The Involvement of a Novel Anion Exchanger, SLC26A3, in Sperm Function

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Abstract of thesis entitled:

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Submitted by **CHEN, Wenying**

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at The Chinese University of Hong Kong in **September 2009**

Abstract

Our previous study has demonstrated the involvement of Cystic fibrosis transmembrane conductance regulator (CFTR) in transporting bicarbonate necessary for sperm capacitation. However, whether its involvement is direct or indirect remains unclear. The present study is design to investigate: (1) the possibility of a Cl^-/HCO_3^- exchanger, solute carrier family 26, number 3 (SLC26A3), operating with CFTR during sperm capacitation, (2) the role and the underlying mechanisms of SLC26A3 in other sperm post-testicular processes and spermatogenesis.

In the first part of study, guinea pig sperm which were incubated in medium with various concentrations of Cl^- resulted in varied percentages of capacitated sperm, in a concentration dependent manner. Depleting Cl^-, even in the presence of HCO_3^-, abolished sperm capacitation and vice versa, indicating the involvement of both anions in the process. Capacitation-associated HCO_3^- dependent events, including cAMP production, protein tyrosine phosphorylation and pHi increase also depend on Cl^- concentrations. Similar Cl^- dependence was observed for sperm hyperactivated motility and sperm-egg fusion. The capacitation-associated events could also be significantly reduced by inhibitors or antibodies of CFTR and SLC26A3, with a more potent effect observed for niflumate, an inhibitor more selective for SLC26A3, over that of DIDS, an inhibitor more selective for SLC4 exchangers. The expression and localization of CFTR and SLC26A3 in guinea pig sperm were also demonstrated using immunostaining and Western blot analysis. Our results indicate that Cl^- is required for the entry of HCO_3^- necessary for sperm capacitation, implicating the involvement of SLC26A3 in transporting HCO_3^- with CFTR providing the recycling pathway for Cl^-.

In the second part of study, GC-1 spg cell line that expresses SLC26A6 but not SLC26A3 was used as a negative control. The cells and sperm were pretreated with anion exchanger inhibitors and SLC26A3 antibody, and then membrane potential and intracellular calcium were measured. Our results showed that DIDS could inhibit the HCO_3^- deficiency induced depolarization of GC-1 spg cells as well as the depolarization induced by Cl^-- or HCO_3^-- deficiency in sperm. Niflumate could inhibit the HCO_3^- induced $[Ca^{2+}]$ i increase of the sperm but not GC-1 spg cells. SLC26A3 antibody had

no effect on the GC-1 spg cells but it could block the depolarization caused by Cl^--deficiency in sperm.

Further *in vivo* functional studies were also performed. The SLC26A3 antibody was injected into the BALB/C mice seminiferous tubules using micropipette. The animals were sacrificed after three days, and CASA, daily sperm production (DSP) were used to evaluate sperm motility and spermatogenesis. The results showed that sperm motility was increased while there was no significant difference between DSP. Our results indicate that SLC26A3 on sperm does not play a dominant role in spermatogenesis, epididymal maturation and sperm motility.

Taken together, our results demonstrate the involvement of SLC26A3 in sperm function, particularly in transporting HCO_3^- necessary for sperm capacitation, which appears to be working with CFTR providing the recycling pathway for Cl^- in parallel. The present results also provide an explanation to the observed subfertility in patients with SLC26A3 mutations. Further *in vitro* and *in vivo* studies also have shown that SLC26A3 does not play a predominant role in spermatogenesis but may affect other post-testicular maturation processes.

摘　要

我們之前的研究證明囊性跨膜電導調節器（CFTR）參與轉運精子獲能所必需的碳酸氫根。但是，它是直接或是間接參與轉運仍然不清楚。我們當前的研究就是爲了闡明: (1) SLC26A3 這個 Cl^-/HCO_3^-陰離子交換器是否和 CFTR 一起參與了精子獲能以及其可能的機制; (2) SLC26A3 是否參與精子發生和除獲能外的其他精子成熟階段及其可能機制。

在第一部分研究中，豚鼠精子在不同 Cl^-濃度培養液中培養，精子獲能隨 Cl^-濃度下降而明顯抑制，並呈濃度依賴關係。甚至在 HCO_3^-（25mM）存在下，精子在缺 Cl^-培養液中仍不能發生獲能，反之亦然，表明兩种離子同時參與了此過程。與獲能相關的 HCO_3^-依賴事件，包括 cAMP 的產生，蛋白酪氨酸磷酸化以及胞內 pH 的升高都依賴于氯離子。同樣，精子超激活運動和精卵融合也依賴于氯離子。這些獲能相關事件可被 CFTR 和 SLC26A3 抑制劑以及 SLC26A3 特異抗體抑制。但是 niflumate 比 DIDS 的作用更強，而前者對 SLC26A3 更特異。精子中 CFTR 和 SLC26A3 的表達和定位也用免疫熒光和 Western blot 免疫印跡法得到證實。

在第二部分研究中， 爲了進一步證實 SLC26A3 在精子功能中的作用，我們用一個只表達 SLC26A6 但不表達 SLC26A3 的細胞係 GC-1 spg 作爲陰性對照。我們用了不同離子交換子抑制劑和 SLC26A3 特異抗體進行對比研究。結果表明 DIDS 可以抑制 GC-1 spg 細胞株在碳酸氫根缺乏下的細胞去極化，但不能抑制碳酸氫根引發的胞內鈣離子的升

高。而 niflumate 和 SLC26A3 特異抗體對這些事件都沒作用。對於精子，DIDS 可以抑制精子在缺氯和缺碳酸氫根時的去極化，SLC26A3 特異抗體則可抑制精子在缺氯時的去極化，而 niflumate 可抑制碳酸氫根引發的胞內鈣離子上升。

BALB/C 小鼠體內睾丸曲細精管 SLC26A3 特異抗體注射實驗表明，SLC26A3 對精子活力有明顯促進作用，但對精子的 DAP 沒有影響。這些結果表明 SLC26A3 在精子發生、附睾成熟和精子活力方面不起重要作用。

綜上所述，SLC26A3 對精子功能，尤其是精子獲能中必須 的碳酸氫根轉運起重要作用。其機制可能是為 CFTR 提供一個氯離子的再循環通路共同轉運碳酸氫根。這也為 SLC26A3 基因突變病人的不育提供了一個解釋。進一步的體內和体外實驗證實，SLC26A3 有可能主要參與成熟精子後期功能，特別是在精子獲能，而不是作用于精子發生，附睾轉運成熟階段和精子活力。

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List of Abbreviations

AE	Anion exchanger
Akt	Protein kinases B
ALH	Average lateral head movement
AMRC	Apical mitochondrial rich cells
ANOVA	Analysis of variance
AR	Acrosome reaction
ARIS	Acrosome reaction- inducing substance
ATP	Adenosine triphosphate
BCECF-AM	2'7-bis(2-carboxyethyl)-5-(^)-carboxyfluorescein-acetoxymethyl ester
BSA	Bovine serum albumin
CA	Carbonic anhydrase
$[Ca^{2+}]i$	Intracellular calcium
CaM	Calmodulin
CaMK	Calmodulin kinase
cAMP	Adenosine-3', 5'-cyclicmonophosphate
CASA	Computer-assisted sperm analysis
Cav2.3 (α_{1E})	Calcium channel, voltage-dependent, R type, alpha 1E subunit
CBVAD	Congenital bilateral absence of the vas deferenses
cDNA	Complementary deoxynucleotic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
ClC-2	Chloride channel 2
CLD	Congenital chloride diarrhea
CNG	Cyclic nucleotide-gated channels
CTC	Chlortetracycline
DAG	Diacylglycerol
DAPI	4',6-duanudubi-2-phenylindole
DEPC	Diethyl pyrocarbonate
DIDS	4', 4'-diisothiocyanostilbene-2', 2'-disulfonic acid
Dlg A	*Drosophila* disc large tumor suppressor
DiBAC4(3)	bis-(1,3-dibarbituric acid)-trimethine oxanol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	N, N-dimethyl sulfoxide
dNTP	Deoxynucleoside 5'-triphosphate
DNA	Deoxynucleotic acid
DPC	Diphenyl-amine 2',2'-dicarboxylic acid
DSP	Daily sperm production
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EnaCs	Epithelial Na^+ channel
Em	Membrane potential
FBS	Fetal bovine serum
HAM	Hyperactivated motility
HBSS	Hank's balanced salt solution

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IP3	inositol-1,4,5-triphosphate
IVF	*In vitro* fertilization
IU	International unit
K-H solution	Krebs-Henseleit solution
LIN	Linearity
MCM	Minimal Capacitating Medium
MgcRacGAP	GTPase activating protein for RhoGTPases
MOT	Percentage motile
mRNA	Messenger ribonucleic acid
MS	Mitochondrial sheath
MSD	Membrane-spanning domain
NBC	Na^+-HCO_3^- cotransporter
NBD	Nucleotide binding domain
NHE3	Na^+/H^+ exchanger 3
NHERF-1	Na^+/H^+ exchanger regulating factor 1
NKCC1	Na(+)-K(+)-2Cl(-) cotransporter
P	Progesterone
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
pHi	Intracellular pH
PI3-K	Protein 1-phsphatidylinositol-3-kinase
PIP2	Phosphatidylinostol-1,4-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMCA4	Ca^{2+}/calmodulin-dependent Ca^{2+}-ATPase
PMSF	phenylmethylsulphonyl fluoride
PROG	Percentage of progressive
P/S	Penicillin/streptomycin
PSD95	Postsynatic density protein 95
PVDF	Polyvinylidene fluoride
RAP	Percentage rapid
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAC (SACY)	Soluble adenylate cyclase
SDS	Sodium Dodecyl Sulfate
SLC26A3, DRA	Solute carrier family 26A3
SLC26A6	Solute carrier family 26A6
SEM	Stand error of the mean
STAS	Sulphate transporter and anti-sigma factor antagonist domain
Tat1 (Slc26a8)	The testis anion transporter 1

TBST	Tris Buffered Saline Tween 20
TRPC	Transient receptor potential cation channel, subfamily C
UV	Ultraviolet
VCL	Velocity curvilinear
VAP	Velocity average path
VSL	Velocity straight line
ZO-1	Zonula occludens-1 protein
ZP	Zona pellucida

List of Publications

1. Chan HC, Shi QX, Zhou CX, Wang XF, Xu WM, **Chen WY**, Chen AJ, Ni Y, Yuan YY. Critical role of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm. ***Mol Cell Endocrinol.*** 2006, 250: 106-113.

2. Xu WM, Shi QX, **Chen WY**, Zhou CX, Ni Y, Rowlands DK, Yi Liu G, Zhu H, Ma ZG, Wang XF, Chen ZH, Zhou SC, Dong HS, Zhang XH, Chung YW, Yuan YY, YANG WX, Chan HC. Cystic fibrosis transmembrane conductance regulator is vital to sperm fertilizing capacity and male fertility. ***PNAS***, 2007, 104(23): 9816-9821.

3. **Chen WY**, Xu WM, Chen ZH, Ni Y, Yuan YY, Zhou SC, Zhou WW, Tsang LL, Chung YW, Höglund P, Chan HC, Shi QX. Cl- is required for HCO3- entry necessary for sperm capacitation in guinea pig: involvement of a Cl-/HCO3- exchanger (SLC26A3) and CFTR. ***Biol Reprod.*** 2009; 80(1): 115-23.

4. Xu WM, Zhang XH, **Chen WY**, Fok KL, Rowlands DK, Chui YL, Chan HC. Immunization with Bin1b decreases sperm motility with compromised fertility in rats. ***Fertil steril.*** 2009, Jan. 7, Epub ahead of print.

5. Yin L, Chung CM, Huo R, Liu H, Zhou C, Xu W, Zhu H, Zhang J, Shi Q, Wong HY, Chen J, Lu Y, Bi Y, Zhao C, Du Y, Ma M, Cai Y, **Chen WY**, Fok KL, Tsang LL, Li K, Ni Y, Chung YW, Zhou Z, Sha J, Chan HC. A sperm GPI-anchored protein elicits sperm-cumulus cross-talk leading to the acrosome reaction. ***Cell Mol Life Sci.*** 2009 Mar; 66(5):900-8.

6. Chan HC, Ruan YC, He Q, Chen MH, Chen H, Xu WM, **Chen WY**, Xie C, Zhang XH, Zhou Z. The cystic fibrosis transmembrane conductance regulator in reproductive health and disease. ***J Physiol.*** 2009 May 15; 587(Pt 10):2187-95. Epub 2008 Nov 17.

Chapter I

Introduction

Mammalian spermatozoa are generated in the testis through a process called spermatogenesis. However, spermatozoa in the testis are immotile and infertile, and they are required to undergo further mature steps in male and female reproductive tracts to gain their fertilizing ability. After release from the testis, the first mature step for the sperm to acquire the motility and fertility occurs in the epididymis. After ejaculation, spermatozoa undergo different processes in female reproductive tract, including capacitation, acrosome reaction (AR) and finally fertilize the egg (Figure I.1).

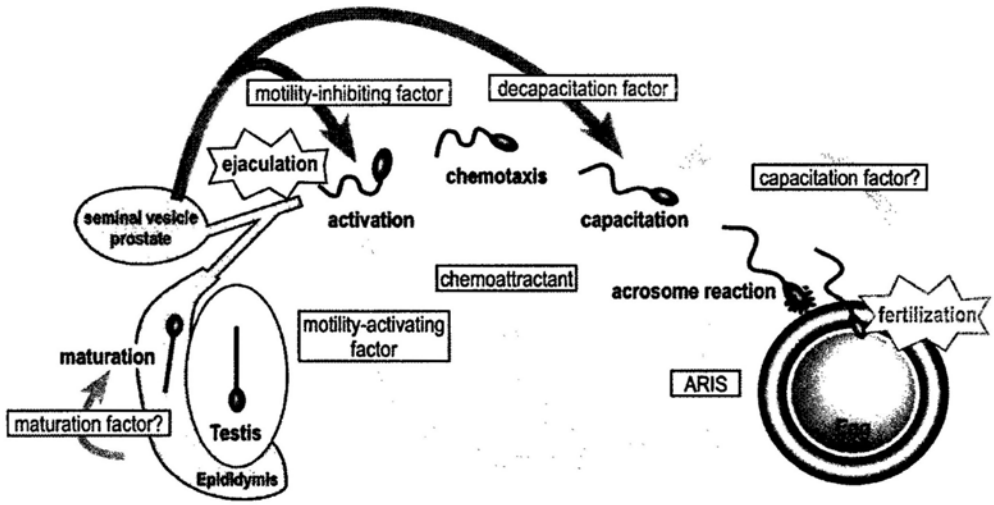


Figure I.1. Schematic drawing of sperm journey and events. ARIS: acrosome reaction- inducing substance. (Yoshida et al., 2008)

I.1 Spermatogenesis

I.1.1 Spermatogenesis process

Spermatogenesis (Figure I.2) is the process that spermatogonia develop into mature spermatozoa. This process starts in puberty and occurs in seminiferous tubules of testes. It takes approximately 35 days in mice and 64 days in human. The entire process can be divided into several stages:

Spermatocytogenesis: In spermatocytogenesis, a diploid spermatogonium divides mitotically to produce two diploid primary spermatocytes. Each spermatocyte then undergoes meiosis I to produce two haploid secondary spermatocytes which includes the chromosomal crossover. It should be noted that some spermatogonia divide to duplicate themselves to maintain the spermatogenesis. **Spermatidogenesis:** The secondary spermatocytes produced earlier undergo meiosis II and divide to produce haploid spermatids. **Spermiogenesis:** In this stage, spermatids are turned into mature spermatozoa. The process includes nuclear and cytoplasmic changes of the cells. During this stage, the spermatids grow the tail and on the other end the Golgi apparatus gradually forms the acrosome. The DNA content also becomes highly condensed and the cytoplasmic residue is removed. At the end of the spermiogenesis, the sperm are released to the lumen of the seminiferous tubules which is called spermiation.

I.1.2. Spermatogenesis and ion channels/transporters

Undoubtedly, different ion channels/transporters in sperm membrane contribute to sperm functions. Besides, in all differentiation stages, spermatogenic cells are surrounded by or attached to epithelial cells. Therefore, ion transport and water absorption/secretion play fundamental roles in the spermatogenesis process. Ion channels are pore-forming proteins that help to establish and control the small voltage gradient across the plasma membrane of all living cells by allowing the flow of ions down their electrochemical gradient. While what and how ion channels/transporters are involved in spermatogenesis remains unclear, there are evidences showing that Cl^- and HCO_3^- transportation may be important. The Na (+)-K(+)-2Cl(-) cotransporter (NKCC1) deficient male mice are infertile because of defective spermatogenesis, as shown by the absence of spermatozoa in histological sections of their epididymes and the small

number of spermatids in their testes(Pace et al., 2000). Anion exchanger 2(AE2) which is responsible for $Cl^-$and HCO_3^- exchange and chloride channel 2 (ClC-2) were involved in the spermatogenesis. Both null mice are sterile due to the blockage of the spermatogenesis caused by mutation of the two genes. (Bosl et al., 2001; Medina et al., 2003). Cystic fibrosis transmembrane conductance regulator (CFTR), which conducts both Cl^- and HCO_3^- (Welsh and Smith, 1993;Poulsen et al., 1994), is related to poor sperm quality besides congenital bilateral absence of the vas deferenses (CBVAD) (van, V et al., 1996). Studies indicate that the frequency of CFTR mutations in the men of non-obstructive azoospermia, oligozoospermia is much higher than general population, with the high prevalence of the 5T allele both in men with non-obstructive azoospermia and in those with CBVAD(van, V et al., 1996;Foresta et al., 2005;Stuppia et al., 2005).Thus it may be concluded that CFTR is involved in spermatogenesis.

Recent studies in our lab have shown that CFTR-mediated HCO_3^- transport plays critical roles in spermatogenesis through sAC mediated cascade. In addition, malfunction of CFTR resulted in abnormal HCO_3^- transport in Sertoli cells which further contributed to the impaired spermatogenesis is observed in CF mice (Xu et al, unpublished data).

Besides Cl^- and HCO_3^- transportation, there is evidence showing that a G-protein-gated inwardly rectifying K^+ channel subunit (Kir3.2), a alternative splicing variant of Kir3.2 (Kir3.2d) may assemble to form a homomultimeric G-protein-gated K^+ channel in the testis. It may be involved in the development of the acrosome during spermiogenesis (Inanobe et al., 1999). Evidence has also been shown that the testis anion transporter 1 (Tat1, Slc26a8) is specifically expressed in spermatocytes and spermatids, and interacts with MgcRacGAP (GTPase activating protein for RhoGTPases) in these cells(Toure et al., 2001). The Tat1 null male mice are sterile due to the complete lack of sperm motility and reduced sperm fertilization potential. These mice sperm also show defects in flagella differentiation indicating this anion transporter is essential for sperm motility and tail differentiation in spermatogenesis.

Taken together, ion channels/transporters appear to play an important role in spermatogenesis. However, SLC26A3 has been reported in the elongating human spermatids (of stages III-VI in human testis), yet the function of this ion transporter remains unknown.

