the initial segment to the cauda. These contractions are dependent on adrenergic, cholinergic and purinergic signalling (Leung and Wong

1994, Mulryan *et al.,* 2000). In the testis, the seminiferous tubules are connected to a tubular complex of 12-15 ciliated efferent ducts, the rete testis. These ducts merge into a single duct, the epididymis at its head. Spermatozoa obtained from the rete testis are almost immotile. Progressive motility acquisition is gradual as the spermatozoa moves along the epididymis (Dacheux and Paquignon 1980). Mature sperm are stored in a viable but quiescent state within the cauda region of the epididymis until ejaculation. The main factor involved in maintaining sperm quiescence during storage is the low pH (Acott and Carr, 1984). Other factors involved are Ca^{2+}, HCO_3^- and the presence or absence of substrates that maintain low spermatozoal adenosine triphosphate (ATP) concentration (Gatti *et al.,* 1993, Wade *et al.,* 2003). During their transit from the caput to the cauda of the epididymis, spermatozoa undergo significant modifications in their plasma membrane composition. This includes changes in lipid content, maturation of flagellar machinery, and modification of plasma membrane proteins (Aveldano *et al.,* 1992, Liu *et al.,* 2000). During the epididymal transit, the main morphological change that occurs in spermatozoa is the migration of the cytoplasmic droplet from the base of the head to the midpiece.

2.1.3 Epididymal cell types

In adult mice, the epididymis consists of six major cell types; principal, basal, narrow, apical, clear and halo cells (Figure 3).

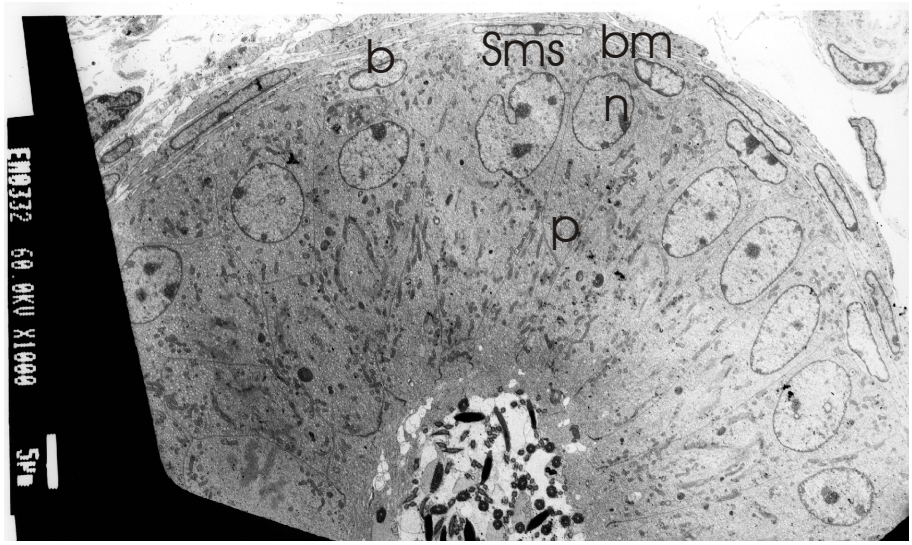

Figure 3. Electron micrograph presenting different cell types found in the initial segment of mouse epididymis: basal cell (b), smooth muscle cell (smc), basal membrane (bm), principal cell (p), nucleus (n). Scale bar (5 µm) at lower left. (Image captured by Ramin Lindqvist)

The main cell type along the epididymal duct is the principal cell. In the initial segment, the principal cells make up 80 % of all cells, while the basal cells contribute 12 %, and halo cells 5 %. The remaining 3 % is made up by a cell type found only in this region of the

epididymis; the narrow cell. Principal cells show nuclei at different levels. Those with a nucleus in the upper half of the cell are referred to as apical cells. In the rat, the principal cells and the structurally related apical cells are derived from a cell type defined as columnar cells. The conversion of columnar cells into principal and basal cells takes place at approximately day 28 after birth. The main functions of these cells are synthesis and secretion of proteins, reabsorption of luminal fluid and androgens, and secretion and transport of small organic molecules (Robaire and Hermo, 1988, Wong, 1988).

Basal cells appear along the entire epididymal duct. These cells, which do not reach the lumen, are adjacent to principal cells at the base of the epithelium, where they show a large area of contact with the basement membrane. The presence of coated and uncoated pits along their cell surface together with multivesicular bodies and lysosomes suggest that these cells are capable of endocytosis. However, the functional role played by this population of cells is not known. One role of these cells could be in the immunological protection of spermatozoa, as they express macrophage antigens. (Robaire and Hermo 1988).

Narrow cells are present in the initial segment and intermediate zone corresponding to segment 1 (Abou-Haila and Fain-Maurel, 1984). The narrow cells of the epididymis are tall columnar cells showing a narrower width than the adjacent principal cells. They are characterised by an apically located nucleus, which is usually elongated, and an apical cytoplasm, which often bulges into the lumen (Palacios *et al.,* 1991). In the mouse caput epididymidis, narrow cells also express carbonic anhydrase II (CA II) and the vacuolar proton adenosine-triphosphatase (H^+V-ATPase) pump (Hermo *et al.,* 2000). These cells produce and deliver protons to the epididymal lumen for acidification, an event that is essential for spermatozoa as they traverse and are stored in the epididymis.

Apical cells are found in the initial segment and in the caput epididymis. The specific function of these cells is still unknown. It has been recently suggested that these cells may be capable of endocytosis from the epididymal lumen (Adamali *et al.,* 1999). Clear cells, also referred to as light or prominent cells, are not found in the initial segment, but are present in the caput, corpus and cauda regions of the epididymis. Clear cells take up cytoplasmic droplets removed from spermatozoa by endocytosis and also some proteins secreted by more proximal principal cells (Robaire and Hermo, 1988). Halo cells are occasionally found in the epithelium of efferent ducts. They are also referred to as lymphocytes and possibly represent a combination of intraepithelial B and T lymphocytes and monocytes. Since monocytes are well known precursors to macrophages and since the latter have been identified in the epithelial lining of the excurrent duct system as well as in the lumen of the duct, the possibility that these cells are in fact monocytes is well founded (Robaire and Hermo, 1988).

2.2 G protein-coupled receptors

In recent years, the epididymis has gained increased attention as a possible target for the action of non-hormonal male contraceptives due to its crucial role in the final steps

of sperm maturation. Disruption of the maturation of spermatozoa in the epididymis provides a promising strategy for generating a male contraceptive. G protein-coupled receptors (GPCRs) are possible targets for such pharmacological approaches, and therefore it is essential to gain more information about the endogenously expressed GPCRs in the epididymis.

Signals triggered by a number of ligands, including hormones and neurotransmitters, are transferred across the plasma membrane by GPCRs. All GPCRs are structurally similar although they recognize different ligands. GPCRs are integral proteins of the plasma membrane with a conserved basic structure, i.e. an extracellular N-terminal portion, seven hydrophobic α-helical segments spanning the plasma membrane, and an intracellular C-terminus (Hertz *et al.,* 1997, Kristiansen *et al.,* 2004). Occupation of GPCRs by various ligands may lead to one or several of the following intracellular responses: stimulation or inhibition of adenylyl cyclases (ACs), activation of phospholipase C (PLC) and generation of inositol-1,4,5-triphosphate (IP_3) which releases Ca^{2+} from intracellular stores, activation of protein kinase C (PKC), activation of phospholipase A_2 (PLA_2), activation of guanylyl cyclases, direct G-protein-mediated activation of inwardly-rectifying K^+ channels, and inhibition or activation of voltage-dependent N-type channels or P/Q type Ca^{2+} channels (Berridge and Irvine, 1984, Neer and Clapham, 1988, Hertz *et al.,* 1997, Kukkonen *et al.,* 2001, Gotow *et al.,* 2007).

2.2.1 Purine nucleotide receptors

Two families of purine nucleotide receptors have this far been characterised; the P2X receptor family of seven subtypes (P2X1-7) consisting of ligand-gated ion channels, and the P2Y receptor family with nine subtypes (P2Y1-9) consisting of GPCRs, established in vertebrates (Abbracchio and Burnstock, 1994 and 1998, Burnstock and King, 1996, Fredholm et al., 1997, Burnstock, 1998, North and Bernard, 1998). Extracellular nucleotides can function as paracrine or autocrine mediators after their release into the extracellular fluid during cell lysis or by excocytosis of nucleotide-concentrating granules, or by efflux through membrane transport proteins (Dubyak and El-Moatassim, 1993). ATP is secreted by skeletal muscle cells, adrenal chromaffin cells, mast cells, blood cells, fibroblasts and endothelial cells (Ralevitc and Burnstock, 1998, Akerman et al., 1998). The role of ATP as a neurotransmitter or co-transmitter is also well established in the peripheral and the central nervous system (Burnstock, 1995, Collo et al., 1996). However, the action of ATP released from intracellular sources is limited by several ecto-ATPases, which keep the levels of extracellular ATP very low (Zimmerman, 1999).

2.3 G-proteins

G-proteins are a large homologous family of trimeric proteins with an α-subunit that binds guanine nucleotides, and β- and γ-subunits that are closely associated with each other. In the basal state, the βγ-complex and the guanosine diphosphate (GDP) -bound α-subunit are associated, and the heterotrimer can be recognized by an appropriate

activated receptor. Coupling of the activated receptor to the heterotrimer promotes the exchange of GDP for guanosine triphosphate (GTP) on the G protein α-subunit. The GTP-bound α-subunit dissociates from the activated receptor as well as from the βγ-complex, and both the α-subunit and the βγ-complex are now free to modulate the activity of a variety of effectors like ion channels or enzymes (Cabrera-Vera et al., 2003). The α-subunits that define the basic properties of a heterotrimeric G protein can be divided into four families; $G\alpha_s$, $G\alpha_i/G\alpha_o$, $G\alpha_q/G\alpha_{11}$, and $G\alpha_{12}/G\alpha_{13}$ (Table 1). The G-proteins are soluble proteins that associate with the plasma membrane through covalently attached fatty acyl groups and they transfer the signal from the receptor to an effector system (see Figure 4).

Table 1. Interaction of GPCRs with G-proteins

G-proteins	Signalling mechanisms associated
G_s	activates adenylyl cyclases (AC) leading to increased formation of cyclic adenosine monophosphate (cAMP) (Sunahara *et al.*, 1996)
$G_{i/o}$	activation leads to inhibition of AC, activation of inward rectified K^+-channels and inhibition of voltage-operated Ca^{2+}-channels (VOCC) (Yatani *et al.*, 1987, Dolphin, 2003)
G_q	couples to phospholipase Cβ (PLCβ) which hydrolyses phosphoinositol-phospholipid-biphosphate (PIP_2) to release diacylglycerol which activates protein kinase C (PKC) and inositol 1,4,5-triphosphate (IP_3) which mobilizes intracellular Ca^{2+} (Berridge and Irvine, 1984)

2.4 Ca^{2+} signalling

Intracellular Ca^{2+} is often considered as a life-giving signal because it is essential both for sperm motility and the acrosome reaction and, in the form of periodic Ca^{2+} oscillations, for fertilization of the egg. Having given life, Ca^{2+} can also take it away through executing apoptosis (Szalai *et al.,* 1999).