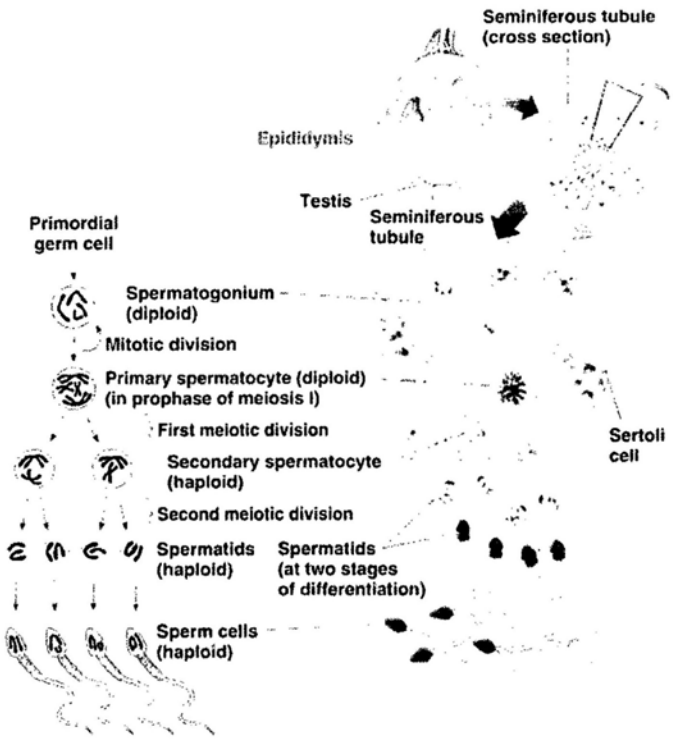


Figure I.2. Schematic diagram of spermatogenesis. (http://iceteazegeg.files.wordpress.com)

I.2 post-testicular sperm maturation and acquisition of the fertilizing ability

I.2.1 sperm maturation in the male reproductive tract

After spermatogenesis, morphologically mature testicular sperm require further maturation in the male and female reproductive tract to finally gain the fertilizing ability. This is known as post-testicular sperm maturation and begins when sperm are transported into the epididymis in the male reproductive tract.

I.2.1.1 sperm maturation in the epididymis

The epididymis is a narrow, coiled tube that connects the efferent duct to the vas deferens. It can be divided into three regions: caput (head), corpus (body) and cauda (tail). The epididymal epithelium consists of several cell types including principal, basal, clear, narrow, apical and halo cells (Figure. I.3) (Cornwall, 2009). Principal cells constitute ~80% of the epithelium and are responsible for most of the protein secretion and HCO_3^- reabsorption that occur in the proximal epididymis (Pastor-Soler et al., 2005). Little is known about the other cell types. Basel cells regulate principal cells by releasing some paracrine factors, specifically prostaglandin E_2 (PGE_2) (Cheung et al., 2005). Narrow, apical and clear cells participant in luminal fluid acidification (Pietrement et al., 2006;Kujala et al., 2007).

Sperm released from the testis are immotile and need further transportation and maturation in the epididymis. The most important function of the epididymis is sperm maturation, which was first demonstrated in 1967 when Orgebin-Crist carried out the experiments in rabbit (Orgebin-Crist, 1967). The epithelial cells secrete different proteins, mediate ion transportation and water absorption in different part of the epididymis to create region-specific micro environments to influence sperm maturation; while spermatozoa migrate through the epididymis, they undergo a series of morphological, biochemical and physiological changes (Cornwall, 2009).

The low pH and HCO_3^- are among key factors in keeping sperm quiescence. High pH can initiate sperm motility and changes in the external pH values would cause the internal pH of the sperm to change in the same direction (Gatti et al., 1993). HCO_3^- plays an essential role in initiating sperm motility and capacitation. It can stimulate sAC and thereby inaugurate the associated downstream signaling pathways. HCO_3^- is also the

major pH buffer. Therefore, low HCO_3^- in the duct lumen is needed to avoid premature capacitation that might lead to impaired fertility (Litvin et al., 2003;Gadella and Van Gestel, 2004).

The table below (Table I.1) shows the pH and HCO_3^- change in rat epididymis. We can see that the luminal pH and HCO_3^- decrease in the distal segments of the efferent ducts and further diminish along the epididymal ducts (Levine and Marsh, 1971;Levine and Kelly, 1978). Newcombe et al have reported that the luminal pH and HCO_3^- concentration increase in the proximal segments of the efferent ducts (Newcombe et al., 2000). *In vivo* studies on *c-ros* knockout mice show that increased luminal pH can affect the sperm maturation, leading to infertility (Yeung et al., 2004;Pastor-Soler et al., 2005), further demonstrating the importance of pH. Yet HCO_3^- transport and the ion channel/transporter involved in these processes are not clearly understood.

Kujala M et al (2007) have reported that SLC26A3, SLC26A6, CFTR, Na^+/H^+ exchanger (NHE3) and Na^+/H^+ exchanger regulating factor 1 (NHERF-1) are expressed on the apical edge of the nonciliated cells (Hihnala et al., 2006;Kujala et al., 2007) which are responsible for the major fluid reabsorption (Hess RA, 2002). They proposed that in human efferent ducts, SLC26A3 and A6 work in conjunction with CFTR to secrete HCO_3^- and absorb Cl^-, and this action is further facilitated by Na^+ absorption by NHE3 (Kujala et al., 2007). In epididymal ducts, the SLC26A6, CFTR, NHERF-1, carbonic anhydrase II (CAII) and Band E subunits of V-ATPase in apical mitochondrial rich cells (AMRC) (Kujala et al., 2007) have been suggested to play a critical role in regulating the luminal pH (Palacios et al., 1991). They may also work as units with SLC26A6 in HCO_3^- transport, with V-ATPase transporting the proton and CAII catalyzing the formation of HCO_3^- and proton and affecting the transporting HCO_3^- activity of SLC26A6 (Kujala et al., 2007). All of these transporters may be regulated and spatially linked by NHERF-1(Kujala et al., 2007).

Other ion transporters, such as SLC26A2, SLC26A7, Na^+/ HCO_3^- transporter (NBC), and Cl^-/ HCO_3^- exchanger AE2 are also expressed in the epididymis (Jensen et al., 1999a;Jensen et al., 1999b;Kujala et al., 2007). While the functions of the former two need to be clarified(Kujala et al., 2007), the two of the latter may be involved in HCO_3^- reabsorption in the initial segments of the epididymis (Pastor-Soler et al., 2005).

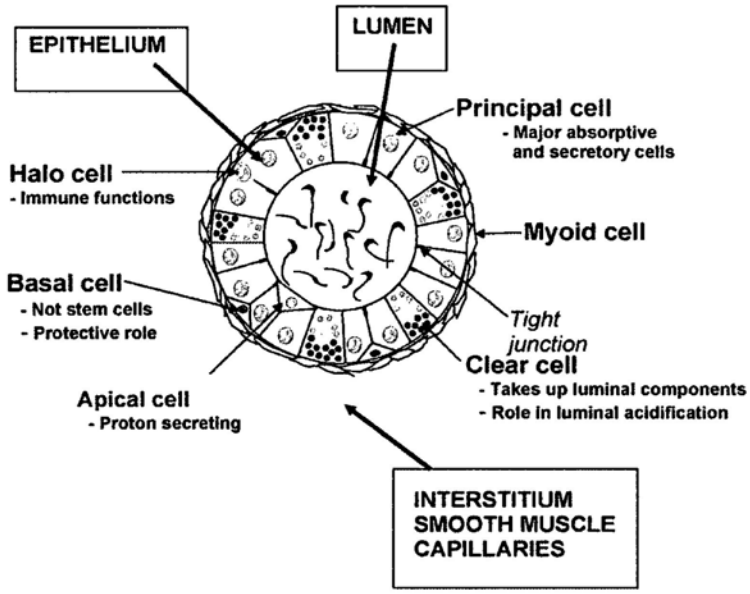


Figure I.3. Schematic diagram of the cellular organization in a representative cross-section of the rat epididymis. Modified and reprinted with permission of the author (Robaire et al., 2003), McGill University and publisher, The Van Doren Co, Charlottesville, VA. (Cornwall, 2009)

Table I.1. Luminal pH (mean ± s.e.m.) in different regions of the rat epididymis and seminiferous tubules measured *in vivo* with antimony microelectrodes

Region	No. of animals	pH	HCO_3^- (m-equiv/l)
Seminiferous tubules	8	7.30±0.11	19.6
Beginning of initial segment	14	7.20±0.06	-
End of initial segment	20	6.79±0.03	-
Intermediate zone	24	6.57±0.08	-
Caput epididymis	14	6.64±0.03	2.7
Cauda and Vas deferens	10	6.85±0.03	6.7
Plasma	6	7.50±0.02	30.1

(Levine and Marsh, 1971; Levine and Kelly, 1978).

I.2.2 The acquisition of fertilizing ability in female reproductive tract

I.2.2.1 Sperm motility

The most important and obvious change in sperm during epididymal maturation is the acquisition of motility. *In vivo*, when sperm enter into the female reproductive tract after ejaculation, they move along and finally reach the ampulla of the oviduct to meet the oocyte.

Spermatozoon is a highly specialized cell that has a long tail, and when it fully matures, it gains the ability to move. Undoubtedly, the normal ultrastructure is critical for function. The tail-flagellum consists of four major subdivisions: connecting piece, mid-piece, principal piece and end-piece (Fawcett, 1975;Turner, 2003;Turner, 2005). The connecting piece is the part attached to the nucleus of the sperm head. The axoneme, with a characteristic "9+2" microtubule arrangement, extends through all four parts from the connecting piece to the end-piece. Outside the outer dense fibers in the mid-piece lies the mitochondrial sheath (MS). The mitochondria in the MS produce ATP that gives the axoneme energy (Turner, 2005). The tail propels sperm through the female reproductive tract in fertilization. Obviously, sperm with morphological defects of the tail would have problems in generating the normal flagella beat.

There are two types of motility. One is activated motility (referred to motility here), as is seen in freshly ejaculated sperm in female reproductive tract or in sperm from cauda epididymis or *vas deference* in physiological media *in vitro*. The other is hyperactivated motility (HAM) as is seen when spermatozoa retrieved from oviductal ampulla at fertilization *in vivo*(Yanagimachi, 1969) or when sperm gained the ability to fertilize oocytes *in vitro*.

Sperm motility generates nearly symmetrical flagella beats and shows a nearly linear trajectory. It is generally accepted that the sAC/cAMP/PKA and calcium signaling pathways are two central regulation mechanisms of sperm motility (Okamura et al., 1985;Suarez et al., 1987;Tash and Means, 1987;White and Aitken, 1989;Yanagimachi, 1994a;Ho et al., 2002;Wennemuth et al., 2003). Soluble adenylyl cyclase (sAC or SACY) is stimulated by Ca^{2+} and HCO_3^-(Chen et al., 2000b;Jaiswal and Conti, 2001;Jaiswal and Conti, 2003), followed thereafter by the production of adenosine-3', 5'-cyclicmonophosphate

(cAMP), on which sperm motility depends. cAMP affects sperm motility through activation of protein kinase A (PKA) (Skalhegg et al., 2002). The serine/threonine phosphorylation of PKA target proteins results in activation of downstream tyrosine kinase or kinases whose targets are primarily located in the flagellum (Leclerc et al., 1996;Si and Olds-Clarke, 2000). This tyrosine phosphorylation of specific flagella proteins results in motility (Figure I.4). Recently, it has been reported that HCO_3^- stimulation of SACY depends on extracellular calcium, which is upstream of the HCO_3^- (Carlson et al., 2007). Based on this understanding, the two signaling pathways are linked together. Therefore, HCO_3^-, Ca^{2+} and their transport mechanisms are important to the sperm motility.

Although sperm have the intracellular calcium stores (largely in the acrosome), there is significant evidence to show that several membrane calcium channels are involved in the transfer of calcium, which causes the intracellular calcium level to form a rise within the flagellum. Yet the mechanism of this rise in sperm is not clearly understood, though it is thought to involve the voltage-gated calcium channels, such as Cav2.3 (α_{1E}), Cyclic nucleotide-gated (CNG) channels (Wiesner et al., 1998; Westenbroek and Babcock, 1999;Wennemuth et al., 2000;Ren et al., 2001;Sakata et al., 2002). Calmodulin (CaM) and Calmodulin kinase (CaMK) are the downstream components of calcium signaling pathways, and there is evidence to show that this pathway is independent of sAC (Turner, 2005).

Despite the importance of HCO_3^-, it is totally unclear of the HCO_3^- transportation in sperm motility process. How ion channels/transporters existing in sperm regulate this process need further investigation.

I.2.2.2 Sperm capacitation

Mammalian sperm remain unable to fertilize the oocytes after maturation in the epididymis. They require additional maturation in the female reproductive tract *in vivo* for a period of time to acquire the fertilizing ability. This was first observed by M.C. Chang (Chang, 1951) and C.R. Austin (Austin, 1951;Austin, 1952). "Sperm that have completed process are referred to as 'capacitated' "(Austin, 1952;Florman and Ducibella T., 2006).

This process consists of morphological, biochemical and physiological changes,

including changes in intracellular ions, metabolism, cAMP/PKA pathways, nucleus, acrosome, plasma membrane and later events- protein phosphorylation(Yanagimachi, 1994a;Florman and Ducibella T., 2006). The changes associated with capacitation in sperm are summarized in Table I.2. It can be mimicked *in vitro* using capacitation medium under capacitating conditions, thus allowing the establishment of convenient *in vitro* models. Over the last few decades, most of our understandings of capacitation are based on these *in vitro* models. Although fertilization is the definitive test of the sperm capacitation, more narrow definition are considered as evaluated point of this process, such as penetration of the zona pellucida (ZP), enhanced tyrosine phosphorylation of sperm proteins, motility pattern. But the most widely used standard is the acquisition of the ability to undergo the acrosome reaction (AR) when stimulated by natural agonists, such as zona pellucida and progesterone.

It is known that capacitation is dependent on the cations and anions present in the capacitation medium, such as HCO_3^-,Ca^{2+}, Na^+ and K^+ (Yanagimachi, 1994a). The clear understanding of mechanisms of those ions involved in the changes of capacitation is still far from understood, though efforts have been made over the decades since the first clarification of this process in the 1950's. Molecular and functional evidence have now shown that voltage-gated K^+ channels (Salvatore et al., 1999), Ca^{2+}-activated K^+ channels(Chan et al., 1998), K_{ATP} channel(Acevedo et al., 2006) are involved in the regulation of sperm hyperpolarization during capacitation. This hyperpolarization changes the inactivation state of the T-type Cav channel to closed state so that they can fully open at the time of ZP3 induction and undergo the AR(Arnoult et al., 1999). There are also some ion channels/transporters that participate in the regulation of capacitation-associated motility change, which will be discussed in I.2.2.3.

What interests us the most is HCO_3^-. It has been clearly shown that HCO_3^- is essential for mammalian sperm capacitation (Shi and Roldan, 1995;Boatman, 1997;Visconti et al., 1999;Gadella and Harrison, 2000;Liu et al., 2005). HCO_3^- contributes to the pH increase during capacitation(Parrish et al., 1989). The major role of HCO_3^- is to regulate the activity of sAC. HCO_3^- induces the activation of sAC, resulting in cAMP synthesis and the activation of downstream PKA signaling pathways. The rapid activation of PKA activates some tyrosine kinases and subsequently leads to

capacitation-associated events, tyrosine phosphorylation (Visconti et al., 1995;Aitken et al., 1995;Visconti et al., 1999;Chen et al., 2000b). PKA also mediates HCO_3^- induced hyperactivated motility, $[Ca^{2+}]i$ increase and cholesterol transport (Nolan et al., 2004;Harrison and Gadella, 2005;Jha et al., 2008).

Although the fundamental role of HCO_3^- has been widely accepted, the transportation of this ion in sperm is far from being clearly understood. Does HCO_3^- pass through ion channels/transporters known to be present in sperm or through others yet to be explored? How do these channels/transporters interact with each other or with the moleculars/messengers or signalling pathways to finally lead to morphological, biochemical and physiological changes? Undoubtedly, understanding of the mechanisms of HCO_3^- transport will give us a greater chance of controlling and regulating the fertilizing event.

CFTR is a cAMP-activated Cl^- channel, mutations of which are related to cystic fibrosis (CF). It is known that CFTR can also conduct HCO_3^-(Poulsen et al., 1994) and undergo a dynamic shift from the primarily Cl^- conductance to the HCO_3^- conductance(Reddy and Quinton, 2003). Recent work in our lab has demonstrated that impaired HCO_3^- secretion by endometrial epithelium reduced capacitation and fertilizing capacity in co-cultured sperm (Wang et al., 2003). Disruption of CFTR function by its inhibitors or antibodies is shown to reduce the number of capacitated sperm, and affect a number of capacitation-associated HCO_3^--dependent events, such as increased intracellular pH (pHi), membrane hyperpolarization and cAMP production, thus indicating the involvement of CFTR in the transport of HCO_3^-. Sperm from CF heterozygous mice also had reduced fertilizing capacity *in vitro* and *in vivo*, confirming an important role of CFTR in determining sperm fertilizing capacity. Although these findings have given us a more detailed picture of the signaling events, much still remains to be elucidated.

Table I.2. Sperm changes associated with capacitation or occurring during incubation under capacitating conditions

Change	Effect	Change	Effect
Surface effects		**Enzyme activities (non-ATPase)**	
Proteins		Adenylyl cyclase	Increase
B-Galactosyltransferase	Unmasked	Protein kinase A	Increase
Fertilin (ADAMI, 2)	Becomes mobile	Protein kinase C	Increase
Various epitopes	Distribution or accessibility changes	ERK kinase	Increase
		Protein tyrosine phosphorylation	
lipids		AKAP3, 4	Increased phosphorylation
Cholesterol, other sterols	Decrease	CABYR	Increased phosphorylation
Phospholipids	Asymmetry decrease	VDAC2	Increased phosphorylation
	Increased diffusion	VCP/p97	Increased phosphorylation
Glycolipids	Redistribution	ODFI	Increased phosphorylation
Surface charge	Less negative	Other proteins	Increased phosphorylation
Membrane potential	Hyperpolarize		
		Other effects	
Ion concentration or content		ATP levels	Decrease
Ca^{2+}	Increase	Reactive oxygen species	Increase
Zn^{2+}	Decrease		
K^+	Decrease, no change	**Functional effects**	
Na^+	Increase	Motility	Hyperactivation
HCO_3^-	Increase		Chemotaxis
pH	Increase	Zona binding	Acquired
		Acrosome reaction	Acquired
Ion channels and transporters			
Na^+/K^+ ATPase	Increased activity		
Ca^{2+}ATPase	Decreased activity		
Ca^{2+} ATPase - PMCA4	Hyperactivation defect in mull sperm		
Na^+/Ca^{2+}exchanger	Increased activity		
CatSper1-4	Hyperactivation defect in null sperm		

(Florman and Ducibella T., 2006), slightly modified.

I.2.2.3 Sperm hyperactivated motility (HAM)

HAM is a type of vigorous nonlinear motion that mammalian spermatozoa exhibit when they get through the female oviduct (Yanagimachi, 1969). It represents a change in the motility pattern, specifically an increase in the amplitude of the flagella bend and average lateral head movement (ALH). HAM allows spermatozoa to detach from the oviductal epithelium and provides increased thrust for the penetration of the cumulus oophorus and zona pellucida *in vivo*(Suarez et al., 1991;Pacey et al., 1995;Stauss et al., 1995;Suarez, 1996). HAM can also be seen after sperm capacitated and in the presence of calcium *in vitro*.

Calcium plays an important role in HAM. As a key second messenger it can trigger the sperm hyperactivated motility. Sperm hyperactivation can be induced by the Ca^{2+} ionophore A23187 (Suarez et al., 1987;Marquez and Suarez, 2007;Xia et al., 2007). Both extracellular calcium brought in through membrane channels and intracellular calcium stores are involved in hyperactivation (Suarez, 2008). Evidence shows that all four CatSper ion channel proteins, which are only specifically expressed in male germ cells and principal piece of the mature sperm are required for the passage of calcium in sperm hyperactivated motility(Carlson et al., 2005;Qi et al., 2007;Jin et al., 2007). All four types of CatSper-/- mice are infertile(Ren et al., 2001;Carlson et al., 2005;Qi et al., 2007). The calcium store in the redundant nuclear envelope (RNE) near the sperm neck provides the source of calcium in the regulation of hyperactivation, and the storage of calcium in the sperm acrosome may also contribute to the hyperactivation(Suarez and Ho, 2003;Suarez, 2008). Apart from this primary second messenger, increased ATP and pH are also required by the axoneme to produce HAM (Ho et al., 2002;Kirichok et al., 2006;Odet et al., 2008) (Figure I.5).

Evidence has shown that the Ca^{2+}/calmodulin-dependent Ca^{2+}-ATPase, PMCA4, is linked to capacitation-associated changes in motility (Okunade et al., 2004). Epithelial Na^+ channel (EnaCs) α and δ subunits have also been reported to exist in mouse sperm and participate in the regulation of sperm hyperpolarization (Hernandez-Gonzalez et al., 2006). A Na^+/K^+ ATPase has been found to localize in bovine sperm. The author has suggested that it acts as a signaling molecule during sperm capacitation (Thundathil et al., 2006).

Despite its importance in sperm motility, the SACY-dependent cAMP/PKA pathway only increase beat frequency and lower flagella bend amplitude which is contrary to the process of HAM (Wennemuth et al., 2003). Furthermore, it has been reported recently that this signaling pathway may be only involved in the promoting hyperactivation. That is to say "sperm must be activated by the SACY signaling pathway before they can be hyperactivated, but this activation is not sufficient for hyperactivation and also it is not involved in the initiation of hyperactivation directly" (Suarez, 2008;Marquez and Suarez, 2008).

I.2.2.4 Acrosome reaction

The acrosome plays a critical role in mammalian fertilization. There are a variety of proteins present in the acrosome, including proteases and other enzymes, bioactive peptides and proteins and some other components with uncertain functions (Eddy, 2006). After capacitation, mammalian sperm gain the ability to undergo the AR and thereby fertilization. During the AR, an event dependent on Ca^{2+}, against stimulation causes the contents of the acrosome to be released, which helps sperm penetrate the oocyte vestments and fuse with the oolema (Yanagimachi, 1994a).

Among the variety of agonists which are capable of initiating the AR, the main one is zona pellucida glycoprotein 3 (ZP_3) (Bleil and Wassarman, 1980;Bleil and Wassarman, 1983) and progesterone(P) (Meizel et al., 1990). Both agonists interact in the initiation of the AR, with the progesterone priming the sperm to respond to the ZP_3 (Roldan et al., 1994;Shi et al., 2005). ZP_3 binds to a specific receptor on the sperm membrane and early events of ZP_3 signal transduction is the activation mechanism leading to Ca^{2+} influx. First the G proteins, G_{i1} and G_{i2} are activated by ZP_3. Next is the fundamental step in this G-protein signaling, namely the elevation of pHi, followed by the stimulation of PLC, PLCδ4. The activation of PLC causes hydrolysis of the phosphatidylinositol-1,4-bisphosphate (PIP_2) to produce inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), thereby activating PKC and finally triggering the transient elevation of $[Ca^{2+}]i$ through a low voltage-activated calcium channel, mostly of the Ca_v3 family(Arnoult et al., 1996a;Arnoult et al., 1999).

The second $Ca^{2+}i$ elevation stimulated by ZP_3 is sustained for minutes (Florman et al., 1989;Florman, 1994;Arnoult et al., 1996b;Shirakawa and Miyazaki, 1999;Fukami et

al., 2003) and is essential for ZP-induced AR. Recently, it has been shown that members of transient receptor potential cation channel (TRPC) family are involved in PLC-dependent Ca^{2+} entry (Clapham, 2003;Montell, 2005;Putney, Jr., 2007). In mouse, this ZP_3-evoked Ca^{2+} influx is conducted by TRPC2 through a store-operated mechanism (Figure I.6) (Jungnickel et al., 2001). Enkurin with its cargo protein 1-phosphatidylinositol-3-kinase (PI3-K) binds to the TRPC channel with its c-terminal, which also interacts with calmodulin. When the TRPC is activated by ZP_3, with extracellular calcium passing through the channel, the PI3-K was also activated, leading to an increase in phosphatidylinositol-3,4,5- triphosphate (PIP3), and thus activation of downstream Akt (protein kinase B, PKB) and PKCξ, the Ser/Thr kinases involved in the ZP_3 signaling in sperm. Evidence has shown that besides TRPC2, TRPC1 and TRPC5 are also involved in the ZP_3- initiated signaling pathways (Sutton et al., 2004;Jungnickel et al., 2007;Florman et al., 2008).

As for P-induced AR, P first binds to its receptor, $GABA_A$ receptor-like/Cl^- channel. The main signaling pathway involved in this process is the activation of PLC and followed by the degradation of PIP_2 into IP_3 and DAG, downstream activation of PKC, thereby the opening of voltage-gated channels and the entry of extracellular calcium leading to an increase in $Ca^{2+}i$ level, and finally the exocytosis (Witte and Schafer-Somi, 2007). The fundamental difference between P- and ZP-induced AR is that the latter is mediated by a G_i-protein, which does not occur in the P-induced AR pathway (Witte and Schafer-Somi, 2007).

Although the signal transduction pathways might differ from each other, ZP_3- and P-induced AR share some common elements, such as the dependence on extracellular calcium and the involvement of DAG and PLA_2. Most importantly, both have long been used as natural agonists for the AR.

I.2.2.5 *In vitro* Fertilization

In order to understand the nature of fertilization, *in vitro* fertilization animal models have been established. From the first definition of sperm capacitation in the early 1950s to 1978, the birth of Louise Brown, the first test-tube baby, indicates that our understanding of fertilization has been beneficial for the human beings. Assisted reproductive technologies including *in vitro* fertilization have been widely used and have

resulted in the birth of over 1,000,000 babies.

Returning to the research, fertilization is undoubtedly the definitive test for sperm capacitation and it can be achieved *in vitro*. It has been reported that lactate, osmolarity, and calcium can influence the *in vitro* capacitation of the BALB/c mice and, the latter two of the three can also affect sperm penetration through the zona pellucida and male pronuclear formation (Kito and Ohta, 2008a;Kito and Ohta, 2008b). In this study, *in vitro* fertilization is also used to evaluate *in vitro* sperm capacitation.

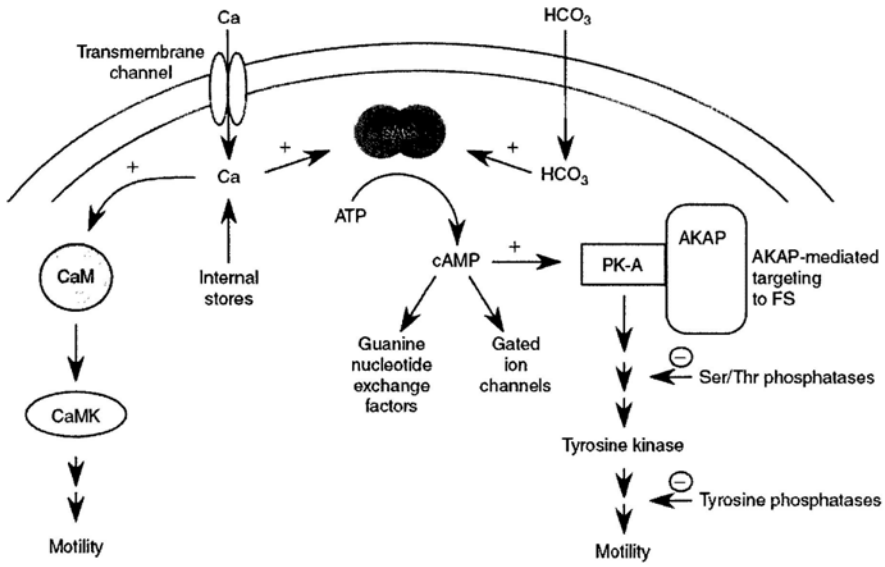


Figure. I.4 Schematic representation of signaling pathways known to be or suspected of being involved in the regulation of mammalian sperm motility. (Turner, 2005).

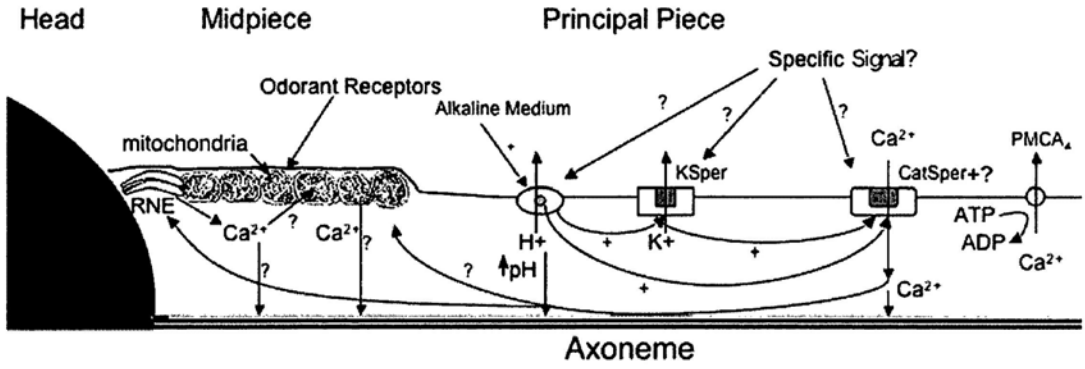


Figure I.5. Schematic representation of mechanisms that may regulate hyperactivation. RNE, redundant nuclear envelope; PMCA4, plasma membrane Ca2t-ATPase4. (Suarez, 2008)

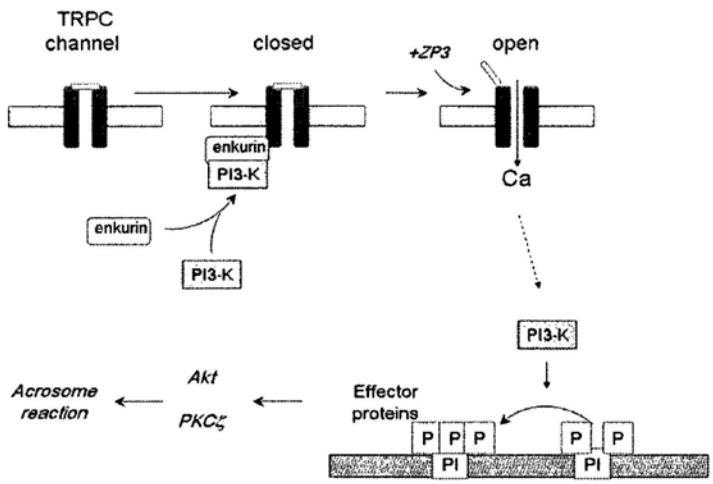


Figure I.6. Model of the late events of the mouse sperm acrosome reaction.

Enkurin is a scaffold protein that binds PI3 kinase to sperm transient receptor potential channels (TRPC). Sperm contact with the zona pellucida results in ZP_3 activation of TRPC channels, leading to both Ca^{2+} entry and to activation of PI3 kinase. The resulting D3-phosphorylation of (PIP_2) (phosphatidylinositol-4,5-bisphosphate) leads to the local accumulation of PIP_3 (phosphatidylinositol-3,4-5-triphosphate). PIP_3 provides docking sites for proteins with appropriate lipid binding domains and results in the activation of the serine/threonine protein kinases, Akt (Protein Kinase B) and PKC . These protein kinases mediate the downstream stages of sperm exocytosis. (Florman et al., 2008)

I.3. CFTR and SLC26A3 in male fertility

Ion channels/transporters are membrane proteins that can efficiently transport ions through the lipid bilayer, allowing cells to exchange information with the external environment or within the cells. It is not surprisingly that numerous ion channels/transporters are involved in this fertilizing process when sperm are in transit through the epididymis and subsequent female tract before finally meeting the oocytes. Ion channels/transporters not only contribute to the determination of the membrane potential, but are also involved in the modulation of the intracellular HCO_3^-, intracellular Ca^{2+}(Ca^{2+}i) and intracellular pH (pHi), which are involved in many sperm functions (sperm motility, capacitation, AR, Figure I.7.) (Darszon et al., 2006).

Although so many ion channels/transporters are involved in sperm functions, only a few whose defects have been implicated in infertility. CFTR and SLC26A3 are among these few. The details are listed below.

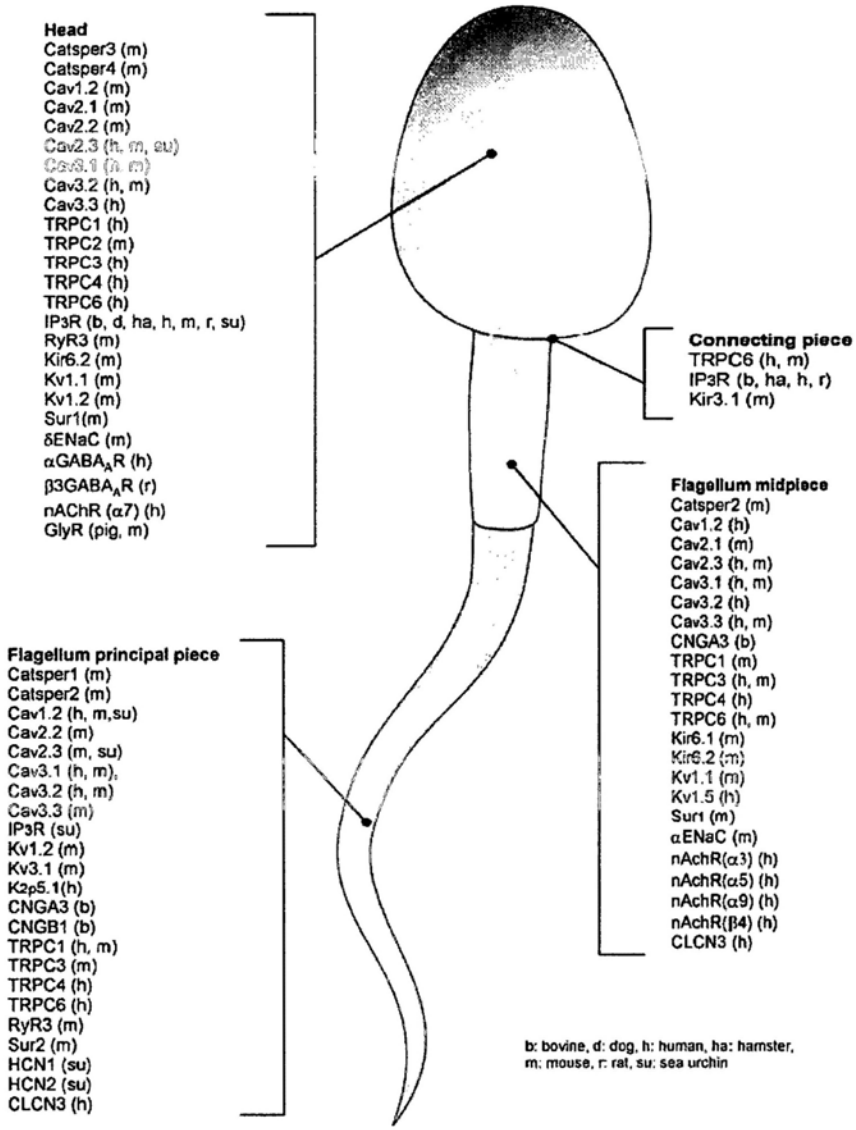


Figure I.7. **Summary of the different ion channels and their spatial distribution in sperm.** The figure includes channels that have been immunolocalized to the region indicated. Additional evidence for the presence of many of these channels such as RT-PCR, in situ hybridization and western blots is available (Darszon et al. 2005). Sea urchin flagella do not have principal and mid pieces, channels found in the flagella of this species are indicated as present in the principal piece. Channels that participate in functions such as volume regulation (not described in the text) have also been included (Yeung et al. 2006). (Darszon et al., 2006)

I.3.1 SLC26A3: introduction

Solute carrier family 26 member 3, SLC26A3, also known as DRA (downregulated in adenoma) is a putative anion transporter and one of the 11 members of the Solute carrier family 26 (SLC26). SLC26 exchangers transport a number of monovalent and divalent anions, such as SO_4^{2-}, OH^-, Cl^-, I^-, formate and oxalate. Four of the SLC26 members including *SLC26A3* have been associated with human disorders, namely *SLC26A2* in chondrodysplasias, *SLC26A3* in congenital chloride diarrhea (CLD), *SLC26A4* in Pendred syndrome and non-syndromic deafness and *SLC26A5* in non-syndromic hearing impairment (Everett and Green, 1999;Markovich, 2001;Liu et al., 2003). These disorders highlight the important roles of the SLC26 family and implore us to study their function.

SLC26A3 is located in 7q22-q31.1. It contains 2881 base pairs, encodes a transmembrane glycoprotein of 764 amino acids and is predicted to contain 12 transmembrane-spanning α-helices and a C-terminal sulphate transporter and anti-sigma factor antagonist (STAS) domain (Figure I.8). cDNA of DRA was first isolated when comparing colon cancer samples with their normal counterparts (Schweinfest et al., 1993). Although studies have shown that DRA has growth suppression function, supporting its role as a tumor suppressor (Chapman et al., 2002), more interest has been given to its anion transport function in the epithelium. Functional studies show that it is a sodium independent anion transporter, which can transport SO_4^{2-}, Cl^-, HCO_3^-, OH^- and oxalate. When it acts as a Cl^-/HCO_3^- exchanger, SLC26A3 mediates a coupled 2 $Cl^-/1HCO_3^-$ exchanger (Shcheynikov et al., 2006). Apart from its expression in the epithelium of the colon and eccrine sweat glands, SLC26A3 is also expressed in the epithelium of the testis, epididymis and seminal vesicles in human (Haila et al., 2000;Hihnala et al., 2006). As mentioned above, mutations of this gene are associated with the autosomal hereditary disease CLD, which is characterized by massive losses of Cl^- in stools with respective defects in intestinal HCO_3^- secretion. The mechanisms of how these mutations of *slc26a3* gene result in protein malfunction are not well understood. Recent study demonstrate that four mutations in the STAS domain which can cause CLD are associated with misfolding and mistrafficking in SLC26A3(Dorwart et al., 2008).

I.3.2 SLC26A3 and fertility

It is known that SLC26A3 mutations are related to the CLD. CLD is a disease inherited in an autosomal recessive manner. Patients with CLD have life-long Cl^--rich, watery diarrhea with low pH, resulting in dehydration, and hypochloremic metabolic alkalosis. If untreated, the patients will die in very early childhood (Holmberg, 1986;Hoglund et al., 2006;Hihnala et al., 2006). With appropriate substitution therapy, patients can live a perfectly normal life. Unlike CLD women, who have normal fertility, CLD men display subfertility, with only 2 of 12 patients fathering a child. The CLD accompanied male subfertility is characterized by oligoasthenozoospermia with normal concentrations of sex hormones, normal testicular histology and a tendency to form spermatoceles (Hihnala et al., 2006). Recently studies have shown that mutations of SLC26A3 in the STAS domain can cause CLD because of protein misfolding and mistrafficking, resulting in a loss of functional proteins in the plasma membrane (Dorwart et al., 2008). Although an exploration of these mechanisms have important implications for potential therapeutic use, its relation to male subfertility remains unclear.