Ca^{2+} is one of the intracellular messengers mediating the action of GPCRs. The rise in cytosolic free Ca^{2+} above 500 nM can activate a variety of cellular responses such as motility, secretion, muscle contraction, gene expression and cell division (Berridge *et al.,* 2000, Ren *et al.,* 2001, Chawla *et al.,* 2001, Bootman *et al.,* 2001). Thus, Ca^{2+} is a second messenger with a wide spectrum of activities. Stimuli are mediated through cell-surface receptors to generate Ca^{2+}-mobilizing signals. Most information is available for receptors coupled to PLC, which catalyse the hydrolysis of a membrane precursor phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) into the Ca^{2+}-mobilizing second messenger inositol 1,4,5-triphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (Berridge and Irvine, 1984). PLC-coupled receptors can be divided into GPCRs and tyrosine kinase-linked receptors (RTKs).

The discovery of Ca^{2+} oscillations is one of the most significant findings in the field of intracellular signalling within the last two decades. Ca^{2+} oscillations are of interest for a variety of reasons. First, they occur in a large number of cell types, either spontaneously or as a result of stimulation by an external signal such as a hormone or a neurotransmitter. Second, it is now clear that, in addition to the rhythms encountered in electrically excitable cells, they represent the most widespread oscillatory phenomenon at the cellular level (Hiroshe et al., 1999). Third, Ca^{2+} oscillations are often associated with generation of Ca^{2+} waves within the cytosol, and sometimes between adjacent cells. Ca^{2+} is a highly versatile intra- and inter-cellular signal that operates over a wide temporal range that is now known to regulate many different cellular processes. Many components of Ca^{2+}-signalling are organised into complexes in which Ca^{2+}-signalling functions are carried out within highly localised environments. These complexes can operate as autonomous units that can be multiplied or mixed and matched to create larger, more diverse signalling systems such as in cardiac Ca^{2+} signalling. Rapid highly localised Ca^{2+} spikes regulate fast responses, whereas repetitive global transients or intracellular Ca^{2+} waves control slower responses. Cells respond to such oscillations using sophisticated mechanisms including an ability to interpret changes in frequency. Such frequency-modulated signalling can regulate specific responses such as exocytosis and differential gene transcription. (Berridge *et al.,* 2000)

2.4.1 Ca^{2+} dynamics

Cellular Ca^{2+} dynamics involves the exchange of Ca^{2+} ions between intracellular stores and the cytosol, the interior and exterior of a cell or between cells, as well as transport by diffusion and buffering due to the binding of Ca^{2+} to proteins. The mechanism of Ca^{2+} oscillations relies on feedback processes that regulate Ca^{2+} levels within the cell. Two classes of oscillations are readily distinguished: those that depend primarily on the influx of Ca^{2+} through channels from the extracellular space, and those that depend primarily on Ca^{2+} release from internal stores (Hiroshe *et al.,* 1999). In this latter class, distinctions can be made on the basis of whether the release of Ca^{2+} is dominated by the ryanodine receptor (RyR), the inositol 1,4,5-triphosphate receptor (IP_3R) or a combination of both. The IP_3Rs are Ca^{2+} channels which are opened by the binding of IP_3, generating a gradient-driven flux of Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol (in non-muscle cells) (Berridge and Irvine, 1984). RyRs are Ca^{2+} sensitive and control the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (in cardiac and skeletal muscle). The autocatalytic release of Ca^{2+} terminates once Ca^{2+} concentration reaches a sufficiently high level. Beyond this level, processes which take up Ca^{2+} from the cytosol dominate the dynamics. These involve transport of Ca^{2+} into the extracellular medium and into the ER/SR by exchangers and pumps (Berridge *et al.,* 2003). This nonlinear feedback process, called Ca^{2+}-induced Ca^{2+} release (CICR), which generates oscillations in the concentration of cytosolic free Ca^{2+}, is believed to underlie the waves that propagate via Ca^{2+} diffusion in a variety of cell types (Bootman *et al.,* 2001).

2.4.2 On mechanisms

Ca^{2+} signals generally result from the opening of Ca^{2+} -channels or the activity of Ca^{2+}-transporters. These are located either in the plasma membrane or inside the cell in the ER or SR. The Ca^{2+} -channels of the plasma membrane can be divided into different types according to their activation mechanisms: voltage-operated, receptor-operated and store-operated channels.

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

Voltage-operated Ca^{2+}-channels (VOCCs) are mainly found in excitable cells such as nerve and muscle cells, and are opened when the membrane is depolarized. Some VOCCs have a specific function to release Ca^{2+} from the internal stores. VOCCs are multimeric complexes composed of a α_1-subunit together with associated subunits (α_2, β and γ). The α_1-subunit, which has the voltage sensor and forms the channel, is the molecular entity that defines the many channel isoforms (Ren *et al.,* 2001, Chawla and Bading, 2001, Bootman *et al.,* 2001). These have different properties and sensitivities to pharmacological agents and toxins (Table 2, for review see Meir *et al.,* 1999).

Table 2. Properties of the classical VOCC functional sub-types, denoted T, N, L and P/Q.

Channel type	T	N	L	P/Q
Voltage threshold	High	Low	Low	Moderate
Inactivation rate	High	Moderate	Low	Low
Pharmacologically bloced by				
Dihydropyridines	-	-	+	-
ω-conotoxin MVIIC	-	+	-/+	-/+
ω-agatoxin IVA	-	-	-	+
Location	Mainly periphery	Neuronal	Multiple	Nerve terminal

2.4.2.2 Receptor-operated channels

Receptor-operated Ca^{2+} channels are located in the plasma membrane and have binding sites for specific transmitters. These include subtypes of *N*-methyl D-aspartate receptors (NMDAR1; NMDAR2A; NMDAR2B; NMDAR2C; NMDAR2D), 5-hydroxytryptamine receptors (5-HT_{3A}, 5-HT_{3B}) an ATP receptor type (P_{2X}), nicotinic acetylcholine receptors, second messenger operated channels (IP_4-receptors) and Ca^{2+} release activated Ca^{2+} -channels (Wang *et al.,* 1996, Walker *et al.,* 2002).

2.4.2.3 Store-operated channels

Store-operated Ca^{2+} channels open following depletion of internal Ca^{2+} stores. The molecular identity of SOCs is still not certain but evidence is accumulating that they may be homologous to the transient receptor potential (trp) channels that are expressed in *Drosophila* photoreceptors (Montell and Rubin, 1989). It has long been recognized that intracellular Ca^{2+} stores exert control over Ca^{2+} influx at the plasma membrane through SOCs. Randriamampita and Tsien first reported the existence of a low-molecular-weight factor in an acid-extracted fraction from a Jurkat cell line which was

capable of activating Ca^{2+} influx in several different nonexcitable cells (Randriamampita and Tsien, 1993). This Ca^{2+} influx factor (CIF), was released from the ER of Jurkat T cells following store depletion with either thapsigargin or an agonist of cell-surface receptors linked to the phosphoinositide pathway. Direct evidence that store depletion activates Ca^{2+} influx was provided by electrophysiological studies which established that the process of emptying the stores activated a Ca^{2+} current in mast cells called Ca^{2+} release-activated Ca^{2+} current or I_{CRAC} (Hoth and Penner, 1992). The Ca^{2+} current I_{CRAC} is the best characterized signalling pathway of store-operated Ca^{2+} entry (Figure 4, for review see Parekh and Putney, 2005).

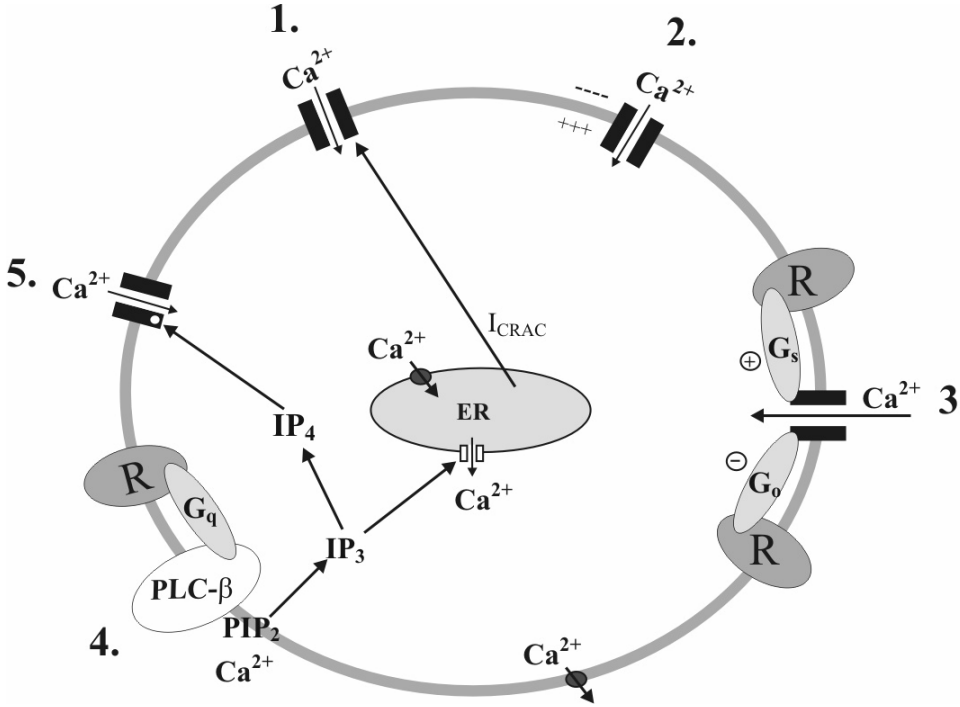

Figure 4. A schematic presentation of some of the best known cellular Ca^{2+}-influx pathways. 1. When emptied the endoplasmic reticulum (ER) store signals to open a Ca^{2+} channel called I_{CRAC}. 2. Voltage gated Ca^{2+} channels open upon depolarization. 3. Direct G-protein coupling to a Ca^{2+} channel. 4. Second messenger activated Ca^{2+} release from ER. 5. second messenger activated Ca^{2+} influx via IP_4 membrane receptor. IP_3, inositol-1,4,5-triphosphate; IP_4, inositol-1,3,4,5-tetrakisphosphate; PIP_2, phosphatidylinositol-4,5-biphosphate, PLC-β, phospholipase Cβ; R, G-protein coupled receptor;, G_s, G_q and G_o, G-proteins.