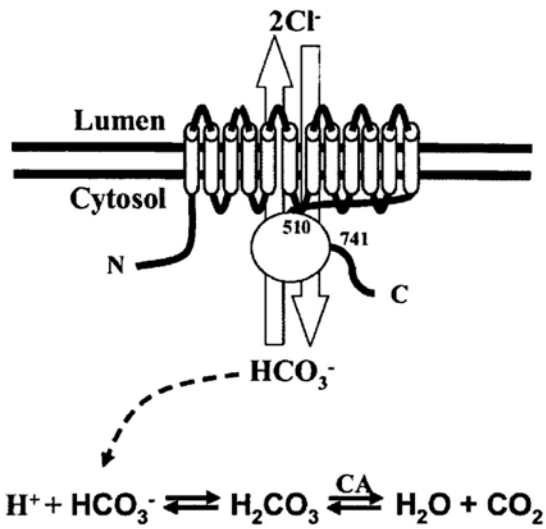


Figure I.8. SLC26A3 structural model.

Schematic model of a predicted SLC26A3 structure, which includes 12 transmembrane-spanning α-helices and a C-terminally conserved STAS domain. The experimentally determined STAS domain boundaries are shown along with the predicted location of the glycosylation site in the second extracellular loop between transmembrane helices 3 and 4. SLC26A3 has been demonstrated to be an anion exchanger with a $2Cl^-/1HCO_3^-$ stoichiometry, and the chemical equation showing how HCO_3^- can affect cellular pH is shown. (Dorwart et al., 2008)

I.3.3 CFTR: introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a channel that transports chloride ions across the epithelial cell membrane. Mutations of CFTR lead to one of the most common autosomal recessive disorders, cystic fibrosis (CF), which is characterized by chronic lung disease, pancreatic exocrine insufficiency, an increase in the concentration of sweat electrolytes and congenital absence of the *vas deferens*. The gene encoding the CFTR is located in 7q31.2. It contains about 170,000 base pairs and encodes 1480 amino acid glycoprotein consisting of five domains (Figure I.9). Two membrane-spanning domains (MSD) form a cAMP-regulated chloride ion channel, and each of them is connected to a nucleotide binding domain (NBD, NBD1 and2) in the cytoplasm. The MSD2 and NBD1 are connected together by a unique regulator domain I. The R domain has a PKA phosphorylation site. When it is phosphorylated by PKA and NBDs bind to ATP, the ion channel opens. The C-terminal of the CFTR is anchored to the cytoskeleton by a PDZ-interacting domain. Over one thousand mutations have been described that can affect the CFTR gene. The most common mutation, ΔF508 results from a deletion (Δ) of three nucleotides which results in a loss of the amino acid phenylalanine (F) at the 508^{th} (508) position (at NBD1) on the protein. As a result the protein does not fold normally and is more quickly degraded(Rowe et al., 2005). Apart from ΔF508, G542X, G551D, N1303K and W1282X are the most common mutations found in Caucasian populations over 1500 mutations (Araujo et al., 2005).

I.3.4 CFTR and SLC26A3 interactions

Not until recently did people pay attention to the function of CFTR other than those as a channel. CFTR contains one PDZ-interacting domain in the c-terminus. The name PDZ is an acronym referring to three proteins: post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg A), and zonula occludens-1 protein (ZO-1), which were first discovered to share the domain. Recent studies have shown that the main defect in cystic fibrosis is the inability of mutant forms of CFTR to activate Cl^-–HCO_3^- exchange, leading to aberrant HCO_3^--driven fluid secretion (Choi et al., 2001). Subsequent studies have found that CFTR specifically up regulated the activity of three members of the SLC26 gene family of multifunctional anion exchangers, namely

DRA, SLC26A6 and pendrin(Ko et al., 2002). It has later been demonstrated that the interaction between CFTR and SLC26 members is mediated by the binding of the regulatory I domain of CFTR to the highly conserved STAS domain of SLC26 (Figure I.10). This interaction is enhanced by phosphorylation of the R-domain through activation of PKA and is mediated by PDZ ligand protein (such as EBP50). The consequence is that SLC26A3 activity was increased with CFTR phosphorylation and CFTR activity is also six-fold higher when expressed with either one of these three SLC26 family proteins compared to when expressed CFTR alone (Ko et al., 2004;Gray, 2004).

I.3.4 CFTR and fertility

About one in every 2500 Caucasians newborns has CF disease. One of the major complications for male CF patients is infertility. Indeed, more than 95% of male CF patients are infertile because of the CBAVD, which is commonly accompanied by absence of the cauda and corpus epididymis (Wong, 1998). It is assumed that CFTR gene mutations on both alleles cause 80% of CBAVD cases. The mechanisms by which CFTR mutations cause CBAVD are not yet fully understood. However Tizzano et al has found that between 10 and 33 weeks of gestation, the epididymis epithelium had low CFTR expression (Tizzano et al., 1994). This may indicate that the development of the vas deferens from the Wolffian duct probably requires CFTR expression.

The question arises as to whether the sterility of CF male patients is due to CBAVD alone or accompanied by the effects on spermatogenesis or sperm maturation. Studies have been done to show the influence of CFTR mutations on spermatogenesis or sperm function. The expression of CFTR on spermatocytes and spermatids have been demonstrated(Wong, 1998;Gong et al., 2001;Hihnala et al., 2006). CFTR are also expressed in the epididymis, *vas deferens* and Sertoli cells(Tizzano et al., 1994;Patrizio and Salameh, 1998;Boockfor et al., 1998). Although there are reports that the frequency of CFTR gene mutations in men with nonobstructive azoospermia, oligozoospermia and asthenozoospermia is similar to those observed in the general population (Slezak et al., 2007), more studies have shown that the frequency of CFTR mutations in men with non-obstructive azoospermia and oligozoospermia is much higher than that observed in the general population, with the high prevalence of the 5T allele both in men with

non-obstructive azoospermia and in those with CBVAD (van, V et al., 1996;Foresta et al., 2005;Stuppia et al., 2005).Thus it can be concluded that CFTR may be involved in normal spermatogenesis. Microarray studies show that CFTR is downregulated in caput epididymis of nonobstructive azoospermic men(Dube et al., 2008). CFTR also plays an important role in HCO_3^- reabsorption and modulation of luminal pH in epididymis (details see I.21.1).

Many studies have been done on the role of CFTR in male fertility. However, the question as to whether CFTR is involved in sperm function is still unanswered, not to mention the exact role of CFTR in male fertility. It is widely accepted that HCO_3^- plays an essential role in sperm functions, such as sperm motility, capacitation, sperm hyperactivated motility and the AR. It has been known for some time that CFTR can also conduct HCO_3^-(Poulsen et al., 1994) and recent studies have demonstrated that CFTR can undergo a dynamic shift from primarily Cl^- conducting to primarily HCO_3^- conducting by a process involving ATP hydrolysis (Reddy and Quinton, 2003). CFTR is also known to act as a regulatory protein interacting with the other ion channels and transporters, such as the EnaC, inwardly rectifying K^+ channel and water channel (Guggino and Banks-Schlegel, 2004). Recent studies have also demonstrated that CFTR interacts with other HCO_3^- transporters, and mutations of CFTR may cause defects in this interaction (Choi et al., 2001;Ko et al., 2002;Ko et al., 2004). Previous studies in our lab have demonstrated a direct role of CFTR in mediating uterine HCO_3^- secretion and that the impaired HCO_3^- secretion by endometrial epithelium due to CFTR inhibition leads to reduced capacitation and fertilizing capacity in cocultured sperm (Wang et al., 2003). Further studies show that CFTR is detected in mature sperm and impaired CFTR function leads to the reduction of capacitation and associated HCO_3^--dependent events. Moreover, the fertilizing capacity of sperm are also reduced due to impaired CFTR function(Xu et al., 2007). Thus CFTR may be involved in the transport of HCO_3^- essential for sperm capacitation, yet it remains unclear whether this transportation is directly or indirectly. Work has also been done on the putative function of CFTR in spermatogenesis (unpublished data). The alteration of the CFTR gene in Sertoli cells caused reduced HCO_3^- secretion and therefore the inhibition of cAMP production through the sAC signaling pathway and resulting in impaired spermatogenesis.

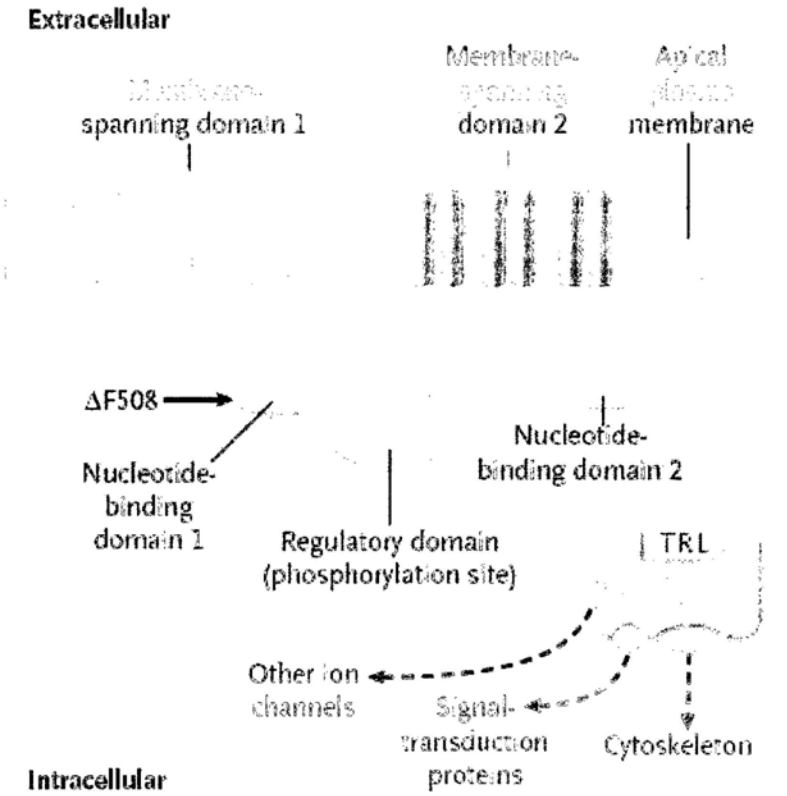


Figure I. 9. Hypothesized Structure of CFTR.

The protein contains 1480 amino acids and a number of discrete globular and transmembrane domains. Activation of CFTR relies on phosphorylation, particularly through protein kinase A but probably involving other kinases as well. Channel activity is governed by the two nucleotide-binding domains, which regulate channel gating. The carboxyl terminal (consisting of threonine, arginine, and leucine [TRL]) of CFTR is anchored through a PDZ-type–binding interaction with the cytoskeleton and is kept in close approximation (dashed arrows) to a number of important proteins. These associated proteins influence CFTR functions, including conductance, regulation of other channels, signal transduction, and localization at the apical plasma membrane. Each membrane-spanning domain contains six membrane-spanning alpha helixes, portions of which form a chloride-conductance pore. The regulatory domain is a site of protein kinase A phosphorylation. The common ΔF508 mutation occurs on the surface of nucleotide-binding domain 1. (Rowe et al., 2005)

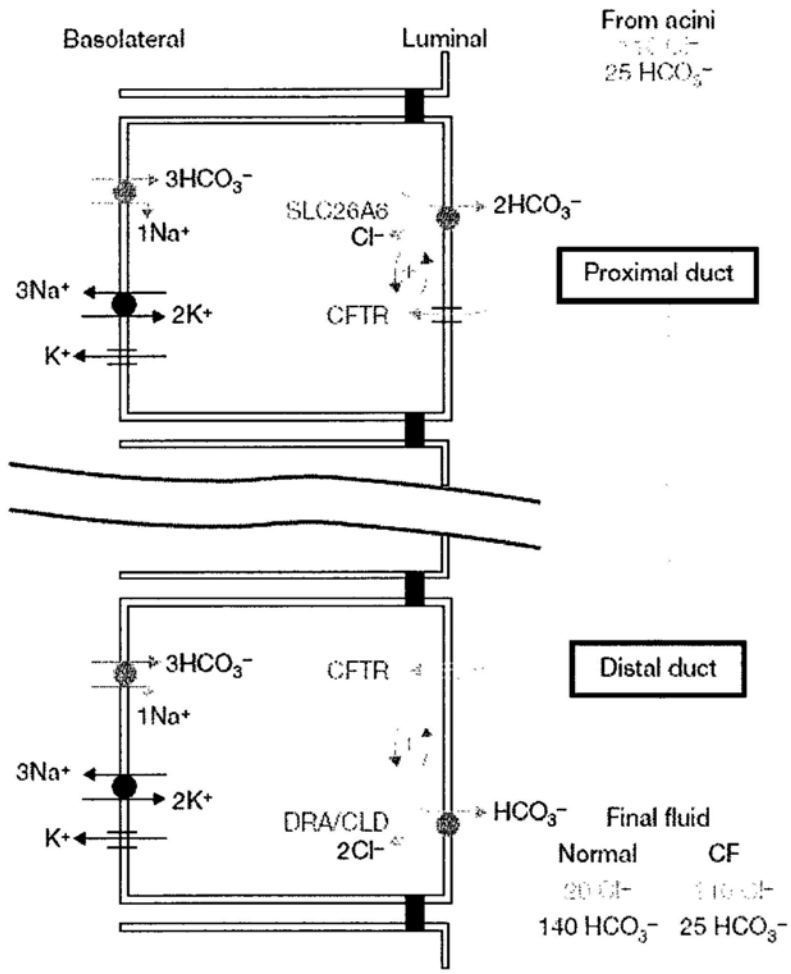


Figure. I.10. A model for ductal chloride absorption and bicarbonate secretion.

SLC26 transporters and CFTR assemble into a bicarbonate-transporting complex with the aid of their PDZ-binding ligands to facilitate an interaction between the CFTR R-domain and STAS domain of SLC26 transporters. This interaction switches on the activity of both proteins. The consequence of this activation is depicted in the model. Bicarbonate-secreting epithelia express different SLC26 transporters in the proximal and distal portion of the duct, with stoichiometries of two bicarbonates to one chloride and two chlorides to one bicarbonate, together with CFTR. In epithelia, an interaction between CFTR and the SLC26 transporters results in stimulation of CFTR and chloride-bicarbonate exchange (Lee et al., 1999). The segment of the epithelia that expresses a two-bicarbonate/one-chloride transporter absorbs the bulk of the chloride and secretes some bicarbonate, whereas the segment that expresses a two-chloride/one-bicarbonate transporter functions to concentrate the bicarbonate. Disruption of this regulation results in aberrant bicarbonate transport in CF (Choi et al., 2001), or to CLD and other chloride and bicarbonate transport-related diseases (Mount and Romero, 2004). (Ko et al., 2004)

I.4. Hypothesis and aim

Accumulating evidence suggests that apart from chloride secretion, the failure of Cl^--HCO_3^- exchange caused by CFTR mutations which lead to aberrant HCO_3^- secretion may be the underlying mechanism for CF pathogenesis. Previous studies in our lab have demonstrated that CFTR mediates HCO_3^- secretion in endometrial epithelium and sperm maturation (Wang et al., 2003;Xu et al., 2007). However, whether the role of CFTR is direct or indirect remains unclear. In somatic cells, as mentioned above, CFTR interacts with SLC26A3 and SLC26A6 to form a complex and thereafter regulate HCO_3^- secretion. Although we know that there is an anion exchanger for Cl^- and HCO_3^- present in mature sperm (Spira and Breitbart, 1992;Zeng et al., 1996;Kaupp and Weyand, 2000), its exact role in male reproduction and its molecular identity are unknown. Based on the above knowledge, we hypothesized that the candidate Cl^-/HCO_3^- anion exchanger maybe SLC26 family members, which may cooperate with CFTR to play a role in post-testicular sperm function. Thus, this study was designed to test this hypothesis.

Chapter II

Cl^- is required for HCO_3^- entry necessary for sperm capacitation in guinea pig: involvement of a Cl^-/HCO_3^- exchanger (SLC26A3) and CFTR

II.1 SUMMARY

Our previous study has demonstrated the involvement of Cystic fibrosis transmembrane conductance regulator (CFTR) in transporting HCO_3^- necessary for sperm capacitation, however, whether its involvement is direct or indirect remains unclear. The present study investigated the possibility of a Cl^-/HCO_3^- exchanger, solute carrier family 26, number 3 (SLC26A3), operating with CFTR during guinea pig sperm capacitation. Incubating sperm in medium with various concentrations of Cl^- resulted in varied percentages of capacitated sperm, in a concentration dependent manner. Depleting Cl^-, even in the presence of HCO_3^-, abolished sperm capacitation and *vice versa*, indicating the involvement of both anions in the process. Capacitation-associated HCO_3^- dependent events, including pHi increase, cAMP production and protein tyrosine phosphorylation also depend on Cl^- concentrations. Similar Cl^- dependence and inhibitors sensitivity were observed for sperm hyperactivated motility and sperm-egg fusion. The expression and localization of CFTR and SLC26A3 were demonstrated using immunostaining and Western blot analysis. Taken together, our results indicate that Cl^- is required for the entry of HCO_3^- necessary for sperm capacitation, implicating the involvement of SLC26A3 in transporting HCO_3^- with CFTR providing the recycling pathway for Cl^-. (The content of this section has been published on *Biol Reprod.* 2009; 80(1): 115-23.)

II.2 INTRODUCTION

Spermatozoa must undergo an activation process called capacitation, which consists of morphological, biochemical and physiological changes in sperm necessary for acquire fertilizing potential (Chang, 1951;Austin, 1952). It has been demonstrated that HCO_3^- is essential for capacitation since prevention of HCO_3^--induced intracellular alkalization results in failure of capacitation (Shi and Roldan, 1995;Boatman, 1997;Visconti et al., 1999;Gadella and Harrison, 2000;Liu et al., 2005). Impaired HCO_3^- secretion by endometrial epithelium also reduced capacitation and fertilizing capacity in co-cultured sperm (Wang et al., 2003).

While HCO_3^- has been demonstrated to activate the soluble adenylate cyclase in the cytoplasm of sperm as the initial step in a series events, including cAMP production and tyrosine kinase phosphorylation, that lead to capacitation(Visconti et al., 1995;Aitken et al., 1995;Visconti et al., 1999;Chen et al., 2000b), how HCO_3^- is transported into sperm remained unanswered. Our recent studies demonstrated a crucial role of the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel known to conduct both Cl^- and HCO_3^- (Poulsen et al., 1994;Reddy and Quinton, 2001), in the process of capacitation (Xu et al., 2007;Hernandez-Gonzalez et al., 2007). Disruption of CFTR function by its inhibitors or antibodies was shown to reduce the number of capacitated sperm, as well as to affect a number of capacitation-associated HCO_3^- dependent events, such as increased intracellular pH (pHi), membrane hyperpolarization and cAMP production, indicating the involvement of CFTR in the transport of HCO_3^-. Sperm from CF heterozygous mice also had reduced fertilizing capacity *in vitro* and *in vivo*, confirming an important role of CFTR in determining sperm fertilizing capacity. However, it was not clear whether CFTR directly conducts HCO_3^- or acts as a Cl^- channel working in parallel with a Cl^-/HCO_3^- exchanger, thereby providing a recycling pathway for the Cl^- necessary to operate the anion exchanger, as is the case of pancreatic ductal HCO_3^- secretion (Steward et al., 2005;Hegyi et al., 2006;Ishiguro et al., 2007). We undertook the present study to test the latter possibility since a Cl^-/HCO_3^- exchanger, has been reported to be present in sperm (Spira and Breitbart, 1992;Zeng et al., 1996;Kaupp and Weyand, 2000). Cl^- dependence would seem to be required for the

operation of the exchanger, which would not be necessary if CFTR were to conduct HCO_3^- directly. Therefore, the primary goal of the present study is to examine the dependence of sperm capacitation and the associated HCO_3^--dependent events on Cl^- concentrations in the capacitating medium, as well as the sensitivity of these events to inhibitors of CFTR and the anion exchanger.