2.4.3 Cellular Ca^{2+} influx pathways in response to activation of GPCRs

Ca^{2+} is one of the intracellular messengers mediating the action of GPCRs. Many GPCRs exert their effects mainly via G proteins that activate the plasma-membrane-bound enzyme PLCβ. Receptors that operate through this signalling pathway mainly activate a G protein called G_q. PLCβ catalyses the hydrolysis of the membrane precursor PtdIns(4,5)P_2 into the Ca^{2+}-mobilizing second messenger IP_3 and diacylglycerol (DAG) (Berridge and Irvine, 1984). When IP_3 reaches the ER, it binds to IP_3R and opens IP_3-

gated Ca^{2+}-release channels. In most of the cells, the fall in Ca^{2+} concentration within the lumen of the ER subsequently activates plasma membrane Ca^{2+} channels. This Ca^{2+} influx across the plasma membrane has been called "capacitative Ca^{2+} entry" or "store-operated Ca^{2+} entry" (Putney 1986). IP_3 is degraded to IP_2 or phosphorylated to inositol-1,3,4,5-tetrakisphosphate (IP_4). The second messenger IP_4, acts via binding to its plasma membrane receptor IP_4R and activates Ca^{2+}-influx.

2.4.4 Off mechanisms

When Ca^{2+} enters the cytosol it is rapidly bound to proteins such as calbindin, calretinin, and parvalbumin. More than 90% of Ca^{2+} entering the cell is bound to these proteins with the small remainder representing the stimulus-evoked elevation of Ca^{2+} responsible for activating the Ca^{2+} sensors. During the recovery from stimulation, cells extrude Ca^{2+} from the cytosol via various mechanisms. The cell plasma membrane extrudes Ca^{2+} to the outside of the cell via Na^+/Ca^{2+} exchanger (NCX) and the ubiquitous plasma membrane Ca^{2+} ATPase (PMCA). The latter is regulated by a variety of factors including calmodulin, acidic phospholipids, and protein kinase A and C (PKA and PKC). The internal stores have a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) which pumps Ca^{2+} back into the SR/ER. The mitochondria also play an important role in Ca^{2+} signalling in that they capture Ca^{2+} rapidly from the cytosol during the recovery phase and then release it slowly back when the cells are at rest. (Berridge *et al.,* 2003)

2.5 Measurement of intracellular free Ca^{2+}

The use of optical methods for Ca^{2+} determination in various solutions is well established. A great variety of compounds undergo spectral changes in the presence of ionized Ca^{2+}. Quantitation of the magnitude of Ca^{2+} signals from changes in the emission of fluorescence relies on assumptions about the indicator behaviour *in situ* (Thomas *et al.,* 2000). Factors such as osmolarity, pH, ionic strength and protein environment can affect indicator properties making it advantageous to calibrate indicators within the required cellular or sub-cellular environment. Fluorescence indicators for intracellular Ca^{2+} can be loaded into cells as acetoxymethylester derivates which are hydrolysed by nonspecific esterases to their respective Ca^{2+} binding derivatives with free carboxyl groups (Thomas *et al.,* 2000). In a recent study with 6 different Ca^{2+} dyes, Thomas and colleagues showed that Fluo-3 proved to be the generally the most applicable Ca^{2+} indicator, since it displayed a large dynamic range, low compartmentalization and an appropriate apparent Ca^{2+} binding affinity. However, fura-2 was not included in the list of dyes studied. Determination of changes in intracellular free Ca^{2+} is most commonly performed using the fura-2 method. This indicator is taken up by cells as a hydrophobic acetoxymethylester derivative (fura-2/AM). Within the cell, the hydrophobic groups are cleaved by nonspecific esterases and the indicator is entrapped. Other indicators with different wavelengths are also available (Berridge *et al.,* 2000). An advantage of the fura-2 method is the shift in the excitation spectrum upon Ca^{2+} binding from 380 nm to 340 nm. This enables the use of dual excitation wavelengths which considerably increases the sensitivity. The measurements are performed using ordinary fluorescence spectrophotometers, either with cell suspensions or cells on coverslips or with micro-

fluorometric methods based on fluorescence microscopy. Cells or membrane sections on coverslips are placed in a fluorescence microscope and excited by alternating wavelengths. Emission is measured by a photomultiplicator, where the fluorescence of a specific cell can be measured by using apertures allowing measurement from for instance only one cell. Emitted light can also be measured using video techniques. In this case images of fluorescent cells are captured and digitized. This allows simultaneous recording from several individual cells.

Some typical optical probes used for Ca^{2+} and other ion measurements are listed in Table 3.

Table 3. Typical optical probes for ion measurements.

Ion	Probe	Excitation Wavelength (nm)	Emission Wavelength (nm)
Ca^{2+}	Fura-2	340/380	510
Ca^{2+}	Fluo-3	488	510
Ca^{2+}	Quin-2	339	400
Ca^{2+}	Fura-Red	488	660
Ca^{2+}	Calcium Green	488	530
Ca^{2+}	Calcium Orange	550	580
Ca^{2+}	Calcium Crimson	593	615
Ca^{2+}	Rhod-2	550	575
Cl^-	SPQ	320	440
Cl^-	SPA	355	490
Cl^-	MQAE	350	460
Na^+	SBFI	340	505

2.6 Green fluorescent protein (GFP)

One of the most exciting recent advances in cell biology is the possibility to use GFP and its various mutated forms as reporter proteins in studies carried out *in vitro* and *in vivo*. GFP can be used as a detection tool for cell sorting, detection of gene expression, analyzing transfection efficiency and measuring cell growth. In addition, GFP and its various mutated forms have become popular reporter proteins in transgenic animals, for evaluating promoter activity *in vivo* in different tissues and cell types (Zhuo *et al.,* 1997, Doherty *et al.,* 1999, Hunt *et al.,* 1999, Nakanishi *et al.,* 1999, Spregel *et al.,* 1999, Srinivas *et al.,* 1999, Lahti *et al.,* 2001). Site-directed mutagenesis of the primary sequence of GFP has offered researchers the possibility to choose among a number of variants of GFPs with different spectral properties.

2.6.1 Applications of GFP

Aequorea victoria GFP has become a convenient and versatile tool as a reporter protein in molecular cell biology and developmental biology in a rather short period of time. First isolated from the jellyfish *Aequorea aequorea* in 1962 (Shimomura *et al.,* 1962), the sequence of GFP remained unknown until 1992, when Prasher cloned it from

Aequorea victoria (Prasher *et al.,* 1992). Since its first use as a reporter gene in 1994 (Chalfie *et al.,* 1994), GFP has been used extensively throughout the biological sciences. GFP has been linked to proteins and promoters from archeae to zebrafish and mice. It can be used to detect and quantite cells, components of cells, or reactions within subcellular spaces. The most common use of GFP has been and remains as a reporter of gene transcription or expression of a protein of interest. While the use of GFP as a fusion protein is common to all biological enterprises, there are several distinctly biotechnological applications of GFP fusions that bear mention. GFP is used to monitor bioprocesses, both on a laboratory scale and on an industrial scale. Poppenborg and co-workers provided one of the first examples of bioprocess monitoring using GFP (Poppenborg *et al.,* 1997). Examples were given for monitoring fermentation as well as downstream processes of protein recovery. An exiting area of research that utilizes GFP fluorescence is the monitoring of non-product information, such as pH (Olsen *et al.,* 2002), oxygen (Albano *et al.,* 2001), temperature (Naik *et al.,* 2001), and nutrient availability (Miller *et al.,* 2001), in a variety of settings. In all of these applications, GFP has been linked to a particular stress or environmental insult-sensitive promoter and the corresponding fluorescence has served as an indicator of the stress level or the toxic compound concentration found after a change in the environmental conditions.

2.6.2 GFP improvements and sensing technology

There are at least three limitations to monitoring biological activity with GFP. One of the dilemmas is its stability. To monitor the expression of molecules with high turnover, it is beneficial to have a signal that is also relatively short-lived. GFP requires up to two hours from transcription to functional fluorescent protein (Tsien, 1998), making monitoring of early developmental events difficult without knowledge of the exact processing time required to make GFP visible. GFP requires oxygen to fluoresce, which is believed to be rate-limiting and makes studies of obligate anaerobes difficult but not impossible (Tsien, 1998). Finally, GFP must be present at very high concentrations in order to be seen above the background. Many biological molecules auto-fluoresce at the same wavelength as GFP, making the background to noise ratio a significant concern, especially when the expression of the gene of interest is low. It has been estimated that at least 1 μmol of GFP is required per mammalian cell to achieve a fluorescence that is twice that of the background whereas only 100 nmol of enhanced GFP (EGFP) is required for the same condition (Tsien, 1998). There have also been attempts to modify GFP to change its emission wavelength, resulting in seven classes of GFPs, green fluorescent proteins, yellow fluorescent proteins, cyan fluorescent proteins and blue fluorescent proteins. Another way to increase the GFP signal is to improve the sensing technology.

3 AIMS OF THE PRESENT STUDY

The purpose of the present thesis was to develop tools for studying the role of the epididymis in sperm maturation.

The specific aims of the present study were:

1. Development of a new technique for measurement of secretion from cells (I)

2. Development of a novel method to analyze GPCR dependent Ca^{2+} signalling in living slices of mouse caput epididymis (II).

3. To identify proper promoters to deliver transgenes into epithelial cells of the epididymis *in vivo* (III).

4. To improve the technique for detecting EGFP in transgenic mice (IV).

4 MATERIAL AND METHODS

4.1 Cell and tissue culture (I, II)

HEL 92.1.7 erythroleukemia cells were grown in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 100 U/ml penicillin G (Sigma Chemical Co., St. Louis, MO, USA), 80 U/ml streptomycin (Sigma) and 7.5% (v/v) fetal calf serum (Gibco) at 37°C in 5% CO_2 in an air ventilated humidified incubator in 260 ml plastic culture flasks (80 cm^2 bottom area, Nunc A/S, Roskilde, Denmark).

PC12 pheochromocytoma cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's Modified Essential Medium (DMEM; Gibco), supplemented with 100 U/ml penicillin G, 80 U/ml streptomycin, 5% (v/v) fetal calf serum and 10% (v/v) horse serum (Gibco) as above. PC12 cells were treated with 50 ng/ml nerve growth factor (NGF; Sigma), for 3-6 days before the measurements were performed. For microfluorometry, the cells were grown on uncoated circular glass coverslips (Ø 25 mm; Knittel, Braunschweig, Germany) in either plastic culture dishes (Ø 35 mm; Nunc) or 6-well plates (Ø 34 mm; Greiner GmbH, Frickenhausen, Germany).

Epididymal epithelia cell lines (mE-Cap27-28) (Sipilä *et al.,* 2004) (Figures 5-6, Table 4) were grown in DMEM/F12 medium (Gibco) supplemented with 10% v/v fetal calf serum (Gibco), 100 U/ml Penicillin G (Sigma), 80 U/ml streptomycin (Sigma) and 100 nM testosterone (Fluka BioChemika, Buchs, Switzerland) in an air ventilated humidified incubator in 800 ml plastic culture flasks (Nunc).

For Ca^{2+} imaging on living cells in epididymal tissue specimens, caput epididymis were dissected under a stereomicroscope and separated from the other parts of epididymis, fat was removed, and the tissues were kept at 37°C as was described for the cultured cells. For sectioning, the tissues were immobilized with 1% low melting point agarose in 0.9% NaCl (FMC BioProducts, Rockland, ME, USA) and glued on the vibratome stage. The stage was placed into a chamber filled with Na^+-based medium (see 4.3). Longitudinal sections, 150 µm in thickness, were cut under a stereomicroscope with a vibratome (752HA Campden Instruments, Leicestershire, UK), and removed carefully from the bath with coverslips, and transferred to petri dishes containing DMEM/F12 medium supplemented with 10% v/v fetal calf serum, 100 U/ml Penicillin G, 80 U/ml streptomycin and 100 nM testosterone (Sigma). Representative sections from different regions of caput epididymides were obtained and kept at 37°C as described above until Ca^{2+} measurements were performed.

All mice were handled in accordance with the institutional animal care policies of the University of Turku (Turku, Finland). The mice were specific pathogen-free and were fed with complete pellet chow and tap water ad libitum in a room with controlled light (12 h light, 12 h darkness) and temperature (21± 1 °C).

4.2 Materials (I, II)

ω-agatoxin IVA, angiotensin II, ATP, bradykinin, ωCTxGVIA (ω-conotoxin GVIA, reduced, synthetic), ωCTxMVIIC (ω-conotoxin MVIIC, reduced, synthetic), dextrometorphane (D-3-methoxy-N-methylmorphine), dopamine, isoproterenol, (–)-noradrenaline, neuropeptide Y, nicotine, phenylephrine, probenecid (p-[dipropylsulfamoyl]benzoic acid), prostaglandin E1, UK14,304 (5-bromo-N-[4,5-dihydro-1H-imidazol-2-yl]-6-quinoxalinamine), progesterone and testosterone were purchased from Sigma. FM1-43, Fura-2 acetoxymethylester, fluo-3 acetoxymethylester and pluronic acid were purchased from Molecular Probes Inc. (Eugene, OR, USA) and nifedipine (1,4-dihydro-2,6-dimethyl-4-[3-nitrophenyl]-3,5-pyridinedicarboxylic acid-2-methyoxyethyl 1methylethyl ester) from RBI (Natick, MA, USA). Membrane filters (Osmonics, Livemore, CA, USA; polycarbonate, ∅ 25 mm, thickness 25 μm, 3 × 106 pores/cm2, pore ∅ 3 μm) were purchased from AKA-filter (Stockholm, Sweden).

4.3 Media (I, II)

The Na^+-based medium consisted of 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 1 mM probenecid, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 20 mM 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulfonic acid (TES) adjusted to pH 7.4 with NaOH. The K+-based medium consisted of 142 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 1 mM probenecid, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $KHCO_3$ and 20 mM TES adjusted to pH 7.4 with KOH (the final [K+] = 158 mM). The high-K^+ medium was a mixture of Na^+- and K^+-media containing 70 mM K^+. In the patch-clamp experiments, the bath solution consisted of 140 mM NaCl, 2.8 mM KCl, 2 mM $BaCl_2$, 2 mM $MgCl_2$, 3 mM glucose and 10 Hepes adjusted to pH 7.2 with NaOH. The internal pipette solution contained 145 mM Cs-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 3 mM Mg-ATP, 2 mM EGTA, 10 Hepes adjusted to pH 7.2 with CsOH. The osmolarity of all solutions utilized in the patch-clamp experiments was 295 ± 5 mosmol/l.

4.4 Ca^{2+} determination and quantitation (I, II)

The coverslips with PC12 cells loaded with fura-2 (4 μM, 30 min, 37°C) were attached on the bottom of a thermostated (37°C) perfusion chamber. For the measurement, the cells were excited by alternating 340 nm and 380 nm light with the use of a filter changer under the control of an InCytIM-2 system (Intracellular Imaging corp. Cincinnati, OH, USA) and a dichroic mirror (DM430, Nikon co, Tokyo, Japan), and the emission was measured through a 510-nm barrier filter with an integrating CCD camera. A new ratio (340/380) image was achieved every second. All the additions were made isotonic with the Na^+-based medium. HEL cells were spun down, medium was removed and then loaded in Na^+-based medium with fura-2 (4 μM, 20 min, 37°C) as described previously (Jansson et al., 1998). After loading, the cells were kept in Na^+-based medium with 100 μM Ca^{2+} at room temperature. For experiments, either an empty coverslip or a coverslip with PC12 cells cultured for 3 days was attached at the bottom of the perfusion chamber and cell suspension of HEL cells was laid over. In

some experiments the Ca^{2+} response of HEL and PC12 cells was recorded simultaneously. For this kind of an experiment, HEL and PC12 cells were loaded separately with fura-2 (as above) and fluo-3 (2 µM, 30 min, 37°C in the presence of 0.02% pluronic acid), respectively, and placed in the chamber as described above. The Ca^{2+} measurements were then performed as described above, except that the excitation wavelengths were 380 nm (fura-2) and 490 nm (fluo-3), the dichroic mirror 500 nm and the band pass filter 520-560 nm**.**

For Ca^{2+} measurements with the epididymal tissue, tissue sections were loaded at 37°C in Na^+-based Tes-bufferred medium (TBM) supplemented with 0.5 mM probenecid and 4 µM fura-2 for 30-60 min. After loading, the sections were washed once with TBM and transferred to a perfusion chamber. In the perfusion chamber the sections were immobilized with a 25 µm -thick membrane filter. The Ca^{2+} measurements were performed using a Zeiss Axiovert microscope and Axon 2.2-image workbench. The cells were kept in TBM at 37°C, excited by alternating wavelengths of 340 and 380 nm using a narrow band filter, and the fluorescence was measured through a 430-nm dichroic mirror and a 510-nm barrier filter with a SensiCam CCD camera. One rationed image was acquired per second.

For Ca^{2+} measurement with epididymal cells (mE-Cap) (Figures 5-6, Table 4), cells were detached from culture dishes with Versene (PBS + 0.2 g/l EGTA) and then loaded in Na^+-based medium supplemented with 4 µg/ml fura-2, 10 mM Glucose, 1 mM $CaCl_2$ and 1 mM probenecid. After loading with fura-2, cells were rinsed twice with Ca^{2+} free Na^+-based medium and stored in a small volume of Na^+-based medium supplemented with only 100 µM $CaCl_2$. For Ca^{2+} measurements, fura-2-loaded cells (corresponding to 3 x 10^5 cells in suspension) were diluted into a final volume of 350 µl of Na^+-based medium supplemented with 10 m*M* glucose, 1 mM probenecid, and 1 mM $CaCl_2$ and were then transferred into a stirred quartz cuvette. This was put into the thermoregulated cuvette holder (at 35.5°C) of a spectrophotometer Hitachi F2000 (Hitachi, Ltd., Tokyo, Japan). Fluorescence was monitored at wavelengths of 340 nm, 380 nm (excitation) and 505 nm (emission). Each experiment was calibrated by treatment of the cells with 60 µg/ml digitonin (Merck, Darmstadt, Germany) followed by 10 mM EGTA. The free Ca^{2+} concentration was calculated as described previously (Holmberg et al., 1998).

4.5 Patch-clamp experiments (I)

Ba^{2+} currents via Ca^{2+} channels were recorded using the whole-cell configuration of the patch-clamp procedure. Cells grown on cover slips were positioned in a perfusion chamber mounted on the stage of a Nikon Eclipse TE200 inverted microscope (Nikon co, Tokyo, Japan). The chamber was constantly perfused at room temperature. With the exception of nifedipine and dextrometorphane which were bath-perfused, the drugs were applied by a puff-pipette placed 30-50 µm from the recording cell. Whole-cell recordings were performed with 2-4 MΩ pipettes (Kimax-51; Kimble, Vineland, NJ, USA) and an EPC-9 patch-clamp amplifier together with Pulse software (Heka, Lambrecht, Germany). Cells were held at a holding potential of –70 mV, and peak inward I_{Ba} were activated by 100 ms step depolarisations to 0 mV. Capacitative

transient and series resistance was continuously monitored and compensated for. Leak correction was applied using the P/4 protocol.

4.6 Data analysis (I, II)

The data were saved and analysed using "Calcalc", a Java program developed using Metrowerks CodeWarior. Briefly, using the Calcalc-program, the background fluorescence values at 340 nm and 380 nm at all recorded time points were first subtracted from all the areas used for analysis. Thereafter, the actual changes in rations after stimulation were calculated. The ratio data were converted to intracellular Ca^{2+} concentration using the principles described in Grynkiewicz et al. (1985). Significances were calculated using paired or non-paired two-tailed Student's *t* test.

4.7 Segment specific expression of P2X and P2Y mRNAs (II)

Total RNA was isolated from the caput, corpus, and cauda epididymides of FVB/N strain adult mice by using a single-step method (Ausbel *et al.,* 1995) as described in detail in (II). Briefly, the expression of different P2X and P2Y receptor mRNAs was analysed by reverse transcriptase-polymerase chain reaction (RT-PCR). Mouse-specific primers were designed and generated for the subtypes present in GenBank that were known to be expressed in epithelial cells. One microgram of DNAse I-treated (Gibco) total RNA was reverse-transcribed with avian myeloblastosis virus RT (Promega, Madison, WI, USA) and amplified using Dynazyme II-polymerase (Finnzymes, Espoo, Finland) in the same reaction tube. Experiments were also performed in the absence of RT to control for possible DNA contamination in the reaction.

4.8 Transgenic mouse lines and RNA analyses (III, IV)

The transgenic mice used in the present study were generated as described in detail in (III). Briefly, a 5.0-kb-long 5′-fragment of the mouse glutathione peroxidase 5 (GPX5) promoter, corresponding to nucleotides -5012 to +24 was used to drive EGFP reporter gene expression (GPX5-EGFP) and a 3.8-kb-long 5′-fragment of the mouse cysteine-rich secretory protein-1 (CRISP-1) promoter, corresponding to nucleotides -3714 to +138 was used to drive EGFP expression (CRISP1-EGFP).