Capacitation is also a prerequisite for the sperm acrosome reaction (AR), an exocytoic process releasing hydrolytic enzymes from the acrosome to enable sperm penetration of the egg investments and cell membrane, in response to natural agonists, such as progesterone (P) and the zona pellucida (ZP) (Roldan et al., 1994;Yanagimachi, 1994b). Therefore, sperm capacitation can be defined as the acquisition of the capacity of sperm to undergo the AR(Yanagimachi, 1989;Kligman et al., 1991), and currently, one of the practical approaches to assess capacitation is to measure the ability of sperm to undergo an induced AR, even if capacitation and the AR are two separate events. This approach exploits the operational definition of capacitation(Florman et al., 1992;Yanagimachi, 1994b), measuring the increase in the number of acrosome reacted-sperm in response to an AR inducer, which in general appears to be highly reliable, when the natural AR inducers, P and ZP, are used. Therefore, the present study investigates the possible involvement of a Cl^-/HCO_3^- exchanger and CFTR in transporting HCO_3^- important for capacitation by assessing the induced AR in guinea pig sperm, which has been used conventionally for its ease in detection of acrosome exocytosis from the sperm head(Huang, Jr. et al., 1985), in conjunction with the use of a number of other biochemical and fluorimetric techniques to monitor different capacitation-associated events.

II.3 MATERIALS AND METHODS

II.3.1 Media preparation

The media used throughout this study were a low calcium Minimal Capacitating Medium (MCM) which consisted of 111.76 mM NaCl, 2.7 mM KCl, 0.49 mM $MgCl_2$, 25.07 mM $NaHCO_3$, 2.78 mM glucose, 10 mM sodium lactate, 1.0 mM sodium pyruvate, 50 μg kanamycin monosulfate/ml, 20mM Hepes, and 4mg/ml bovine serum albumin (Fraction V, A3059, Sigma, St. Louis, MO, USA). Its pH was 7.9 and it had a final

osmolality of 300-305 mOsm/kg. It contained no added Ca^{2+} although, when measured as described previously(Wang CN et al., 1996;Yuan et al., 2003), Ca^{2+} concentration was 23 μM. This low Ca^{2+} MCM medium induces capacitation of guinea pig spermatozoa under *in vitro* conditions but does not support the AR(Barros, 1974;Chen et al., 2000a;Yuan et al., 2003). Since the AR can occur only upon the addition of calcium(Yanagimachi and Noda, 1972;Yanagimachi and Usui, 1974), therefore, 2mM $CaCl_2$ was added when induction of the AR. This medium contained 115.3±1.7 mM Cl^- (n=3) and was termed complete MCM throughout the text. The Cl^- in this medium was replaced with gluconic to yield Cl^--deficient MCM (26.0±0.4 μM, n=3).

II.3.2 Isolation and preparation of ZP

Ovaries were obtained from female guinea pigs (age, 21-22 days) and homogenized in cold buffer as described previously(Yuan et al., 2003). Briefly, the homogenate was washed through two-step Percoll gradient (10%-20%) and the zonae were isolated by examining the samples of various layers under a phase-contrast microscope. The band containing the majority of ZP was located in the interphase between the homogenate and the 10% Percoll. Zona were stored at –20°C until the day of use when they were solubilized at 60°C for 1 h which were heat-stabilized.

II.3.3 Evaluation of sperm capacitation

White and black retired male breeder guinea pigs were purchased from the Laboratory Animal Service Center of The Chinese University of Hong Kong and the Experiment Animal center of Zhejiang Province. All the animal experiments were approved by the animal experimentation ethics committee of the Chinese University of Hong Kong (Ref 07/085/MIS) and the Local Ethics Committee of Zhejiang Province. All the guinea pigs were housed and euthanatized in humane ways. The spermatozoa from guinea pig vas deferens and cauda epididymis were washed for 18 min at 600xg through a Percoll gradient (85%-55%-35%) and adjusted to $2\text{-}3x10^7$ cells/ml (unless indicated otherwise), re-suspended in complete or Cl^--deficient MCM medium and incubated at 38.5°C for 6 hours(Yuan et al., 2003;Chen et al., 2005). Sperm capacitation was evaluated by the induction of a rapid synchronization of the AR following the addition of 2mM Ca^{2+} and P or ZP(Shi and Friend, 1983;Yuan et al., 2003). To assess the capacitation-dependent spontaneous or induced AR, spermatozoa were first exposed to

reagents or their solvents as controls for 6 hours to capacitate and then washed, re-suspended in desired medium, treated with 2mM Ca^{2+} or other agonists for 15 min before assaying the AR using phase-contrast microscopy. The percentage of AR was counted among 600 motile spermatozoa. The spermatozoa that had lost their acrosomal caps without losing motility were recorded as acrosome reacted.

II.3.4 Measurement of intracellular pH in spermatozoa

Level of [pH]i increase was determined by cells loaded with 2',7-bis-2 (carbosyethyl)- 5-(and-b)-carboxyfluorescence, acetoxymethyl ester (BCECF), as previously described (Xu et al., 2007), with minor modifications. Briefly, caudal epididymal spermatozoa were obtained from retired breeder guinea pigs and adjusted to ~2×10^6 cells/ml in complete MCM and Cl^--deficient MCM. Spermatozoa were then incubated in a 5% CO_2 incubator at 38.5°C. When needed, 5μM BCECF was added and the incubation continued for a further 30 minutes. Following this, the cells were pelleted and washed twice to remove free dye before pHi measurement. The fluorescence was detected by excitation ratio of 490nm/440nm (emission 520nm) using a luminescence spectrometer (PERKIN ELMER, LS50B). Calibration was performed according to (Fraire-Zamora and Gonzalez-Martinez, 2004) with modification. Briefly, at the end of each trace, 10 μl 5mM nigericin was added to permeablize the cells, then, the pH was acidified with 10 μl HCl and the measured pH values (determined with a conventional pH meter) were compared with the corresponding ratio values. Usually we add three times before the ending of the experiment. These data were analyzed with Prism 3.0 software to convert ratios to pH values.

II.3.5 Intracellular cAMP measurement

Spermatozoa were incubated in the complete MCM or Cl^--deficient MCM as well as HCO_3^- -deficient MCM for 6 hours. 50 μL of sperm suspension (~10^6 cells/ml) was added to an equal volume of the same medium. Incubations were ended by the addition of 5 volumes of ice-cold 100 mM HCl in 100% ethanol. Samples were kept on ice for 30 min, then lyophilized and assayed for cAMP (Assay Designs, Ann Arbor, MI) using the manufacturer's protocol for acetylated samples.

II.3.6 Protein tyrosine phosphorylation of spermatozoa

Incubated sperm were centrifuged at 5,000g for 3min at room temperature,

washed once in 1 ml of phosphate buffered saline (PBS), re-suspended in sample buffer without mercaptoethanol and boiled for 5 min, and then centrifuged and boiled in the presence of 5% 2-mercaptoethanol for 5min. SDS-PAGE was performed in 10% gels with constant 100V for 1.5 hour followed with transfer to PVDF membrane for 1 h at 4°C.The membrane was blocked and then incubated with primary antibody (1:2000), a monoclonal mouse antibody against phosphotyrosine (p-Tyrosine,4G10, Cell Signaling) at 4°C overnight. After washing with TBST for 4 times, membrane was incubated with the secondary antibody (1:7500) (Peroxidase conjugated affinity purified anti-mouse IgG, Rockland) for 1 hour at room temperature and then washed for 4 times with TBST. Positive immunoreactive bands were detected using the Lumi-Light chemiluminescence kit (ECL^{TM} western blotting detection regents, RPN2106, GE healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

II.3.7 Assessment of sperm fertilizing capacity

To ascertain whether Cl^- is involved in the sperm fertilizing ability, we used a zona free hamster egg assay as a test system for the appraisal of the fertilizing ability of guinea pig spermatozoa as described previously(Yanagimachi et al., 1976;Shi et al., 1992).

II.3.8 Assessment of sperm hyperactivated motility

Sperm hyperactivated motility (HAM) accompanies sperm capacitation. HAM is a change in motility pattern, specifically, an increase in the amplitude of the flagella bend and average lateral head movement (ALH). No software for analysis of guinea pig sperm motility is available. In this study, the movements of three characteristic patterns were designated as hyperactivated motility as described previously(Shi and Friend, 1983) with some modifications: A. sperm head exhibited marked lateral placement with forward motility and tail whiplash movement; B. sperm forward progressive movement with a non-linear trajectory; C. sperm head equatorial segment attached to the slide surface displaying quick swinging with flagella movements which are much greater in the flagellum than in the head without cell progression(Yanagimachi, 1994a;Suarez, 1996).

II.3.9 Immunofluorescent staining

For indirect immunofluorescence studies of CFTR and SLC26A3 localization,

spermatozoa washed by centrifugation through a three-layer gradient of Percoll as mentioned above. Sperm were fixed in 4% paraformaldehyde overnight at 4°C and then were smeared on slide previously coated with poly-L-lysine, air dried and after washing with PBS for three times, the slides were subsequently blocked with 10% normal goat serum at room temperature for 1 hour. After further washing in PBS twice for 5min each, sperm were incubated with primary CFTR antibody (CF3, Abcam, Cambridge, UK, ab2784, 1:500) or SLC26A3 antibody (a gift from Dr. Höglund P., 1:250) at 4°C overnight. Sperm were washed with PBS three times and incubated with secondary antibody (anti-mouse IgG+IgM-FITC, Abcam, Cambridge, UK, ab47830, 1:1000; Alexa-488 conjugated goat anti rabbit IgG, Invitrogen, A11008, 1:1000) in dark room for 1 hour at room temperature. Unbound antibody was removed by washing with PBS three times. The slides were then stored in a dark box before visualization.

II.3.10 Western blot

Proteins are resolved by SDS-PAGE on polyacrylamide gels followed by transfer onto nitrocellulose membranes. Membranes are blocked with 4% milk in TBST for 1 hour before being probed with primary mouse anti-human CFTR monoclonal antibody (CF3, Abcam, Cambridge, UK, ab2784, 1:2000) or SLC26A3 antibody (Santa Cruz, sc34943, 1:500) at 4°C overnight. Membranes are washed three times in TBST followed by incubation with 1:10000 anti-mouse IgG-HRP (Abcam, Cambridge, UK, ab6006, 1:10000) or 1:5000 donkey anti-goat IgG–HRP (Santa Cruz, sc2033) in TBST containing 1% BSA for 1 hour at room temperature. Following three washes in TBST, proteins are detected using an enhanced chemiluminescence kit (ECL plus western blotting detection, RPN2132, GE healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

II.3.11 Statistical analysis

Results are means ± S.E.M. For statistical analyses, percentage data were transformed [arcsin $\sqrt{}$(percent of cells÷100)] and comparisons were made with one-way ANOVA followed with Tukey's or Dunnett's post-hoc tests. Values of $P<0.05$ were regarded as statistically significant.

II.4 RESULTS

II.4.1 Dependence of sperm capacitation on Cl^- concentrations

Using the sperm AR for assessing capacitation, two separate events, we were obligated to distinguish the effect of Cl^- on sperm capacitation from its effects on the AR. To do that, Cl^- replacement was made separately in either the capacitating Medium (Minimal Capacitation Medium, MCM) or the MCM in which the AR was induced. When washed sperm were incubated initially in the complete MCM for 6 hours followed by thorough washing and re-suspension in the Cl^--deficient MCM, there was a significant increase in the percentage of the AR as compared to the control in response to stimulation with 10 μM P or 1ZP/μl ZP for 15min (Figure II.1 A). However, when sperm were incubated in the Cl^--deficient MCM but re-suspended in complete MCM during the AR induction, the percentage of AR was greatly reduced with no significant difference between all treatments of sperm regardless the stimulus used (Figure II.1 B), indicating that Cl^- plays an essential role in sperm capacitation but not in the AR itself.

II.4.2 Sperm capacitation requires both Cl^- and HCO_3^-

Since HCO_3^- had been shown to initiate sperm capacitation, we then tried to determine whether or not the effect of Cl^- on sperm capacitation could be independent of HCO_3^-. Two sets of experiments were carried out. One set was conducted using capacitating media at different HCO_3^- concentrations with a normal Cl^- concentration (115 mM). As shown in Figure II.2A, sperm capacitation was reduced as the concentration of HCO_3^- in the capacitating medium decreased, with total abolishment when HCO_3^--deficient MCM was used for incubation, confirming an essential role of HCO_3^- in the process of capacitation as previously reported.

The other set of experiments was performed using MCM of various Cl^- concentrations with a constant HCO_3^- concentration (25mM). After incubation for 6 hours, sperm were incubated in complete MCM and stimulated with P for 15 min before assessing the AR. The results showed that sperm capacitation decreased with decreasing Cl^- concentrations in the MCM used despite the presence of sufficient amount of HCO_3^- (Figure II.2 B), indicating that sperm capacitation requires Cl^- as well as HCO_3^-, with the possibility that Cl^- may be required for the transport of HCO_3^- into sperm.

II.4.3 Effect of Cl^- Intracellular pH increase during capacitation

An increase in intracellular pH of sperm due to the entry of HCO_3^- is an early hallmark event in the process of capacitation. Indeed, when sperm were incubated in complete MCM, the intracellular pH increased with time (Figure. II.3); however, it remained almost unchanged in the Cl^--deficient MCM throughout the whole period of capacitation (Figure II.3), consistent with the observed Cl^- dependence of sperm capacitation.

II.4.4 Effect of Cl^- and HCO_3^- on sperm cAMP levels

If Cl^- is required for transporting HCO_3^- into sperm, such as in the case of an anion exchanger, depleting Cl^- should affect HCO_3^--dependent events that are known to be involved in sperm capacitation. We first examined whether the cAMP production resulting from HCO_3^- activation of the soluble adenylate cyclase could be affected by Cl^- depletion. Sperm were incubated separately in complete Cl^-/HCO_3^- MCM or Cl^--deficient or HCO_3^-- deficient MCM for 6 hours. The intracellular cAMP levels in sperm measured by ELISA (Figure II.4) showed that the mean level of cAMP in sperm incubated in complete Cl^-/HCO_3^- MCM for 6 hours was significantly elevated as compared to that observed in the control (0 hour). However, this elevation of intracellular cAMP was completely abolished when sperm were incubated in Cl^--deficient or HCO_3^- -deficient MCM for the same amount of time ($P<0.05$). Interestingly, the effect of Cl^- depletion on sperm cAMP level was similar to that of HCO_3^- depletion, indicating the possible involvement of a similar mechanism in both cases, the most likely candidate being an anion exchanger. Depletion of either one ion ceases the operation of the exchanger, resulting in the failure of HCO_3^- entry, and thus, cAMP production.

II.4.5 Effect of Cl^- on capacitation-related tyrosine protein phosphorylation

To further confirm whether Cl^- is involved in mediating capacitation-associated HCO_3^- dependent processes, its effect on protein tyrosine phosphorylation during sperm capacitation was examined. Washed sperm were separately incubated in the complete Cl^-/HCO_3^- or Cl^--deficient MCM for 0, 2, 4 and 6 hours. The results showed that sperm incubated in complete Cl^-/HCO_3^- MCM were accompanied by a time-dependent increase in the protein tyrosine phosphorylation of a subset of proteins of Mr. 25

KDa-120 KDa normally observed during sperm capacitation. However, sperm incubated in Cl^--deficient MCM showed reduced protein tyrosine phosphorylation with only two phosphorylated proteins about 23 KDa and 50 KDa observed (Figure II.5). These two protein bands were also attenuated with the incubation time in the absence of Cl^- in the capacitating medium, suggesting that Cl^- is also required for protein tyrosine phosphorylation, a well-known event downstream of HCO_3^- activation of cAMP production.

II.4.6 Effect of Cl^- on sperm fertilizing capacity

In vitro fertilization may measure the actual physiological function of the sperm capacitated status. To determine whether the sperm fertilizing ability is affected by Cl^-, we used a zona-free hamster egg as a test system for the assessment of the fertilizing capacity in guinea pig spermatozoa(Yanagimachi et al., 1976;Aitken et al., 1986;Shi et al., 1992). Washed sperm were pre-incubated in complete or Cl^--deficient MCM for 6 h. Zona-free hamster eggs were in the complete or deficient Cl^- BWW medium, introduced separately into sperm suspension (1:1, 50 μl each) in the complete or Cl^--deficient MCM for co-incubation for 3 h. Sperm were exposed to a constant concentration of complete Cl^- (~ 115 mM), a half concentration of Cl^-(~56 mM) or Cl^- -deficient medium (both MCM and BWW of Cl^--deficient). As shown in Figure II.6, the penetration rate of sperm declined significantly with a decrease in concentration of Cl^- in the medium. Sperm-egg fusion almost ceased when spermatozoa and eggs were exposed in both types of Cl^--deficient medium. To evaluate whether Cl^- directly affects the fertilizing ability of zona-free hamster eggs, we pre-incubated the eggs in 1/3 Cl^- BWW medium for 3 h and then washed them with complete BWW medium and re-suspended in the same medium for co-culture for 3 h. Similarly, sperm were pre-incubated in the complete MCM for 6 h, and then mixed with eggs for 3 h as mentioned above. The fertilizing capacity of the zona-free hamster eggs was not significantly affected and was similar to that of those in the complete medium (data not shown).

II.4.7 Effect of inhibitors and antibody of SLC26A3 and CFTR on capacitation-associated HCO_3^- dependent events

Although we had previously demonstrated an important role of CFTR in transporting HCO_3^- into sperm necessary for capacitation(Xu et al., 2007), the currently

observed Cl^- dependence of capacitation-associated HCO_3^- dependent events suggested additional involvement of an exchanger for Cl^-/HCO_3^-, Since SLC26A3 mutations in men had been reported to exhibit sub-fertility(Hoglund et al., 2006), the exchanger may be involved in sperm capacitation, perhaps working together with CFTR for the transport of HCO_3^-. We tested this hypothesis by examining the effects of inhibitors of CFTR and the candidate anion exchanger, SLC26A3, as well as its antibody on a number of capacitation-associated events in the following experiments.

II.4.7.1 CFTRinh-172, niflumate and SLC26A3 antibody inhibit sperm capacitation

CFTRinh-172 in various concentrations (5nM to 100nM) was added to sperm suspensions at the beginning of capacitation and incubated for 6 hours, and then washed, re-suspended in inhibitor-free MCM challenged with 10 μM P for 15min before measurement of the AR. As shown in Figure II.7 A, the percentage of AR decreased significantly with increased concentrations of CFTRinh-172 in the MCM, as compared to the P-induced AR ($P < 0.001$), indicating the involvement of CFTR in sperm capacitation. When sperm were capacitated in HCO_3^--deficient MCM, there was no significant difference between the P-induced and CFTRinh-172 groups. Similarly, niflumate, an inhibitor more selective to SLC26A3, at various concentrations (1-100μM) or 4,4'- diisothiocyanatostilbene-2, 2'-disulfonic acid (DIDS, 1-100μM), an inhibitor of a number of anion exchangers which sensitive to SLC26A6 and SLC4 family, was added to the MCM but not in the MCM for inducing AR. As shown in Figure II.7 B, when compared the concentration-dependent effects between niflumate and DIDS, it showed a more potent effect of niflumate over that of DIDS. There was no significant difference between the two treatments when sperm were incubated in HCO_3^--deficient MCM (Figure II.7 C). Since the inhibitors used may not be exclusive for SLC26A3, its specific antibody was also employed. Adding various concentrations of SLC26A3 antibody (1:100-1:1000) to sperm suspensions for capacitation for 6 hours and washed, re-suspended in antibody-free medium, challenged with 10 μM P for 15min before assessment of the AR. As shown in Figure II.7 D, the AR was markedly reduced with increased concentrations of antibody in the medium as compared to the P-treated group ($P<0.001$), indicating the involvement of SLC26A3. Also, when capacitated in HCO_3^--deficient MCM, there were no significant differences between different

treatment groups.