For RNA analyses, total RNA from various tissues was isolated by using the single-step method (Ausbel *et al.*, 1995) as described in detail in (III). Briefly, the endogenous expression of GPX5, CRISP-1 and GPX5-EGFP and CRISP-1 -EGFP transgenes were studied by RT-PCR. One microgram of DNase I (Gibco) treated total RNA was reverse-transcribed (10 min, 50 °C) using AMV Reverse Transcriptase (Promega) and amplified using Pfu- (Promega) or Dynazyme II -polymerase (Finnzymes,) in the same reaction tube. The endogenous expression of the mouse ß-actin gene was used to generate a control for the amount of RNA used in the RT-PCR reactions. Southern Blot analyses were performed for the RT-PCR products from the GPX5-EGFP and CRISP-1 -EGFP lines. For Northern-blot analysis, 20 µg of the denatured total RNA were resolved on a 1 % denaturing agarose gel and transferred

onto nylon membranes (Hybond™-XL, Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were hybridised with the [^{32}P]αCTP labelled full-length cDNA for the EGFP using standard techniques. Hybridisation signals were detected by autoradiography using a Fuji film or a PhosphoImager (Fuji Film Ltd., Tokyo, Japan).

4.9 Detection of EGFP in tissue sections and squash preparations (III, IV)

The GPX5-EGFP and CRISP-1 –EGFP reporter mice were sacrificed by CO_2 asphyxia and the tissues were transferred to a Petri dish containing PBS. Caput epididymides were dissected out, separated from the other parts of the epididymides and fat was removed. Testes were dissected out, punctured with a needle and then the tissues were fixed for 2 hours in 4% paraformaldehyde (PFA) in PBS at room temperature.

For vibratome sections, fixed tissues were embedded in 1% low melting point agarose in 0.9% NaCl at 40 °C, and chilled to room temperature. Alternatively, the fixed tissues were supported in agarose and glued onto the vibratome stage. The stage was put into a chamber filled with PBS, 70 µm-thick sections were cut with a vibratome. The sections were mounted in PBS or fluorescent mounting medium (FMM, Dako, Carpinteria, CA, USA) and kept in the dark at 4 °C until analysed.

For cryo-sections, fixed tissues were rinsed once in PBS and embedded in Tissue-Tek O.T.C (Sakura Finetek, Torrance, CA, USA). The embedded tissues were then quickly frozen by immersing the specimens in isopentane at –70 °C or kept in the dark at 4 °C for 24 hours, and then slowly frozen at –70 °C. Before sectioning, the tissues were kept for 30 min at –20 °C. Then, 10- to 50-µm-thick sections were cut with a cryomicrotome (Leica CM 3050, Leica, Germany) and were collected on poly-l-lysine (1%) -treated microscope slides. During sectioning, the material was kept at room temperature to avoid several freezing and thawing cycles. Finally, the sections were rinsed in PBS and mounted in PBS or FMM. The sections were kept in the dark at 4 °C until analysed. When PBS was used as the mounting medium, the cover slips were sealed with rubber cement (Sanford, Bellwood, Washington, USA). For squash preparation, PFA-fixed tubules were separated from each other and the specimens were mounted in PBS or FMM.

The fluorescent light emitted by EGFP was evaluated using FITC optics (Leica,) and a research microscope (Leica DM RBE), and by using a confocal laser microscope setup (Leica TCS SP scanner and DMRE microscope; Leica Lasertechnik, Heidelberg, Germany). In the research microscope, the sections were exposed to ultraviolet light through a 450–490-nm BP filter, via a 510-nm DM dichroic mirror and the emission was captured through a 520-nm LP long pass filter with a digital camera (Leica DC 100). In the confocal laser microscope the sections were exposed to a 488-nm excitation wavelength and emission was obtained at 500–520 nm. Ten images with 2-µm intervals in the Z-axis were collected with a confocal scanner (Leica TCS SP), equipped with an Argon-Krypton-ion laser system (Omnichrome, Chino, CA, USA) coupled with a LeicaSCANware 4.2a program.

Transillumination under a stereomicroscope and preparation of cell squashes for phase contrast microscopy were performed as described earlier (Parvinen and Partula 1972, Parvinen and Hecht 1981). Phase contrast and fluorescent pictures were captured using a Kappa CF 8/1 FMC CCD black/white video camera (Gleichen, Germany) attached to a research microscope (Leica DM RBE). For fluorescence experiments, the tubules and cell monolayer were exposed by ultraviolet light through 450-490-nm BP filter, 510-nm DM dichroic mirror and the emission was captured through 520-nm LP filter with a Kappa CF 8/1 FMC CCD black/white video camera using 10 x, 40 x and 100 x objectives.

4.10 Immunohistochemistry (II, IV)

Tissues from GPX5-EGFP and CRISP-1 –EGFP reporter mice were fixed for 2 hours in 4% PFA, followed by 1–2 hours in 4% PFA - 5% sucrose, and finally incubated overnight in PBS - 20% sucrose. Frozen sections (10 μm) were prepared using the rapid freezing technique and immunostained with rabbit anti-GFP antibodies (Living Colors™ Peptide Antibody, Clontech Laboratories, Palo Alto, CA, USA), using 1:500 to 1:5000 dilutions in PBS supplemented with 1% normal goat serum (Vector laboratories Inc, Burlingame, CA, USA). The antigen-antibody complexes were visualised by using biotinylated anti-rabbit antibody (Vector Laboratories) combined with streptavidin-FITC (Dako, Glostrup, Denmark) complex.

For detection of purinergic receptors in the epididymis, FVB/N strain adult male mice were killed with CO_2 asphyxiation and the epididymides were removed and fixed in 4% PFA in PBS at room temperature for 12–16 h. Two- to three μm-thick paraffin sections were prepared for immunohistochemistry that was performed using polyclonal antibodies against rat P2X1, P2X2, P2X4, and P2X7 receptor subtypes (Sigma). The antibody binding was visualised using the avidin-biotin technique (ABC Kit, Vector Laboratories,) and Liquid DAB-Plus Substrate Kit (Zymed Laboratories, San Francisco, CA, USA).

5 RESULTS

5.1 Establishment of a novel technique for detection of secretion from cells (I)

5.1.1 Monitoring neurotransmitter release and identification of release sites (I)

Neurotransmitter release from PC12 pheochromocytoma cells was monitored using fura-2 loaded HEL 92.1.7 cells dispersed among Fluo-3 loaded NGF-differentiated PC12 cells. Depolarisation of the PC12 cells with 70 mM K^+ caused an increase in fura-2 340/380 nm fluorescence ratio in HEL cells, whereas the HEL cells alone did not respond to 70 mM K^+. The response of the HEL cells in contact with PC12 cells occurred after a delay. A subsequent application of the α_2-adrenoceptor agonist UK14,304 (10 μM) after a 5-min recovery period caused an immediate rise in the intracellular Ca^{2+} concentration in the same cells, indicating that the delayed response to depolarisation was due to slow communication between the PC12 and HEL cells. Hence, upon depolarisation, NGF-differentiated PC12 cells release factors, which activate nearby randomly distributed HEL cells. HEL cells alone, also responded to noradrenaline, dopamine, neuropeptide Y and prostaglandin E_1. The potency of dopamine was low, as 1 μM dopamine did not cause any significant increase in the intracellular Ca^{2+} concentration and 100 μM dopamine increased it to a level that was less than 50% of the response to noradrenaline or UK14,304. The α_2-adrenoceptor antagonist RX821002 reduced the secondary response in HEL cells to elevated K^+ but did not abolish it. The response to the α_2-adrenoceptor agonist UK14,304 and dopamine were, however, almost totally abolished by the antagonist. Thus the catecholamines, most likely noradrenaline, are partially responsible for the HEL cell signal.

For identification of the release sites, NGF-differentiated PC12 cells were depolarised by increasing external K^+ from 5.4 to 70 mM in the presence of the membrane dye FM1-43. In order to visualise the sites where retrieval of membrane had occurred during the depolarisation period, the fluorescence, after subsequent washing of the cells with perfusion, was monitored. The wash-resistant staining with FM1-43 was strong in areas containing cell processes but a signal could also be observed in certain cell bodies, demonstrating that the release sites are relatively randomly distributed among cell bodies and processes.

5.1.2 Role of VGCC in communication between PC12 and HEL cells (I)

Different voltage-gated Ca^{2+} channel blockers were at first tested on the K^+-induced fura-2 response in PC12 cells. The response was partially blocked by the N-type channel blocker ω-conotoxin GVIA (ωCTxGVIA, 2 μM). The L-type channel blocker, nifedipine (2 μM) reduced the response by about 50%. The nonspecific L- and N-type VGCC blocker dextromethorphan (100 μM) and the P/Q-type VGCC blockers ω-conotoxin MVIIC (ωCTxMVIIC, 2 μM) and ω-agatoxin IVA (ωAgaTxIVA, 200 nM)

both caused an even greater reduction of the Ca^{2+} response in these cells. The effects of the ω-conotoxin GVIA on Ba^{2+} currents, as determined by the patch-clamp method, was reduced by about 50% by the blocker. The other blockers of VGCC also reduced the Ba^{2+} current with a pattern similar to their effects on the fura-2 responses.

The secondary response in HEL cells dispersed among the PC12 cells was totally abolished by dextromethorphan and ωCTxMVIIC, considerably reduced by ωCTxGVIA and ωAgaTxIVA, but was relatively resistant to nifedipine. Thus, the release is a Ca^{2+} dependent exocytotic event.

5.2 Establishment of a novel technique for Ca^{2+} imaging on living cells in epididymal tissue specimens (II)

We developed a novel method to analyse Ca^{2+}-signalling in living slices of mouse caput epididymides. Our results revealed that ATP (100 μM) induced a rapid transient Ca^{2+}-signal in the epithelial cells of the different sub-regions of the mouse caput epididymis. Pre-incubating the sections in Ca^{2+}-free media did not affect the amplitude of the ATP induced Ca^{2+}-signal, indicating the presence of P2Y type purinergic receptors and PLC activity. However, extracellular Ca^{2+} was also found to be responsible for the ATP induced Ca^{2+}-signal. This was confirmed by the results showing that when 1.5 mM $CaCl_2$ was added after ATP stimulation in Ca^{2+}-free media, there was an influx of Ca^{2+} into the cells. This influx is likely to consist mainly of store-evoked Ca^{2+} (SOC) entry, which opens Ca^{2+} channels in the plasma membrane as the Ca^{2+} stores are depleted with ATP in a Ca^{2+}-free buffer, but is likely to be also partially dependent on P2X channels. Immunohistochemistry and RT-PCR analyses further revealed the expression of several P2X and P2Y receptor subtypes in epididymal epithelial cells. Immunohistochemistry revealed that all the P2X subtypes (P2X1, P2X2, P2X4 and P2X7) were expressed in the clear cells of corpus and proximal cauda epididymides, but not in the distal cauda. In the caput region, however, there were differences between the expression patterns of the subtypes: P2X1 and P2X7 were not expressed at all in the caput region, and P2X4 was only expressed in the principal cells of the proximal caput, and not in the initial segment. Interestingly, in contrast to the other subtypes, P2X2 receptor was expressed in the narrow cells of the intermediate zone of the caput epididymidis, and was the only subtype analysed which was also expressed in the initial segment. RT-PCR analyses further revealed the expression of P2X4, P2Y1 and P2Y2 in all regions of the epididymis, while P2Y4 transcript was not detected in the cauda epididymis. Hence, this implies that ATP induced Ca^{2+}-signal is due to activation of both P2X and P2Y purinergic receptors.