II.4.7.2 Sperm hyperactivated motility (HAM)

HAM is a capacitation-associated event that may also serve as a potential assay for capacitation(Yanagimachi, 1994a;Suarez, 1996). First, we chose to confirm whether Cl^- would have a similar effect on HAM as it did on other capacitation-associated events (see above). Washed sperm were incubated separately in Cl^--deficient or the complete MCM with or without CFTR inhibitor, CFTRinh-172, for 6 hours and then stimulated with P (10μM) or ZP (1ZP/μl) for 15min before assessment for sperm HAM. As shown in Figure II.8 A, sperm in the complete MCM in response to P or ZP exhibited a significantly higher HAM than that of the sperm in Cl^--deficient MCM. When CFTRinh-172 was added, the ZP induced AR was inhibited. Similar to the results observed with induced AR, the inhibitors of anion exchangers, as well as its antibody, also significantly inhibited sperm HAM (Figure. II.8 B, D), further confirming the involvement of SLC26A3 in the process of sperm capacitation. When comparison of concentration-dependent effects between niflumate and DIDS were also made, it showed a more potent effect of niflumate over that of DIDS ($P<0.05$-$P<0.001$, Figure II.8 B). Similarly, there were no differences between niflumate and DIDS treatments or among different concentrations of SLC26A3 antibody when sperm were capacitated in HCO_3^--deficient MCM (Figure II.8 C, D).

II.4.8 Expression and localization of CFTR and SLC26A3 in sperm

The above functional studies clearly demonstrated the involvement of CFTR and an anion exchanger, which is likely to be SLC26A3, however, their expression and localization have not been demonstrated in guinea pig sperm. In fact, SLC26A3 has not been shown to be expressed in sperm of any species. Using indirect immunofluorescence staining we demonstrated that CFTR was localized to the sperm equatorial segment, as previously demonstrated in human and mouse sperm(Xu et al., 2007), whereas, SLC26A3 was localized over the acrosomal anterior area of the sperm head (Figure II.9 A,B). The expression of CFTR and SLC26A3 was further demonstrated by Western blot analysis, which confirmed the presence of a 170KDa protein of CFTR, as shown previously(Xu et al., 2007) and for the first time the presence of a 75 KDa protein of SLC26A3 in sperm (Figure II.9 A, B).

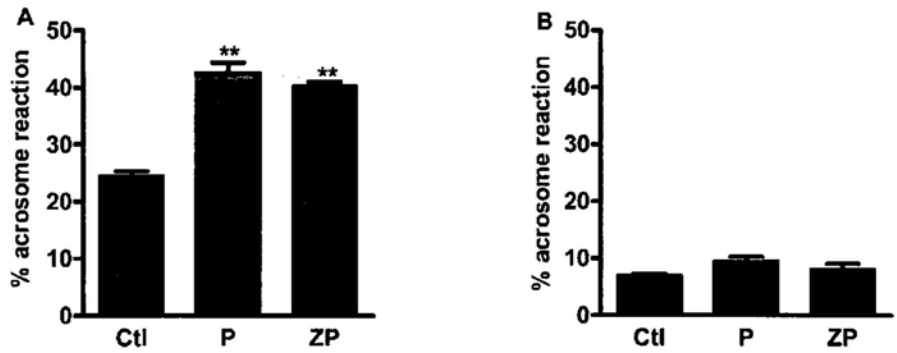


Figure II.1 Dependence of sperm capacitation on Cl^- in the capacitating medium

Capacitation in guinea pig sperm was assessed by their ability to undergo the agonist-induced acrosome reaction (AR) after incubation for 6 hours in different capacitating media with or without Cl^-. Results are means ± SEM (n=3). **A.** Sperm were initially incubated in MCM for capacitation and then washed and resuspended in Cl^--deficient MCM (Ctl) and stimulated with progesterone (P, 10 μM) or zona pellucida (ZP, 1ZP/μl) for 15 min before the AR assessment; **B.** The P or ZP-induced AR was greatly suppressed when sperm were incubated in Cl^--deficient MCM for 6 hours and switched to a complete MCM for AR induction, indicating the dependence of capacitation but not the AR on Cl^-. **, as compared to control and Cl^--deficient capacitating medium respectively, $P< 0.001$.

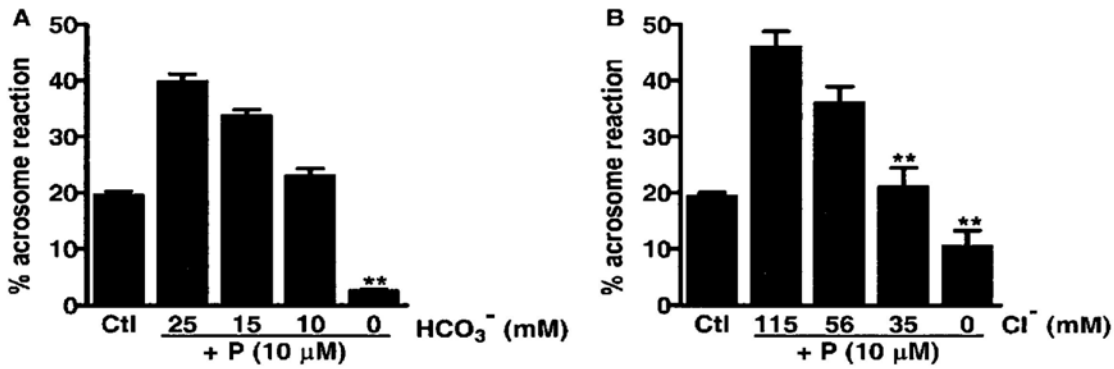


Figure II.2 Sperm capacitation requires both Cl^- and HCO_3^-

A. Effect of varying HCO_3^- concentrations on capacitation. Washed sperm were incubated in MCM with different concentrations of HCO_3^- but constant Cl^- concentration (115mM) for 6 hours and then switched to a complete Cl^-/HCO_3^- MCM for AR induction (n=5); **B.** Effect of varying Cl^- concentrations on capacitation. Sperm were incubated in MCM with different concentrations of Cl^- but constant HCO_3^- concentration (25 mM) for 6 hours and then switched to a complete Cl^-/HCO_3^- MCM for AR induction. Depletion in either HCO_3^- or Cl^- prevented capacitation (n=3). Results are means ± SEM. **, as compared to highest anion concentration, $P<0.001$. The MCM incubation without progesterone (P, 10μM) induction is as control (Ctl).

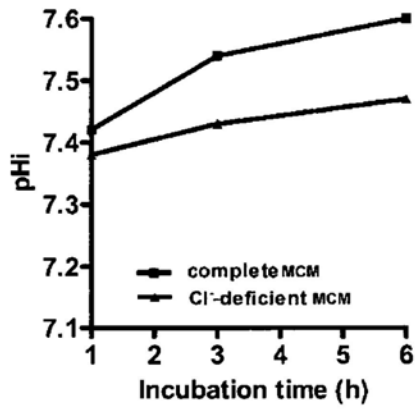


Figure II.3 Cl^- dependence of sperm intracellular pH (pHi)

Washed spermatozoa were incubated in Cl^--deficient or complete MCM. 5μM 2', 7-bis-2 (carbosyethyl)- 5-(and-b)- carboxyfluorescence, acetoxymethyl ester (BCECF) was loaded for 30 minutes prior to the determination of the pHi. The representative time-course changes in pHi during the capacitation showed suppressed increase in pHi when sperm were incubated in Cl^--deficient MCM as compared to those incubated in complete MCM.

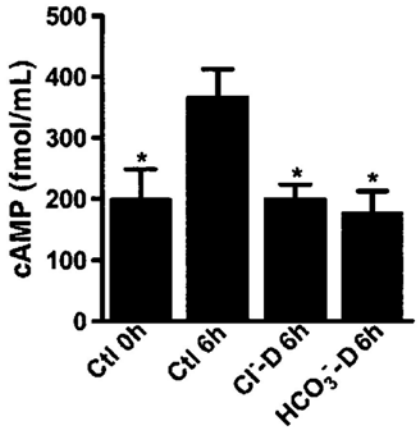


Figure II.4 Effect of Cl^- and HCO_3^- on capacitation-associated increases in sperm intracellular cAMP levels

Washed sperm were incubated separately in MCM (Ctl) for 0 hour and 6 hours, or Cl^--deficient (Cl^--D) and HCO_3^--deficient (HCO_3^--D) MCM for 6 hours before intracellular cAMP measurement (n=3). Results are means ± SEM. *, as compared to Ctl 6 h, $P<0.05$.

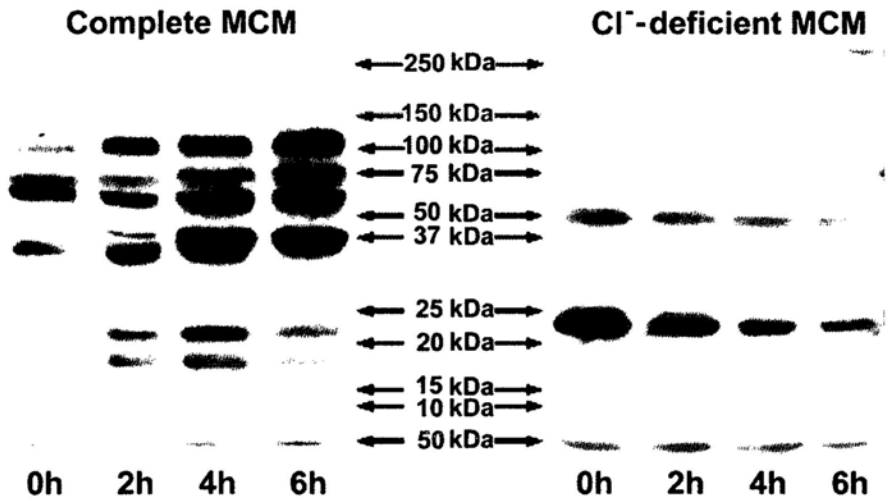


Figure II.5 Effect of depleting Cl^- on sperm protein tyrosine phosphorylation

Washed spermatozoa were incubated under capacitation conditions at 0, 2, 4, 6 hours and then proteins were extracted for Western blot. The primary antibody, a monoclonal mouse antibody against phosphotyrosine (p-Tyr0sine, 4G10, Cell Signaling) 1:2000 and the second antibody, Peroxidase conjugated affinity purified anti-mouse IgG (Rockland) 1:7500 were used. Left: Protein tyrosine phosphorylation in guinea pig sperm incubated in complete MCM; Right: Protein tyrosine phosphorylation in sperm incubated in Cl^--deficient MCM, showing reduced phosphorylated proteins with time.

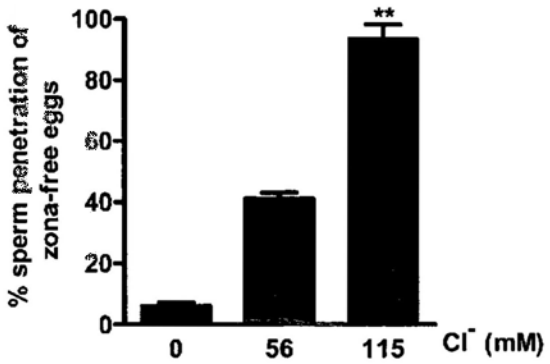


Figure II.6 Effect of Cl^- on sperm-egg fusion

Washed sperm were pre-incubated separately in the complete MCM (A) under capacitation conditions for 6 h, and then co-cultured with 20-30 hamster zona-free eggs in complete BWW (C; i.e. AC) and Cl^--deficient BWW (D, i.e. AD) respectively for 3 h. In addition, sperm were incubated in the Cl^--deficient MCM (B) for 6 h, washed and then co-cultured with 20-30 hamster zona-free eggs in Cl^--deficient BWW (D, i.e. BD) for 3 h. The percentage of spermatozoa penetration into eggs was assessed (n=3). Results are means ± SEM. ** as compared to 56 mM Cl^-(AD) and Cl^--deficient medium (0mM Cl^-, BD), $P<0.001$.

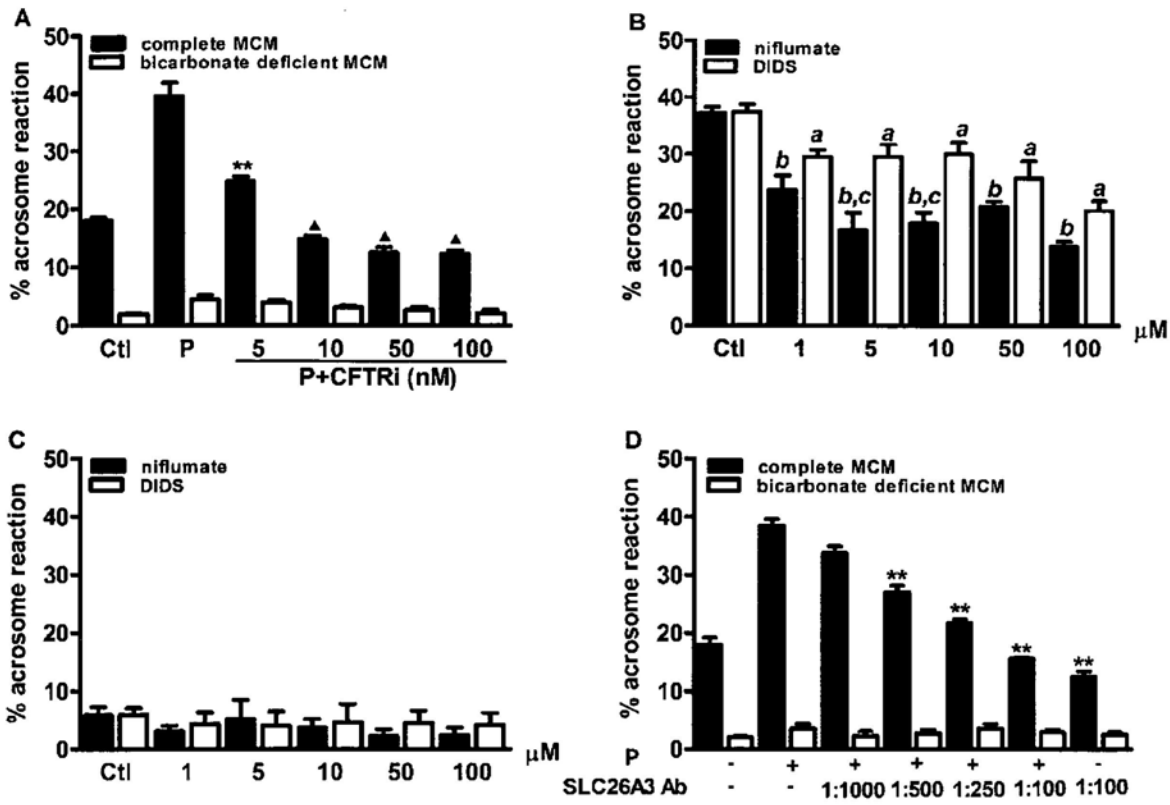


Figure II.7 Effects of inhibitor of CFTR (CFTRi) and anion exchanger inhibitors as well as SLC26A3 antibody on capacitation with or without HCO_3^-

Washed sperm were incubated in complete MCM or HCO_3^-- deficient MCM without (Ctl) or with CFTRiinh-172 (CFTRi, 5-100nM), niflumate (1-100 μM), DIDS (1-100μM) or SLC26A3 antibody (1:1000-1:100) separately for capacitation and then washed and challenged with progesterone (P, 10μM) for 15 min before AR assessment. Results are means ± SEM. **A.** Inhibitory effect of CFTRi on capacitation (n=6-8). **, as compared to the P treated group, P<0.001; ▲, as compared to CFTRi 5nM group, P<0.001; **B.** Comparison of concentration- dependent inhibitory effects of niflumate and DIDS on capacitation in complete MCM (n=3). (*a*, as compared to niflumate control, P<0.001, *b*, as compared to DIDS control, P<0.05-0.001, *c*, as compared to correspondence niflumate treatment, P<0.01); **C.** Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on capacitation in HCO_3^--deficient MCM(n=3). **D.** Effect of antibody of SLC26A3 on capacitation. (n=3). **, as compared to the P-treated group, P<0.001.

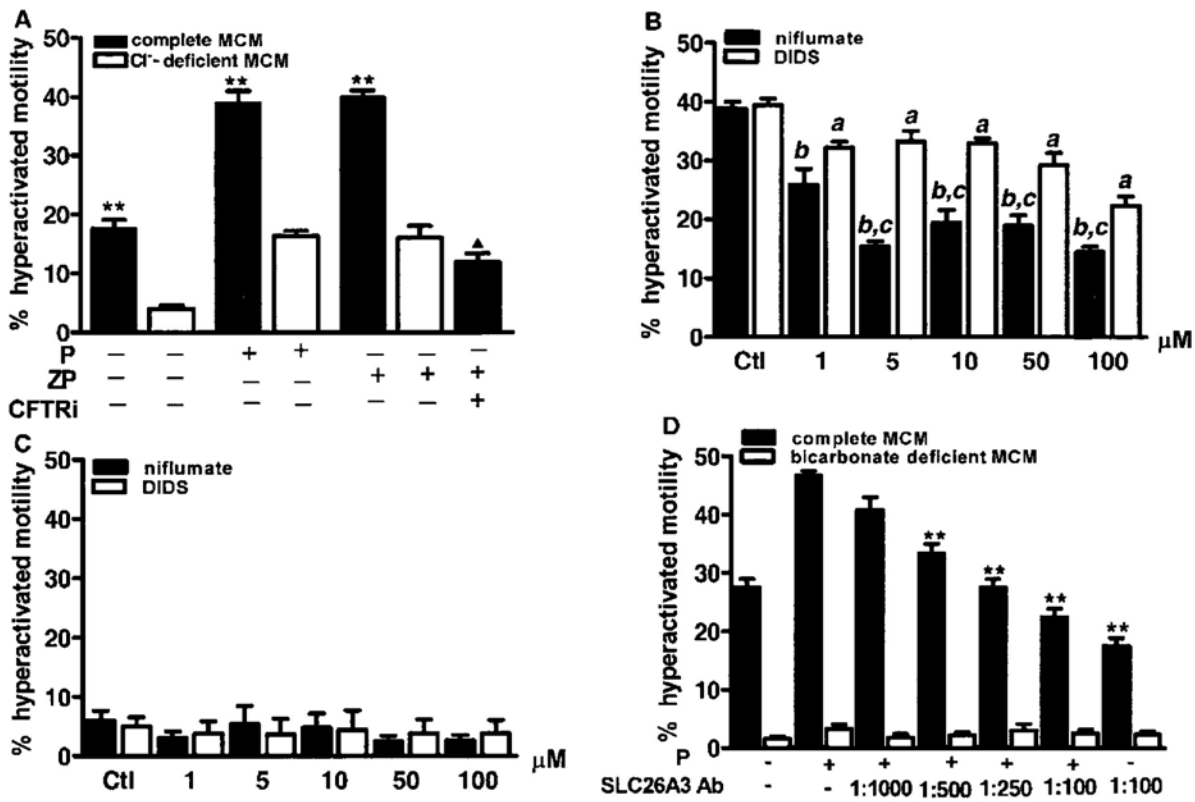


Figure II.8 Effects of Cl^- and transport inhibitors as well as SLC26A3 antibody on sperm hyperactivated motility (HAM) with or without HCO_3^-

Washed sperm were incubated in Cl^--deficient or complete MCM or HCO_3^--deficient MCM without or with CFTRiinh-172 (CFTRi, 100nM), niflumate (1-100 μM), DIDS (1-100μM) or SLC26A3 antibody (1:1000-1:100) separately for capacitation and then washed and challenged with progesterone (P, 10μM) or zona pellucida (ZP, 1ZP/μL) for 15 min before HAM assessment. Results are means ± SEM. A. Depleting Cl^- and CFTR inhibitor reduced sperm HAM (n=3). **, as compared to Cl^--deficient MCM, respectively, or compared to ZP treatment, P<0.001. B. Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on HAM in complete MCM (n=3). (*a*, as compared to niflumate control, P<0.001, *b*, as compared to DIDS control, P<0.05-0.001, *c*, as compared to correspondence niflumate treatment, P<0.05-0.001). C. Comparison of concentration-dependent inhibitory effects of niflumate and DIDS on HAM in HCO_3^-- deficient MCM (n=3). D. Effect of SLC26A3 antibody on sperm HAM (n=3). **, as compared to P-treated group, P<0.001.

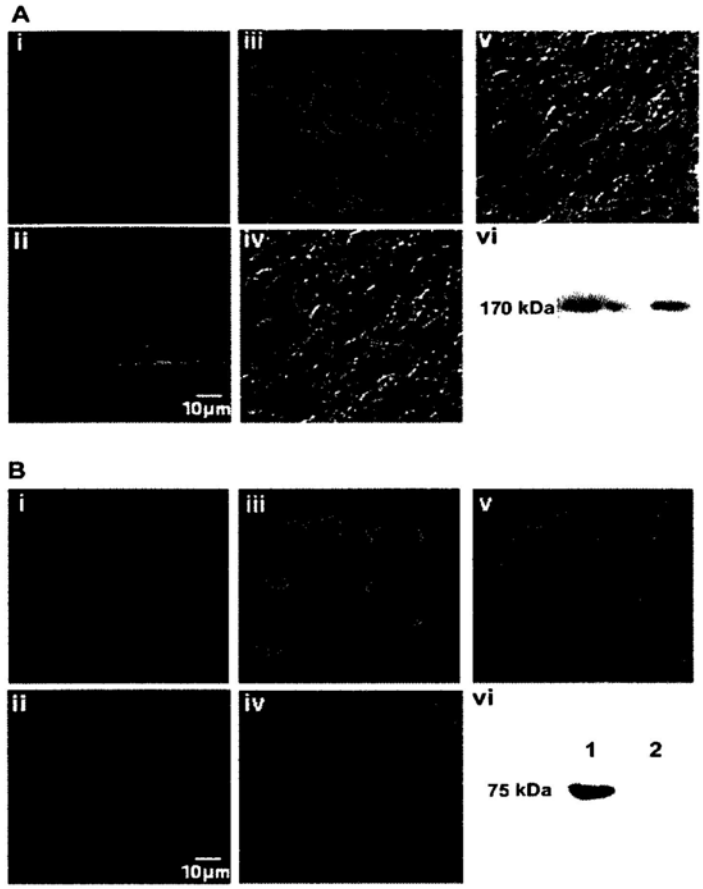


Figure II.9 Localization and expression of CFTR and SLC26A3 in guinea pig sperm

A. Immunostaining and Western blot of CFTR in guinea pig sperm using a Mouse Monoclonal antibody (CF3) to CFTR (Abcam, Cambridge, UK, ab2784, 1:500 or 1:2000) and the second antibody anti-mouse IgG+IgM-FITC (Abcam, Cambridge, UK, ab47830 1:1000) or anti-mouse-HRP (Abcam, Cambridge, UK, ab6006, 1:10000). **i.** Negative immunostaining control of CFTR with the mouse isotype specific IgM (Rockland Co, Gilbertsville, PA, USA); **ii.** Corresponding differential interference contrast image (scale bar, 10μM); **iii.** Immunofluorescence of CFTR localized to the equatorial segment region of the sperm head; **iv.** Corresponding differential interference contrast image of guinea pig sperm; **v.** Superimposed image of CFTR staining and differential interference contrast image (×630); **vi.** Western blot of CFTR in guinea pig sperm, recognized a specific band of about 170 kDa. Left to the right, protein loading 30μg, 10μg, 20μg.

B. Immunostaining and Western blot of SLC26A3 in guinea pig sperm using an antiserum against SLC26A3 (gift from Dr. Höglund P., 1:250) and Alexa-488 conjugated goat anti rabbit IgG (Invitrogen, A11008, 1:1000) or goat anti-mouse antibody (Santa Cruz, sc 34943, 1:500) and donkey anti-goat IgG-HRP (Santa Cruz, sc2020, 1:5000): **i.** Negative control without primary antibody; **ii.** Corresponding differential interference contrast image of guinea pig spermatozoa (scale bar, 10μM); **iii.** Immunofluorescence of SLC26A3 in guinea pig spermatozoa which is localized in the anterior acrosome region; **iv.** Corresponding differential interference contrast image; **v.** Superimposed image of SLC26A3 staining and differential interference contrast image (×630); **vi.** Western blotting of SLC26A3 in guinea pig spermatozoa. lane 1:SLC26A3 antibody, lane 2: negative control with blocking peptide(Santa Cruz, sc34942p).

II.5 DISCUSSION

While it had been established that HCO_3^- plays an essential role in sperm capacitation(Shi and Roldan, 1995;Boatman, 1997;Visconti et al., 1999;Gadella and Harrison, 2000;Liu et al., 2005), the present study demonstrates an equally important role of Cl^- in the process by mediating the entry of HCO_3^- required for capacitation. To initiate capacitation, HCO_3^- has to first enter into sperm and act on a HCO_3^- sensor, the soluble adenylate cyclase, which in turn triggers the signaling cascade leading to capacitation(Chen et al., 2000b). Some of the key events in this cascade, such as increases in intracellular pH, cAMP production and tyrosine kinase phosphorylation, which have been previously shown to be dependent on HCO_3^-, are also found to be dependent on Cl^- in the capacitating medium as proved in the present study. While the assessment of sperm capacitation by the induced AR in the present study also supports the Cl^- dependence of capacitation, but not the AR. It should be noted that previous studies by others have suggested a Cl^- dependence of the AR process. Replacing Cl^- with Br^- in the sperm-suspending medium was shown to result in inhibition of the zona-initiated hamster sperm AR(Yoshimatsu and Yanagimachi, 1988). Others also reported that Cl^- was required for the P-initiated AR in human and porcine sperm (Melendrez and Meizel, 1995). It should be noted that sperm capacitation and the AR are sequential but separable processes (Yanagimachi, 1994b) and that in those reported studies, effort was not made to differentiate these two processes. In the present study, Cl^- depletion in the capacitating medium, but not the AR inducing medium, reduced acrosome reacted sperm, indicating that Cl^- is required for capacitation but not the AR. Indeed, the agonist-induced HAM, which is an event associated with sperm capacitation, was also found to be dependent on Cl^- in the present study. Together with the demonstrated Cl^- dependence of the capacitation-associated key events, the present results confirm that Cl^- is required for capacitation.

The dependence of the capacitation-associated events on Cl^-, in addition to HCO_3^-, suggests the involvement of a Cl^-/HCO_3^- exchanger in mediating the entry of HCO_3^-. Indeed, an inhibitor of the anion exchanger was shown to reduce the HCO_3^- dependent sperm capacitation assessed either by the AR or HAM in the present study. This is in

agreement with previous reports demonstrating the presence of an anion exchanger in sperm (Spira and Breitbart, 1992;Zeng et al., 1996;Kaupp and Weyand, 2000). Although it has been known for years that an exchanger for Cl^- and HCO_3^- is present in mature sperm, its exact role in sperm function and its molecular identity are unknown. The present study not only has confirmed the involvement of an anion exchanger in mediating HCO_3^- entry necessary for capacitation, but also provided evidence indicating that the exchanger could be SLC26A3 with its expression in sperm demonstrated by both immunofluorescence staining and Western blot analysis. The involvement of SLC26A3 is further supported by pharmacological evidence demonstrating a more potent effect of niflumate, an inhibitor more selective for SLC26A3(Ko et al., 2002;Chernova et al., 2003), over that of DIDS, an inhibitor known to inhibit a wide spectrum of anion exchangers/cotransporters, including SLC4 member, SLC4A2 (also known as AE2)(Shmukler et al., 2005), It is interesting to note that SLC4A2 is also expressed in mature mammalian sperm(Parkkila et al., 1993) and shown to be essential in spermiogenesis(Medina et al., 2003). However, the observed more potent effect of niflumate over DIDS suggests that SLC26A3 plays a more primary role in sperm capacitation. This notion was further supported by the observed inhibitory effect of its specific antibody on sperm capacitation. The specificity of the antibody against SLC26A3 has been previously reported(Haila et al., 2000;Hihnala et al., 2006), and thus, its interference with sperm capacitation observed in the present study clearly indicates the involvement of SLC26A3 in the process. A sodium HCO_3^- cotransporter has also been previously proposed to be involved in mouse sperm capacitation(Demarco et al., 2003). While this mechanism may be a possible pathway for HCO_3^- entry into sperm during capacitation, it cannot explain the Cl^- dependence of capacitation observed in the present study. Taken together, the present results suggest that SLC26A3 plays a primary role in transporting HCO_3^- into sperm, on which capacitation depends.

Patients with congenital chloride diarrhea (CLD) with mutations in SLC26A3 exhibit reduced fertility(Hoglund et al., 2006;Hihnala et al., 2006), although the exact cause is unknown as far as we known. In the present study we found that the capacity of spermatozoal penetration of oocytes greatly reduced with decreased Cl^- concentrations. Fertilizing ability was almost abolished in the Cl^- deficient MCM as compared to the

complete MCM, indicating that Cl^- is required for fertilization. Based on the current finding, it is highly possible that defective SLC26A3 impairs HCO_3^- entry into sperm and affects the capacitation process leading to reduced fertilizing ability and thus reducing the fertility rate, as seen in CLD patients. Therefore, SLC26A3 could be a potential molecular target for diagnosis of male infertility.

The present results also indicate that the operation of the anion exchanger requires the involvement of CFTR as a Cl^- channel to provide a recycling pathway for Cl^-. Without CFTR working properly, the entry of HCO_3^- would be disrupted, due to lack of Cl^- in exchange, resulting in capacitation failure as demonstrated in the present study using the CFTR inhibitor. The importance of CFTR in the process of sperm capacitation as well as in male fertility has also been demonstrated in our previous studies using CF heterozygote sperm, although it was not clear whether it played a direct or indirect role(Xu et al., 2007). The present study not only confirms the importance of CFTR in capacitation, but also demonstrates an indirect role of CFTR in transporting HCO_3^- into sperm, by providing a recycling pathway for Cl^- obligated by the operation of the anion exchanger. The parallel working of CFTR with a Cl^-/HCO_3^- exchanger is best established for pancreatic HCO_3^- secretion(Steward et al., 2005;Ishiguro et al., 2007). Surprisingly, CFTR and SLC26A3, though localized in sperm head, are not localized to the same region, suggesting that CFTR protein in sperm does not directly interact with the anion exchanger protein as it does in the pancreas(Ko et al., 2002;Ko et al., 2004). Nevertheless, the two sites in the sperm head, to which CFTR and SLC26A3 are localized, are close enough to allow ion flux exchange and the present study provides clear evidence for a functional interaction between the CFTR and SLC26A3 exchanger leading to sperm capacitation, which is illustrated in a working model shown in Figure II.10. The demonstrated involvement of SLC26A3 in sperm capacitation, in addition to CFTR, may shed new light into our understanding of molecular mechanisms regulating male fertility and provide an explanation to some cases of unexplained male infertility.

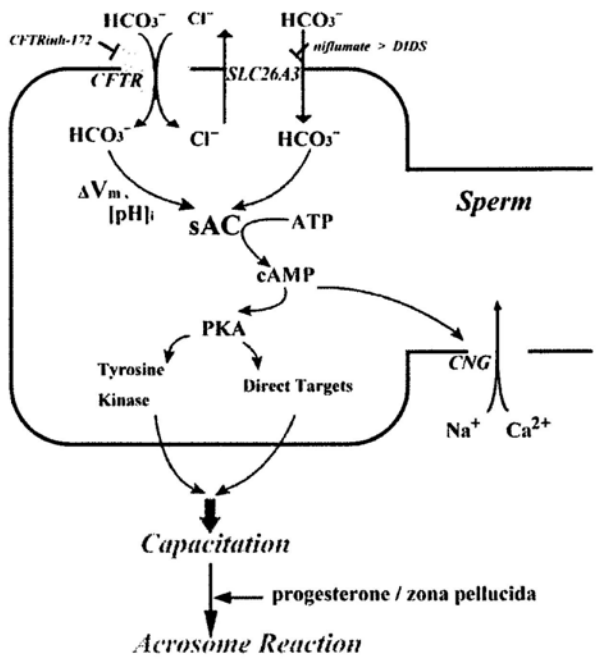


Figure II.10 Schematic illustration of involvement of SLC26A3 and CFTR in HCO_3^- entry and the HCO_3^--dependent events involved in sperm capacitation.

HCO_3^- influx is mediated by SLC26A3 with an exchange of $2Cl^-/HCO_3^-$. CFTR, apart from its reported role in conducting HCO_3^- directly, may act as a Cl^- channel to provide a recycling pathway for Cl^- which is required for the operation of SLC26A3. The sites of action for inhibitors, CFTRinh-172, niflumate and DIDS, as well as the intracellular HCO_3^-- dependent events are also shown.

Chapter III

Further demonstration of SLC26A3 in sperm function: in comparison to GC-1 spg cells expressing SLC26A6

III.1 SUMMARY

Our previous work has demonstrated that Cl^- is required for the entry of HCO_3^- necessary for sperm capacitation, implicating the involvement of SLC26A3 in transporting HCO_3^- with CFTR providing the recycling pathway for Cl^-. Since SLC26A6 is also expressed in mice sperm, and the anion exchanger inhibitors used in the previous study are not very specific to SLC26A3, this part of study was designed to further investigate the role of SLC26A3 in sperm function. GC-1 spg cell line that only expresses SLC26A6 was used as a negative control for comparison. The cells and sperm were pretreated with anion exchanger inhibitors and SLC26A3 antibody, and then membrane potential (Em) and intracellular calcium ($[Ca^{2+}]i$) were measured. Our results showed that DIDS could inhibit the HCO_3^- deficiency-induced depolarization of GC-1 spg cells as well as the depolarization induced by Cl^-- or HCO_3^-- deficiency of the sperm. Niflumate could inhibit the HCO_3^- induced $[Ca^{2+}]i$ increase in sperm but not GC-1 spg cells. SLC26A3 antibody had no effect on the GC-1 spg cells but it could block the depolarization caused by Cl^--deficiency in sperm.

Taken together, these results indicate that SLC26A3 in sperm has a role in HCO_3^--induced $[Ca^{2+}]i$ but does not play a dominant role in determining sperm Em.

III.2 INTRODUCTION

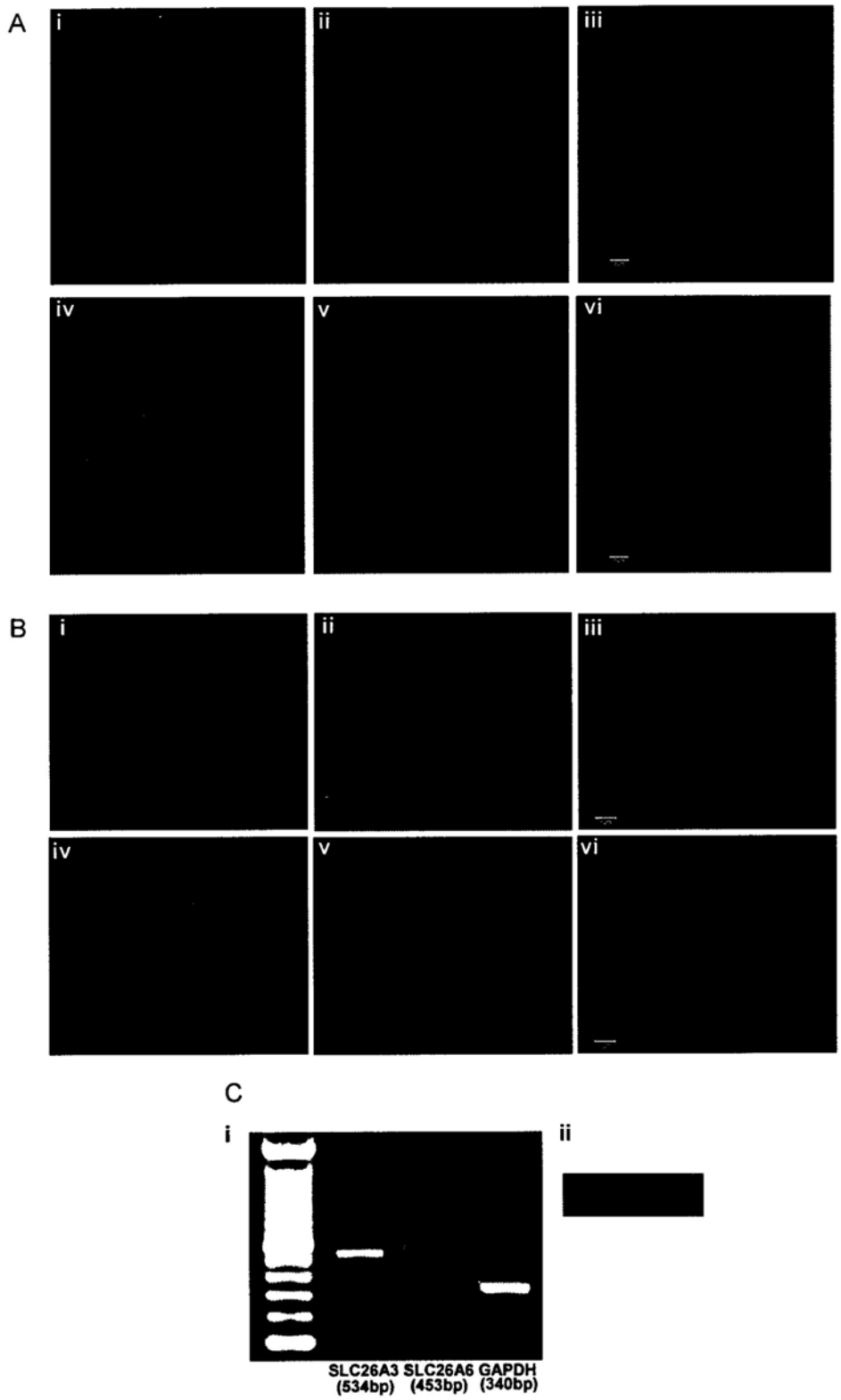
HCO_3^- plays an essential role in capacitation, as prevention of HCO_3^--induced intracellular alkalization results in failure of capacitation (Shi and Roldan, 1995;Boatman, 1997;Visconti et al., 1999;Gadella and Harrison, 2000;Liu et al., 2005). Impaired HCO_3^- secretion in endometrial epithelium also reduced capacitation and fertilizing capacity of co-cultured sperm (Wang et al., 2003). Our recent studies demonstrated a crucial role of cystic fibrosis transmembrane conductance regulator (CFTR) in the transport of HCO_3^- in capacitation (Xu et al., 2007;Hernandez-Gonzalez et al., 2007). Sperm from CF heterozygous mice also had reduced fertilizing capacity *in vitro* and *in vivo*, confirming an important role of CFTR in determining sperm fertilizing capacity. Whether this conductance is directly or indirectly via CFTR is not clear. Our further work has demonstrated that Cl^- is required for the entry of HCO_3^- necessary for sperm capacitation, and that SLC26A3 is involved in transporting HCO_3^- with CFTR providing the recycling pathway for Cl^-. Inhibition of SLC26A3 could lead to impaired guinea pig sperm hyperactivated motility and sperm capacitation(Chen et al., 2009). Yet the results obtained in the study (Chen et al., 2009) are indirect results. Although SLC26A3 antibody could abolish the guinea pig sperm hyperactivated motility and sperm capacitation, the anion exchangers used in the study are not specific to SLC26A3.