5.3 Ca^{2+} measurements with epididymal epithelial cell lines

Established epididymal epithelial cell lines (Sipilä et al., 2004), were also characterised for the expression of functional GPCR signalling via elevation of intracellular Ca^{2+} (fluorescence spectrophotometer-based system). The data showed the expression of purinergic, cholinergic, adrenergic and bradykinin receptors in the cell lines examined (Figures 5, 6 and Table-4).

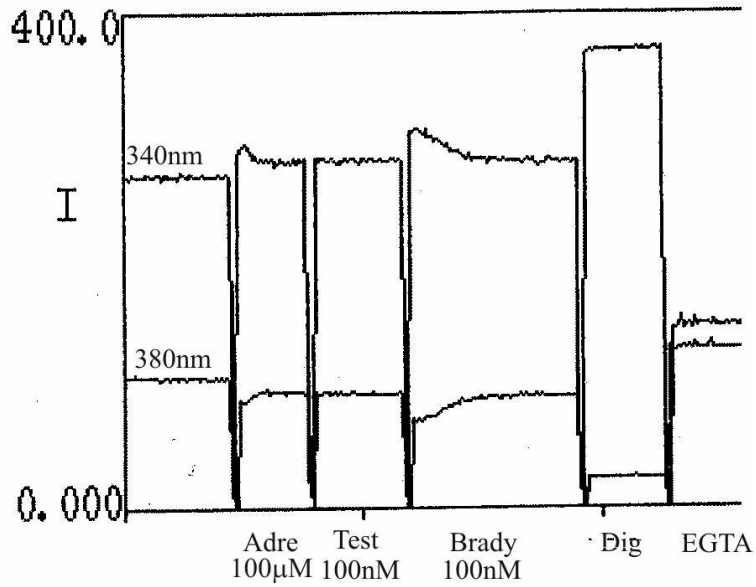

Figure 5. A representative experiment revealing the expression of functional adrenergic and bradykinin receptors in epididymal epithelial cell line mE-Cap27. The agents applied: adrenaline 100 µM (Adre), testosterone 100 nM (Test), bradykinin 100 nM (Brady), digitonin 60µg/l (Dig) and EGTA 10 mM. (Lindqvist et al., unpublished data)

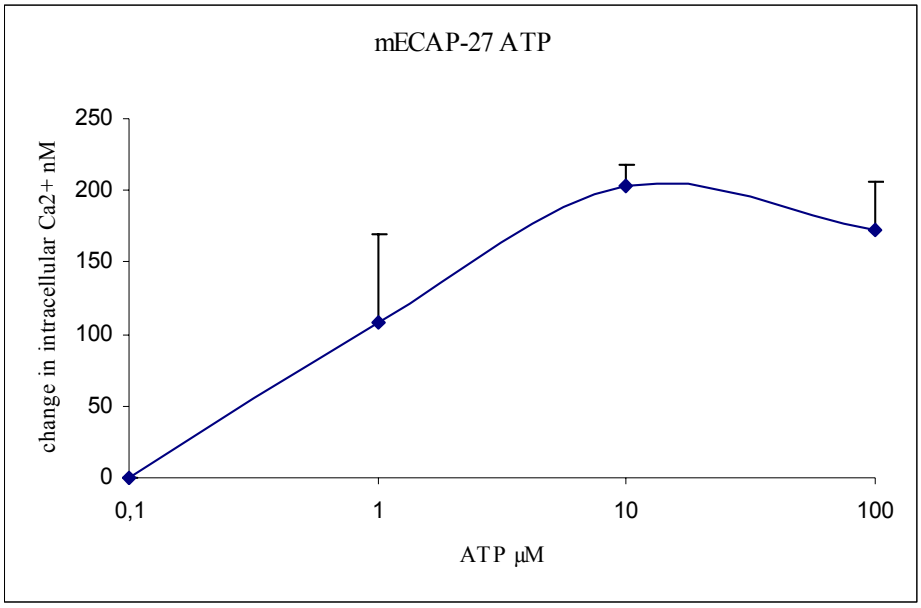

Figure 6. Intracellular Ca^{2+} elevation after stimulation of mE-Cap27 cells with different concentrations of applied ATP, n=9. (Lindqvist et al., unpublished data)

Table 4. Ligands used for the stimulation of epididymal epithelial cell lines (mE-Cap27-28), fluorescence spectrofluorometer-based system. The concentration of the ligands tested are the following; Nicotine 100 µM, Carbamyl choline chloride 100 µM, Adenosine triphosphate 100 µM, Adrenaline 10 µM, Phenylephrine 1 µM, UK14,304 1 µM, Isoproterenol 1 µM, Bradykinin 100 nM, Dopamine 5 µM, Testosterone 100 nM, Angiotensin II 100 nM, Progesterone 1µM and Human chorionic gonadotropin 100 nM. + indicates elevation of intracellular Ca^{2+} upon stimulation with ligand and -, for no elevation. (Lindqvist et al., unpublished data)

Ligand	Receptor	Response
Human chorionic gonadotropin (100 nM)	Luteinizing hormone receptor	-
Bradykinin	B_1, B_2 receptor	+
Angiotensin II	AT_1, AT_2 receptor	-
Nicotine	Nicotinic acetylcholine receptor	+
Carbamyl choline chloride	Nicotinic and muscarinic AChR	+
Adenosine triphosphate	P2X, P2Y –type purinergic receptors	+
Adrenaline	α and β-adrenergic receptors	+
Progesterone	Progesterone receptor	-
Testosterone	Androgen receptor	-
Dopamine	D_1-D_5 receptors	-
Isoproterenol	β_1, β_2, β_3 -adrenergic receptors	-
UK14,304	α_{2A}, α_{2B}, α_{2C} -adrenergic receptors	+
Phenylephrine	α_{1A}, α_{1B}, α_{1D} -adrenergic receptors	+

5.4 Tissue and cell-specific expression of GPX5-EGFP and CRISP-1 -EGFP (III)

Two epididymis-specific promoters were analysed for transgene delivery into the epididymis. The idea was to use a tissue-specific promoter to establish epididymal epithelial cell lines from immortalised epididymal cells by expressing the Simian virus 40 T-antigen in all regions of the epididymis (Sipilä *et al.,* 2004). For this purpose, we tested the 5.0 kb GPX5 and 3.8 kb CRISP-1 promoters. For visualisation, EGFP was used as the reporter gene. RT-PCR analyses of endogenous CRISP-1 gene expression confirmed that this gene is highly expressed in the epididymis, vas deferens and salivary gland. Unexpectedly, Northern blot analysis of the CRISP-1 –EGFP mice implied that the 3.8 kb long CRISP-1-promoter was highly expressed only in the testis. This was further confirmed by Southern blot analysis of the RT-PCR products. In GPX5-EGFP mice the 5.0 kb long GPX5 promoter was able to direct the reporter gene expression into the epididymis. In these mice, using Southern blot analyses of the RT-PCR products the strongest signal for EGFP mRNA was found in the epididymis.

However, when analysed, a low expression level of the reporter gene was detected in all tissues studied, similar to the level of the endogenous GPX5 gene. In the CRISP-1 - EGFP mice, EGFP fluorescence was restricted to the tubular compartment of the testis. EGFP fluorescence was detected at all stages of the seminiferous epithelial cycle between pachytene spermatocytes at stage VII to elongated spermatids at step 16. The highest fluorescence intensity was observed in steps 10-16 spermatids. Stage VII pachytene spermatocytes showed a low level of EGFP fluorescence, whereas stages X-XI pachytene spermatocytes showed an increasing EGFP fluorescence. No specific EGFP fluorescence could be detected in Sertoli cells, Leydig cells, stem cells, preleptotene, leptotene, zygotene or early pachytene spermatocytes. As expected, in the GPX5-EGFP mice, EGFP fluorescence was detected in principal cells of the distal caput region, corresponding to segment four, as defined previously by Abou-Haila & Fain-Maurel (1984). However, also a few cells expressed EGFP in the cauda region of the epididymis.

5.5 EGFP preservation and detection (IV)

Tissues obtained from transgenic mice were prepared by several procedures and the preservation of EGFP fluorescence was evaluated using conventional fluorescence and confocal microscopy. When specimens were prepared as squashes, EGFP expression could not be localized to a specific region of the caput epididymis. In addition, the specimens were difficult to seal, which led to quick drying and loss of EGFP fluorescence. Furthermore, with conventional fluorescence microscopy, EGFP detection was difficult due to high unspecific background fluorescence. In the vibratome sections and frozen sections prepared by the slow freezing protocol, EGFP fluorescence could be detected and was seen to be strictly localised to the cells expressing the protein. Conversely, when the tissues were processed using the rapid freezing protocol, all of the EGFP fluorescence was lost. Furthermore, with the slow freezing technique, EGFP fluorescence was detectable at the same intensity even after storing the specimens at –70 °C for 6 weeks or even 6 months (data not shown). The high sensitivity achieved with the slow freezing protocol developed was equal to that found using a routine immunohistochemistry method (rapid freezing protocol) involving the biotin-streptavidin-FITC technique. Furthermore, when using confocal microscopy, it was found that both in the vibratome and cryomicrotome sections, the amount of fluorescent cells increased rapidly when the fluorescence was analysed at about 5 µm below the cutting surface. Similar results were obtained with 10-µm-thick frozen sections prepared by the slow freezing protocol (data not shown).

6 DISCUSSION

6.1 The importance of developing tools for studying the role of the epididymis in sperm maturation (I-IV)

With all the information available today, it is evident that the epididymis provides the local environment needed for spermatozoa to mature. Above all, the epididymis appears to be essential for the spermatozoa to gain the ability of *in vivo* fertilisation. Therefore, contraception based on interference with epididymal function may be a good strategy for developing a male contraceptive. However, little is known about the proteins involved. For drug development, it is essential to have tools to study the function of these proteins *in vitro,* too. One approach to validate the function of certain target proteins for fertility is the generation of transgenic animals with possible defects in post-testicular sperm maturation and fertility (Yeung *et al.,* 2000, Sipilä *et al.,* 2002, Gottwald *et al.,* 2006, Davis et al., 2007). Such *in vivo* models provide new tools to study the role of the epididymis in sperm maturation and to test strategies of non-hormonal male contraceptives.

An additional approach for screening novel targets is to study the secretory products of the epididymis or the GPCRs that are involved in the maturation process of the spermatozoa. The modified Ca^{2+} imaging technique to monitor release from PC12 pheochromocytoma cells can also be applied to monitor secretory products involved in the maturational processes of spermatozoa (I).

An ideal drug compound for the pharmaceutical industry should have a relatively small molecular size, and the target protein should have a specific binding site for the drug. Proteins with enzymatic activity or receptor functions are feasible targets for this approach. Recently, this view was supported by targeted disruption of the epididymis-specific HE6/Gpr64 gene in mice. These knockout mice were infertile but histologically there was no epididymis-specific phenotype observed. Instead, the reabsorption of the testicular fluid along the efferent duct was strongly impaired. At the molecular level, many genes that belong to the cystatin, lipocalin and β-defensin families were down-regulated (Davies *et al.,* 2004, Gottwald *et al.,* 2006, Davies *et al.,* 2007). There is still uncertainty regarding the functions of these genes, but many studies have suggested that these genes are important for sperm maturation and/or protection (Kim *et al.,* 2006, Nishimura *et al.,* 2007). The development of a novel method to analyse GPCR-dependent Ca^{2+} signalling in living slices of mouse caput epididymis is an additional tool for screening for drug targets (II).

Searching for epididymis-specific promoters for transgene delivery into the epididymis is of key importance for the development of specific models for drug development. EGFP is often recombinantly coupled to other proteins (Cao et al. 2001) and the location of the resulting fusion proteins is visualised in living cells using standard fluorescence microscopy or confocal fluorescence microscopy. We used EGFP as the reporter gene to identify proper promoters to deliver transgenes into the

epithelial cells of the mouse epididymis *in vivo* (III, IV). Our results revealed that the 5.0 kb murine GPX5 promoter can be used to target transgene expression into the epididymis while the 3.8 kb CRISP-1 promoter can be used to target transgene expression into the testis (III, IV).

Although the visualisation of EGFP in living cells in culture usually poses few problems (Baumann *et al.,* 1998, Endow 2001), the detection of EGFP in tissue sections can be more difficult because soluble EGFP molecules can be lost if the cell membrane is damaged by freezing, sectioning, or permeabilisation. Furthermore, the fluorescence of EGFP is dependent on its conformation (Brejc *et al,*. 1997). Therefore, fixation protocols that immobilise EGFP may also destroy its usefulness as a fluorescent reporter. Hence, we developed a novel tissue preparation and preservation techniques for EGFP. We demonstrated that the fluorescence of EGFP is maintained in frozen sections of fixed tissues (IV). The technique is based on sections of tissues being slowly frozen in cryoprotective agent.

6.2 Cell to cell communication (I, II)

In living organisms, cells interact, communicate and signal with each other. Many cell signals are carried by molecules that are released by one cell and move to make contact with another cell. In multicellular organisms, a multitude of different signal transduction processes are required for coordinating the behaviour of individual cells to support the function of the organism as a whole. Analysis of cell signalling requires a combination of experimental and theoretical approaches, including the development and analysis of simulations and modelling. In this study, we demonstrated the development of a novel technique for measurement of secretion from cells (I).

For understanding the function of the epididymis in sperm maturation, it is of key importance to know the factors that are secreted from the epididymal cells into the lumen of the epididymis in a region-specific manner. Some of these factors can activate or inhibit ion channels on the sperm surface or even direct gene expression in the more distal parts of the duct. It has been reported that the sperm-specific cation channels CatSper 1 and CatSper 2 are required for the motility of spermatozoa in mice (Ren *et al.,* 2001, Quill *et al.,* 2003). These cation channels could be one possible target for the development of a male non-hormonal contraceptive. It has long been postulated that the main factor involved in maintaining sperm quiescence during storage is a low pH (Acott and Carr, 1984). However, other factors are also probably involved. One can postulate that the epididymis secretes factors that could inhibit the motility of the spermatozoa by inhibiting the sperm-specific ion channels. This hypothesis is possible to test in theory by combining the technique developed for investigation of functional GPCRs in the ductus epididymis with the technique for measurement of secretion from cells (I, II). The results from experiments with established epididymal epithelial cell lines revealed the expression of several GPCRs coupled to the elevation of intracellular Ca^{2+} (Table. 4). One can assume that activation of these receptors could activate the release of factors from epididymal epithelial cells that could interfere with sperm motility *in vitro*. Spermatozoa in the cauda epididymis are sub-motile. However, they are motile if isolated from the cauda epididymis and

transferred into physiological medium *in vitro*. This means that the composition of the luminal fluid is the key element in keeping the sperm in a sub-motile state. One can prepare tissue slices from different regions of the epididymis as described in (II) and keep the isolated spermatozoa in near contact to these tissue slices with the membrane (I, II). Thereafter, epididymal epithelial cells can be stimulated with different agonists and the delayed response, hopefully "inhibition of sperm motility", could be recorded. This is a feasible approach as our results with NGF-differentiated PC12 cells and HEL cells revealed that communication between randomly dispersed cells is possible even if the actual quantities of released factors are extremely small (I).

This technique could further be applied for the detection of the endogenous ligand for HE6/GPCRs. HE6/GPCRs activate G_s- and G_q-type G-proteins when overexpressed in *Xenopus* melanophores (Foord et al., 2002). Constitutive activity allows the screening for antagonists as well as for inverse agonists. However, for finding the site of secretion of the natural ligand one could stably transfect HEL cells with HE6/GPCR and apply the technique developed (I, II). Recently, even more complicated cell to cell communication was reported between gametes. The first finding was that mature sperm from dog possess odorant receptors (ORs) (Vanderhaeghen *et al.,* 1993). ORs belong to the superfamily of GPCRs, which are the most important class of target proteins for the pharmaceutical industry. The assumption is that the sperm ORs direct the migration of the sperm after ejaculation towards the oocyte. This discovery was supported by the finding that human sperm also express the odorant receptor hOR17-4 (Spher *et al.,* 2003).

In olfactory neurons, odorants bind to ORs, which activate G-proteins and initiate signal transduction *via* AC. This leads to the opening of cyclic-nucleotide-gated (CNG) channels and Ca^{2+} influx. Some of the components in the olfactory pathways, such as $G\alpha_{olf}$, AC type III and CNG channels, are also expressed in the testis and sperm (Gautier-Courteille *et al.,* 1998, Wiesner *et al.,* 1998), suggesting that testicular ORs can recruit the same cAMP-Ca^{2+} signalling cascade as in the olfactory epithelium. Pharmaceutical interference with sperm proteins involved in chemotaxis thus offers the possibility of developing drugs for contraception.

6.3 Imaging technique developed to monitor secretion from cells (I)

The development of techniques to explore cell to cell communication is of great interest in the field of cell biology. The imaging technique developed to measure secretion from NGF-differentiated PC12 cells is simple and allows real-time recordings from several regions close to the effector cells (I). The present approach, where the response of the target cells is monitored, gives information complementary to amperometry studies that measure transmitter release (Chen *et al.,* 1994, Zerby and Ewing, 1996a).

The results of this study revealed that upon depolarisation, NGF-differentiated PC12 cells release factors which trigger nearby randomly distributed HEL cells (I). This means that during the release process the released ligands reach concentrations high enough to activate receptors even some distance from the release sites.

Neurotransmitter release from NGF-differentiated PC12 cells was induced by application of 70 mM K^+. Upon application of 70 mM K^+, there was an instant elevation of Ca^{2+} in NGF-differentiated PC12 cells and a secondary Ca^{2+} elevation observed within a lag period of approximately 20 seconds in HEL cells. Stimulation with norepinephrine or UK14,304 resulted in almost instant Ca^{2+} elevation in HEL cells. The lag time in the Ca^{2+} response of HEL cells to 70 mM K^+ was therefore caused by the lag time of neurotransmitter release. This was further confirmed as the HEL cells alone did not respond to 70 mM K^+. Our results with FM1-43 and VGCC antagonists further implied that transmitter release from differentiated PC12 cells primarily takes place in cell processes and that the release is a Ca^{2+} dependent exocytotic event (I). The nature of the transmitters released from the PC12 cells is still uncertain since the sensor cells chosen may react to many transmitter substances, *e.g.* at least to noradrenaline and neuropeptide Y (Daniels et al., 1989, Michel et al. 1989, Feth et al., 1992, Musgrave and Seifert, 1995). However, the result obtained using the α_2-adrenoceptor antagonist RX821002 suggests that the catecholamines, most likely noradrenaline, are partially responsible for the HEL cell signal. The data obtained in this study suggest that the use of a filter with defined pores to prevent cellular movement makes it possible to reconstruct a communication pathway using any combination of effector and target cells providing that the affinity of the sensor cell receptors is high enough.

6.4 Development of a novel technique to investigate the presence of functional GPCRs in the ductus epididymidis by analysing intracellular Ca^{2+} in living tissue slices (II)

Measurement of intracellular ion concentrations is a conventional method in cell biology, and researchers in the field of neuroscience have consistently used brain slices for optical Ca^{2+} imaging and electrophysiological studies (Ito *et al.,* 1991, Jaffe and Brown, 1994, Yuste and Denk, 1995). Conversely, in the field of male reproduction, only cultured or isolated cells have been used this far (Leung, 1993, Wong, 1998). This, however, provides only partial information about the mechanisms involved *in vivo*.

Accumulating evidence has indicated the versatile role of extracellular ATP in regulating a number of functions of the male reproductive tract. While the extracellular ATP may be derived from the nerves innervating the reproductive tract, the epithelium or the sperm cells themselves, the actions of ATP may be multi-faceted in signal transduction (Wong, 1998, Gerendi *et al.,* 2001, Marostica *et al.,* 2001). Nevertheless, we should not exclude the possibility that extracellular purine nucleotides in the epididymis would co-operate with other ligands, similarly to that shown in other model systems (Sneddon and Burnstock, 1984, Sneddon and Westfall, 1984, Burnstock, 1995, Akerman et al., 1998). Whether ATP released from spermatozoa in connection with such a ligand is capable of stimulating the epididymal epithelial cells remains to be analysed. A major problem in demonstrating the physiological roles of different purinergic receptor subtypes is due to lack of potent selective antagonists. However, from the findings from P2X1-knockout mice, it is apparent that these receptors may be

considered as putative targets for male contraception (Mulryan *et al.,* 2000). Even if the only direct phenotypic consequence observed in these mice was a reduction of the contractions of the vas deferens, this led to low sperm count in the ejaculate and infertility. This clearly shows that purinergic co-transmission has a physiological role in the vas deferens.

This study was initiated to evaluate the technique developed for detection of GPCRs *in vitro* (II). Purinergic receptors were chosen for the evaluation of this technique as previous findings indicated that ATP-induced rises in intracellular Ca^{2+} concentrations in epididymal cells activate Cl^- channels in the apical cell membrane, leading to increased transepithelial Cl^- secretion *in vivo* and *in vitro* (Leung, 1993, Wong, 1998). However, the purinergic receptor subtypes responsible for Cl^- secretion *in vivo* are still unclear.

The results of this study demonstrate the wide expression of different types of purinergic receptors, and the evidently cell-specific expression of the receptor subtypes in the caput and initial segment of the epididymis. Our results with Ca^{2+} imaging revealed the presence of P2Y-type ion-gated purinergic receptors in the epithelial cells of the mouse caput epididymidis (II). This is in line with the results obtained using immortalised epididymal epithelial cell lines (Sipilä *et al.,* 2004). Immunohistochemical and RT-PCR analyses further implied the expression of at least P2X1, P2X2, P2X4 and P2X7, and P2Y1 and P2Y2 receptors in the epididymis. In the corpus and proximal cauda regions, all P2X receptor subtypes analyzed (P2X1, 2, 4 and 7) were expressed in the clear cells, while in the distal caput, only P2X2 and P2X4 receptors were expressed in a cell-specific manner. The P2X4 receptors were expressed in the principal cells, while P2X2 receptors were expressed in narrow cells in the initial segment and intermediate zone.

The cell-specific expression of P2X2 and P2X4 receptors is interesting as these isoforms do not desensitise as readily in neurons, muscle cells or heterologous expression systems as do P2X1 or P2X3 receptors (Buell and Collo, 1996, Collo 1996). The function of P2XRs in epithelial cells may not be limited to stimulation of Cl^--secretion across the epithelial cell membrane. Changing of membrane potential caused by P2XR-mediated fluxes and capacitative Ca^{2+} entry through P2XR channels may affect many epithelial functions through intracellular Ca^{2+} signalling such as vesicle trafficking, protein secretion and signal transduction pathways that depend on Ca^{2+}. Taken together, it is apparent that purinergic receptors play an essential role in the epididymis. However, the details regarding the roles of different receptor subtypes involved in producing the proper epididymal environment for post- testicular sperm maturation require further investigation.

6.5 GPCRs expressed in the epithelial cells of the caput epididymis

Even if there have been many reports of primary cultures of epididymal epithelial cells, their use has been limited due to their short life span. Research in this field would be greatly facilitated by the establishment of cell lines that would offer a permanent and constant supply of epididymal epithelial cells. We have established transgenic mouse

lines GPX5-Tag1, expressing SV40 Tag under a 5-kb murine GPX5 promoter (Lahti *et al.,* 2001). Eighteen epithelial cell lines were generated from the caput region, using epididymal tumours from 4-month-old mice, and were named mE-Cap11-28 (Sipilä *et al.,* 2004). Ca^{2+} imaging revealed the expression of functional purinergic receptors in these cell lines, similar to that shown on vibratome slices of living ductus epididymidis (II). In addition, a spectrophotometer-based method revealed the expression of purinergic, adrenergic, cholinergic and bradykinin receptors in the tested cell lines (mE-Cap27-28). In line with our results, muscarinic acetylcholine receptor (mAChR) subtypes M1-M5 have been reported to be expressed in the epithelial cells of the ductus epididymis (Marostica *et al.,* 2001, Siu *et al.,* 2006). In addition, functional studies implied the involvement of these subtypes in epididymal duct contraction. Thus, cholinergic neurotransmitter mechanisms in the epididymis may be among the factors controlling contractility and/or the composition of the luminal fluid.

In our experiments, we used carbamyl choline chloride for the screening of possible mAChRs. Since this agonist activates both nicotinic and muscarinic AChRs, it is impossible for us to conclude which receptor subtypes are expressed in epididymal epithelial cells. However, further experiments showed the expression of nAChRs in the cells examined. This is interesting, as this is the first report to reveal the expression of functional nAChRs in epididymal epithelial cells. There have been many reports suggesting that components of tobacco smoke can reduce the motility of spermatozoa *in vitro* (Vine, 1996, Arabi, 2004). However, other findings have implied that nicotine alone at physiological concentrations does not affect sperm kinetic parameters *in vitro* (Gandini *et al.,* 1997). Therefore, the cell-specific expression of the various mAChR subtypes and nAChRs in the efferent duct and epididymis suggests that these receptors may play roles in the modulation of luminal fluid composition and smooth muscle contraction.

6.6 Characterisation of GPX5 and CRISP-1 promoters *in vivo*

Previous studies have shown that the mouse GPX5 gene is expressed at a high level in the caput epididymidis (Fauer *et al.,* 1991, Vernet et al., 1996), but also at low levels in the kidney and liver (Dufaure et al., 1996). In contrast, the mouse CRISP-1 gene is highly expressed in the corpus and cauda epididymis, but also to a lesser extent in the male salivary gland and vas deferens (Haendler *et al.,* 1993, Eberspracher *et al.,* 1995). In the present study, we used EGFP as the reporter gene to identify proper promoters to deliver transgenes into epithelial cells of the mouse epididymis *in vivo* (III, IV). Our results demonstrate that the 5.0 kb murine GPX5 promoter can be used to target transgene expression into principal cells of the segment 4 of the caput epididymis, while the 3.8 kb CRISP-1 promoter can be used to target transgene expression into all stages of the seminiferous epithelial cell cycle between pachytene spermatocytes at stage VII to elongated spermatids at step 16 in the testis (III, IV). Hence, the 5.0 kb 5'-flanking segment of the GPX5 gene can be used for expressing genes of interest mainly in the distal caput region of epididymis, whereas the 3.8 kb 5'flanking region of the CRISP-1 promoter may be used for expressing genes in late meiotic and post-meiotic spermatogenic cells.

6.7 Preservation and detection of EGFP in tissues from transgenic male mice

We used EGFP as the reporter gene to identify proper promoters to deliver transgenes into the epithelial cells of the mouse epididymis *in vivo* (III, IV). Although the visualisation of EGFP in living cells in culture usually poses few problems (Baumann *et al.,* 1998, Endow 2001), the detection of EGFP in tissue sections can be more difficult because soluble EGFP molecules can be lost if the cell membrane is damaged by freezing, sectioning, or permeabilization. Furthermore, the fluorescence of EGFP is dependent on its conformation (Brejc *et al,.* 1997). Therefore, fixation protocols that immobilize EGFP must also preserve its conformation. We therefore developed a novel tissue preparation and preservation techniques for EGFP. The technique is based on sections of tissues being slowly frozen in cryoprotective agent. Our results indicate that the sensitivity of EGFP detection is clearly dependent on the specimen preparation technique used, and when using frozen sections, the type of tissue freezing protocol is of key importance. In line with our results, Kusser and Randall showed that EGFP was preserved in thick sections, but they found that the technique developed did not equally well maintain the brightness of EGFP in thin sections when a combination of 4 % formalin and 7 % picric acid in PBS was used (Kusser and Randall, 2003). In contrast to our results, Manfra and colleagues showed that GFP can be detected in frozen sections without post-fixation (Manfra *et al.,* 2001). However, they did not report the freezing protocol used.

Among the various preparation procedures tested, the best morphological and histological preservation and reproducibility were obtained using frozen sections and a slow tissue freezing protocol. In addition, our results implied that the fluorescence of EGFP is maintained in frozen sections of fixed tissues and was detectable at the same intensity even after storage of the specimens at –70 °C for 6 weeks (IV) and still even after 6 months of storage (unpublished data). Thus, the technique developed offers the possibility to collect tissue samples and perform the analyses several weeks or even months later. An additional advantage of this method is that prior to sectioning, the collected samples are already in the correct orientation as positioned by the investigator.

6.8 Concluding remarks and future directions

The present series of investigations developed tools for studying the role of the epididymis in sperm maturation. We developed a new technique to analyze GPCR dependent Ca^{2+} signalling in living slices of the mouse caput epididymis. In addition, we improved the method of detecting reporter gene expression in the epididymis. Furthermore, we characterised two epididymis-specific gene promoters and developed an novel technique for the measurement of cell to cell communication. Together, these new techniques will certainly help us to explore the functions of the epididymis more efficiently. An important future direction would be to further analyse the role of GPCRs in sperm maturation, in order to find potential target candidates for male contraception.

7 SUMMARY AND CONCLUSIONS

In recent years, the epididymis has gained increased attention as a possible target for the action of non-hormonal male contraceptives due to its crucial role in the final steps of sperm maturation. An ideal male post-testicular contraceptive should allow normal sperm production in the testis, but block the maturational event of spermatozoa in the epididymis. Hence, disruption of the maturation of spermatozoa in the epididymis provides a promising strategy for generating a male contraceptive. GPCRs are possible targets for such pharmacological approaches. The present series of investigations focused on developing tools to detect, study and measure cell to cell communication, reporter gene expression and functional GPCRs in endocrine cells and reproductive tissues *in vivo* and *in vitro*. The following conclusions were reached:

1. Upon depolarisation, NGF-differentiated PC12 cells release factors which activate nearby randomly distributed HEL cells. This means that during the release process the released ligands reach concentrations high enough to activate receptors even some distance from the release sites. Thus, communication between randomly dispersed cells is possible even if the actual quantities of transmitter released are extremely small. The results obtained using blockers of VGCC suggest that the release is a Ca^{2+} dependent exocytotic event.

2. A novel method was developed for intracellular Ca^{2+} imaging on living cells of epididymal tissue. The method provides a novel tool for observing dynamic changes in epididymal cells *in vitro*, and also in other reproductive tissues.

3. Epididymal epithelial cells express functional purinergic, adrenergic, cholinergic and bradykinin receptors *in vitro*.

4. The 5.0 kb murine GPX55 promoter may be used to deliver transgene expression in the epithelial cells of the caput epididymidis while the 3.8 kb CRISP-1 promoter can be used to deliver transgene expression in late spermatids of the testis. EGFP may be used as a reporter gene when the tissue slices are prepared at room temperature.

5. With confocal imaging techniques, it is currently possible to detect EGFP fluorescence in cells which do not show fluorescence in conventional fluorescence microscopy. The highest sensitivity for detection of EGFP was obtained by applying confocal microscopy to frozen sections prepared according to the slow freezing protocol described in the present report. In addition, our results implied that the fluorescence of EGFP is maintained in frozen sections of fixed tissues and was detectable at the same intensity even after storage of the specimens at –70 °C for six weeks.

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