The immunofluorescence staining and RT-PCR results have shown that mouse sperm also have SLC26A6 expression (Figure III.1). SLC26A3 and SLC26A6 both belong to the SLC26 family. Unlike SLC26A6, which is one of the more widely expressed members of the family with particularly abundant transcript in kidney, pancreas, intestine, heart, muscle, and placenta (Mount and Romero, 2004), SLC26A3 has a more strict tissue specific expression pattern. So far, SLC26A3 is only found to express in the epithelium of the colon and eccrine sweat glands, the epithelium of the testis, epididymis and seminal vesicles in the human (Haila et al., 2000;Hihnala et al., 2006). Mutations of SLC26A3 are the cause of CLD, while no pathology has been reported related to defect in SLC26A6. Although both are Cl^-/HCO_3^- transporters, their coupling modes and stoichiometries are different. SLC26A3 is a $2Cl^-/1HCO_3^-$ exchanger and SLC26A6 a $1\ Cl^-/2HCO_3^-$ exchanger. As SLC26A3 and SLC26A6 are electrogenic,

changes in membrane potential (Em) were investigated in this study to further demonstrate SLC26A3 involvement in sperm function. Em plays an important role in sperm function. Alteration in Em is involved in the later processes of post-testicular sperm maturation with the former hyperpolarization and the latter depolarization (Yanagimachi, 1994a;Arnoult et al., 1999).

Apart from HCO_3^-, many sperm functions such as motility, capacitation, hyperactivated motility, AR, are also dependent on Ca^{2+} (Baldi et al., 1991;Yanagimachi, 1994a;Chen et al., 2000b;Jaiswal and Conti, 2003). Several types of voltage-gated Ca^{2+} channel proteins have been detected in mouse sperm (Serrano et al., 1999;Wennemuth et al., 2000;Ren et al., 2001;Quill et al., 2001). The depolarizing stimuli open some of these channels to allow Ca^{2+} entry, and this evoked channel activity increases after conditioning of sperm with HCO_3^- (Wennemuth et al., 2000). Thus, impaired HCO_3^- transport caused by defective function of SLC26A3 may also play a role on HCO_3^- induced calcium flux and change of Em.

Therefore, our present study, using GC-1 spg cell line, which is originated from BALB/C mice spermatogonia that only express SLC26A6 but not SLC26A3, as a negative control, was designed to compare Em and HCO_3^--induced $[Ca^{2+}]i$ in GC-1 spg cells with the mature sperm which express both SLC26A3 and SLC26A6 (Figure III.1&2).


A
i
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C
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SLC26A3 (534bp)
SLC26A6 (453bp)
GAPDH (340bp)

Figure III.1 RT-PCR and immunostaining of SLC26A3 and SLC26A6 expression in mouse mature sperm.

A. Immunostaining of SLC26A3 in BALB/C mouse sperm using an antiserum against SLC26A3 (gift from Dr. Höglund P., 1:50) and Alexa-488 conjugated goat anti-rabbit IgG (Invitrogen, A11070, 1:500). i. Negative control without primary antibody; ii. Corresponding immunofluorescence of DAPI of i; iii. Merged image of i and ii; iv. Immunofluorescence of SLC26A3 which is localized in anterior acrosome region; v. Corresponding immunofluorescence of DAPI of iv; vi. Merged image of iv and v.
B. The immunostaining of SLC26A6 in BALB/C mouse sperm using an antiserum against SLC26A6 (gift from Dr. Höglund P., 1:50) and Alexa-488 conjugated goat anti rabbit IgG (Invitrogen, A11070, 1:500). I. Negative control immunostaining of SLC26A6; ii. Corresponding DAPI staining of ii; iii. Merged image of i and ii. iv. Immunostaing of SLC26A6; v. Corresponding DAPI staining of iv; vi. Merged image of v and vi.
C. RT-PCR of SLC26A3 and SLC26A6 and western blot of SLC26A3 in mature mouse sperm. i. RT-PCR of SLC26A3 and SLC26A6 in mouse sperm; ii. Western blots of SLC26A3 in sperm (~80kDa).

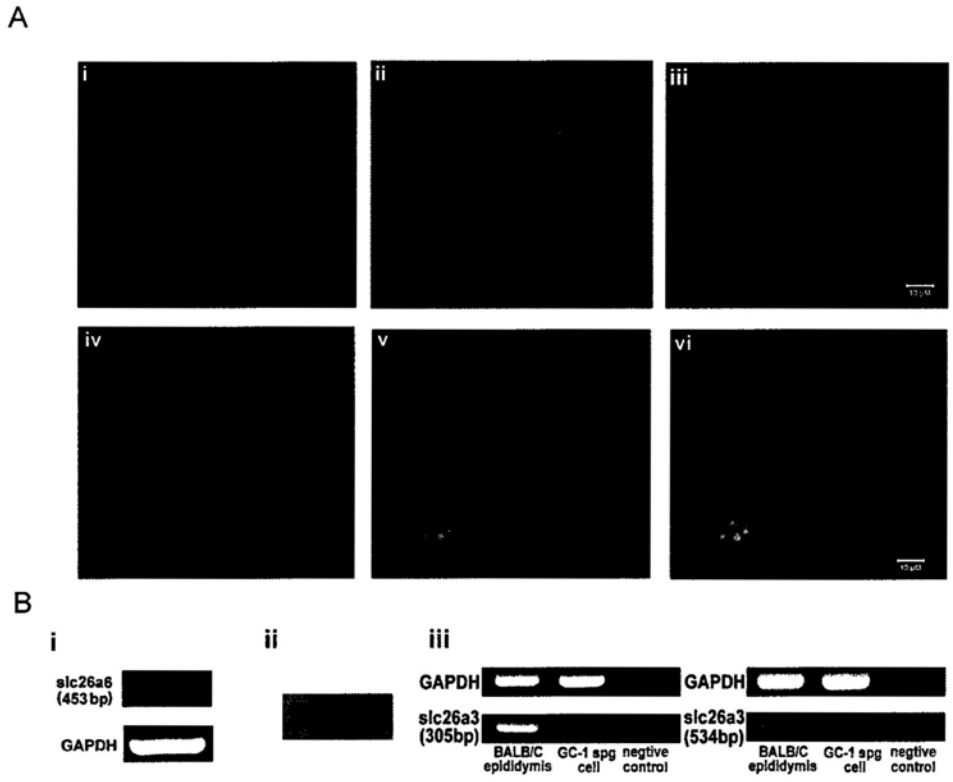


Figure III.2. Expression and localization of SLC26A6 in GC-1 spg cells

A. Immunostaining of SLC26A6 on GC-1 spg cells using an antiserum against SLC26A6 (gift from Dr. Höglund P., 1:100) and Alexa-488 conjugated goat anti rabbit IgG (Invitrogen, A11070, 1:500). I. Negative control without primary antibody; ii. Corresponding immunofluorescence of DAPI of i; iii. Merged image of i and ii; iv. The immunofluorescence of SLC26A6 (1:100) in GC-1 spg cells which is localized in cell membrane; v. Corresponding immunofluorescence of DAPI of iv; vi. Merged image of iv and v.

B. RT-PCR and western blot results of GC-1 spg cells. i. RT-PCR of SLC26A6 in GC-1 spg cells; ii. Western blot of SLC26A6 in GC-1 spg cells (~85kDa); iii. RT-PCR result of SLC26A3 on GC-1 spg cell with two different primers (BALB/C epididymis as positive control).

III.3 MATERIAL AND METHODS

III.3.1 Reagents, cell line and animal

Glucose, sodium HCO_3^-, calcium gluconate, hydrochloric acid, sulfuric acid, calcium chloride, magnesium chloride, magnesium sulfate, potassium chloride, sodium chloride, sodium dehydrogen phosphate, L-cystein, sodium pyruvate, sodium lactate, Bovine serum albumin (BSA) and penicillin/streptomycin were obtained from Sigma Aldrich (St. Louis, MO, USA). Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antifade reagent, Tris base, TRIZOL Reagent, RNase inhibitor, bis-(1,3-dibarbituric acid)-trimethine oxanol (DiBAC4(3)) and all the primers used in RT-PCR were from GIBCO BRL/Invitrogen (USA). Modified sperm washing medium was purchased from Irvine Scientific (Santa Ana CA). Percoll, dNTPs, ECL Western Blot Detection Reagent and Hybond-ECL nitrocellulose membranes were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Fluo-4-AM was purchased from Molecular Probes (Eugene, OR). RX X-ray film was purchased from Fuji (Japan). The SLC26A3 antibodies were from Santa Cruz (USA). The SLC26A3 and SLC26A6 antiserum were gifts from Dr. Höglund P (Haila et al., 2000;Lohi et al., 2003). Alexa-488 conjugated goat anti-rabbit IgG was from Invitrogen and the anti-rabbit IgG-HRP was from GE Life Sciences. The normal goat IgG was from Santa Cruz (USA). The normal rabbit serum was from the laboratory animal service center (LASEC), The Chinese University of Hong Kong (CUHK). GC-1 spg cell line was from ATCC (ATCC® number, CRL-2053™). Sexually mature (about 10-12 weeks old) BALB/C male mice were obtained from LASEC, CUHK. All experiments were conducted in accordance with the guidelines on the use of laboratory animals laid down by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (Ref 07/062/MIS).

III.3.2 Medium

For GC-1 spg cells, KH solution was used for normal condition. When in HCO_3^-- or Cl^--deficient conditions, the HCO_3^- or the Cl^- was substituted by equivalent gluconate. The medium used for BALB/C sperm was mT-25 (when used in membrane potential measurement, no BSA was added; Table III.1) or modified sperm washing medium).

When in HCO_3^-- or Cl^--deficient conditions, the HCO_3^- or the Cl^- was substituted by equivalent gluconate.

Table III.1 contents of different medium or solution used in the experiments

Normal KH Solution

	[stock] M	Volume (ml)	Final (mM)	g per liter	MW
NaCl	1.17	100	117	6.837	58.44
$NaHCO_3$	0.248	100	24.8	2.083	84.01
KCl	0.47	10	4.7	0.35	74.55
$MgCl_2$	0.1	12	1.2	0.244	203.3
KH_2PO_4	0.12	10	1.2	0.163	136.09
$CaCl_2.2H_2O$	0.256	10	2.56	0.376	147.02
Glucose			11.1	2	

Make up to 1L (Osm=283), Gas with 95% O_2, 5% CO_2

Chloride free solution

	[stock]	Volume (ml)	Final (mM)	g per liter	MW
Na-gluconate			117	25.52	
$NaHCO_3$	248mM	100	24.8	2.083	
K-gluconate			4.7	1.10	
$MgSO_4.7H_2O$	120mM	10	1.2	0.296	
KH_2PO_4	120mM	10	1.2	0.163	
Ca-gluconate			2.56		
Glucose			11.1	2	

Make up to 1L (Osm=286), Gas with 95% O_2, 5% CO_2 Use D-Mannitol to adjust Osm

Margo Ringer's solution

	[stock]	Volume (ml)	Final (mM)	g per liter
NaCl			130	7.597
KCl			5	0.373
HEPES			20	4.766
Glucose			10	1.802
$CaCl_2.2H_2O$	250mM	10	2.5	0.368
$MgCl_2$	100mM	10	1	0.203

MT-25 medium

Components	mM	MW	mg/100ml
NaCl	104.80	58.44	
KCl	2.68	74.56	
$MgCl_2 \cdot 6\ H_2O$	0.49		
$NaHCO_3$	25.07	84.01	210.6
$NaH_2PO_4 \cdot 2\ H_2O$	0.36		
$CaCl_2 \cdot 2\ H_2O$	1.8	147.03	(180μl/1M)
D-glucose	2.78		50
HEPES	20		2 ml (1M)
Phenol red			100μl
P/S			1ml
BSA	4mg/ml		400
Na-Pyruvate	1.0		11.0
Na-Lactate	10	112.1	0.16ml

Adjust pH to 7.5, Sterilized through a Millipore filter (0.22μM).

III.3.3Membrane potential measurement

GC-1 spg cells were passaged on 25 mm diameter cover glass in 35 mm culture dish using 2ml DMEM at low density. After one day, the cells were treated with or without DIDS or niflumate 10μM for 30 min or the SLC26A3 antibody or normal IgG for 1 hour. Then the cells were washed and 5 nM DiBAC4(3) was added for 5 min. After that, the measurement was started using a microscope (NICON ECLIPSE Ti-U) at 495nM. The cells were then changed to Cl^- deficient or HCO_3^- deficient medium (with the same concentration of dye) to see if there are any membrane potential changes. The control group was no pre-treatment with anion blocks or antibody.

For sperm, 10-week old BALB/C male mice were sacrificed and the cauda and vas deferens were taken out and chopped with scissors. 1 ml sperm washing medium was added and after 15 min, the sperm suspension was collected and adjusted to 5×10^6 cells/ml. Sperm were treated with niflumate or DIDS for 30min or SLC26A3 antibody or normal goat IgG for 1h. After that, 0.5 ml sperm suspension were added to the well containing poly-L-lysine coated 25mm cover glass for 5 min, then washed and changed

to medium with 5 mM DiBAC4(3). After 5 min of loading, the measurement was started using a microscope (NICON ECLIPSE Ti-U) at 495nM. The sperm were then changed to Cl^- deficient or HCO_3^- deficient medium (with the same concentration of dye) to see if there are any membrane potential changes.

III.3.4 Intracellular calcium measurement

GC-1 spg cells were passaged on 25 mm diameter cover glass in a 35 mm culture dish using 2ml DMEM at low density. When measured, DMEM medium was discarded, and then the cells were washed with HBSS. 2 ml of Margro's solution was added and followed by loading with 3 μM Fluo-4 and 3 μM pluronic F127 for 45min at 37°C. Meanwhile, 10μM DIDS or niflumate was added for 45min. The cells were then washed, changed to Margro's solution, and measurement was started using a microscope (NICON ECLIPSE Ti-U) at 495nM. The cells were then stimulated with 25 mM final concentration HCO_3^-.

For sperm, 10-week old BALB/C male mice were sacrificed and the cauda and the vas deferens were taken out and chopped with scissors. 1ml modified sperm washing medium was added and after 15 min, the sperm suspension was collected and adjusted to 5×10^6 cells/ml. After that, the sperm were first incubated for 1 h and then loaded with 5 μM Fluo-4 and 5 μM pluronic F127 for 30 min at 37°C. Meanwhile, 10μM DIDS or niflumate was added for 30min and the cells were washed with centrifuge at 1000 rpm for 5 min. Sperm were resuspended in modified sperm washing medium and measurement was started using a microscope (NICON ECLIPSE Ti-U) at 495nM. Sperm were then stimulated with 20 mM HCO_3^-.

III.3.5 RT-PCR

The adherent cells were digested using 0.25% Trypsin (Invitrogen, USA) for 1 min in 37°C and the reaction was stopped and centrifuged at 1000 rpm for 5min. After washing with PBS twice, the cells were lysed with TRIZOL with pipetting up and down (1ml for 5×10^6-10×10^6 cells) until no cell pellet could be seen. The samples were incubated at room temperature for another 5 min and 0.2 ml chloroform per 1ml TRIZOL was added. The tubes were then shaken vigorously by hand for 15 seconds. The samples were then centrifuged at 12000 g for 15 min at 4°C and the upper aqueous layer was transferred to 1.5 ml eppendorf tubes. To precipitate the RNA, 0.5 ml isopropanol

per 1ml TRIZOL was added and incubated in –20°C for 10 min. After centrifugation at 12000 g for 10 min at 4°C, the RNA pellets were washed with 1 ml 75% ethanol. The RNA was air-dried and dissolved in DEPC- treated water at 58°C for 10 min. The concentration was measured using a Nanovue spectrophotometer (GE, USA). All the tubes used were treated with DEPC.

5-10 μg total RNA was added in a 0.5 ml tube with 1μL Oligo dT (0.5μg/μL), and the volume was adjusted to 10μL with DEPC-treated water. The tubes were put into a PCR cycler at 70°C for 10 min and then ice-cooled for 3 min. 7μL RT mix (2μL 10× PCR buffer, 2μL 25 mM $MgCl_2$, 2μL 0.1M DTT, 1μL 10mM dNTP) was added to each tube, mixed and incubated for 5 min at 42°C. After that, 1μL MMLV enzyme 200U/μL was added and programmed for 50 min at 42°C and for 15min at70°C. The cDNA was stored at –20°C for further use.

The 0.2 ml PCR tube containing template DNA (1μL), 2.5 μL 10× PCR buffer, 25 pmol of each of the primers, 0.5μL 10 mM dNTP, 0.65 unit Taq DNA Polymerase(Promega, USA) and sufficient DEPC-treated water to adjust the volume of 25μL were put into PTC-200 peltier thermal cycler (MJ research). The reaction program was as follows: 94°C for 5 min, 30-34 cycles at 94°C for 30 seconds, 55-58°C for 30 seconds (the annealing temperature varied for different primers), 72°C for 30 seconds each and at last 72°C for 10 min. The primer sequences are shown in Table III.2.

Table III.2. The primers used in RT-PCR

NAME	ACCESSION No.	SEQUENCE	EXPECTED SIZE
mSLC26A3			
Forward :	NM_000111	5' – TGG ATC ATG ACC TTC ATC – 3'	534 bp
Reverse:		5' – GAG GCT GTG GAG GCT GAT – 3'	
mSLC26A3			
Forward :	NM_000111	5' – CTCTGCTGGTCAACATCCCTC – 3'	305bp
Reverse:		5' – TATCACCACAAAGCCGACCTG – 3'	
mSLC26A6			
Forward :	NM_134420	5' – TTC GGC TTC GTT GTC ACC – 3'	453bp
Reverse:		5' – CAC AGC AAT GGC AAA GGC – 3'	
mSLC26A6			
Forward :	NM_134420	5' –GAGGCTTCTTCCAGTGCTTCC- 3'	307bp
Reverse:		5' –CCAATGTCCAGGTTCAGCAGG- 3'	
mGAPDH			
Forward :	NM_008084	5' – GAC CAC AGT CCA TGC CAT CAC TGC – 3'	340bp
Reverse:		5' – GCT GTT GAA GTC GCA GGA GAC AAC – 3'	

III.3.6 Western blot

The flask was washed with PBS and the cells were scraped off from the flask, resuspended in 1 ml PBS and then transferred to the eppendorf tube and washed twice with centrifuge at 13, 000 rpm, 4°C for 5min using PBS. The cell pellet was resuspended in membrane protein (MP) extraction buffer (10mM EDTA in PBS), of a volume nine times that of the pellet with 1:200 protease inhibitor mixture and 1:100 0.1M PMSF. The homogenate was centrifuged at 1,000 x g for 10 min, 4°C and supernatant was transferred to a new centrifuge tube. The supernatant was centrifuged at 25,000 x g for 60 min, 4°C. The supernatant was removed and the pellet was re-suspended in MP buffer. The protein concentration was measured using Bradford protein assay system (BIO-RAD, USA). In brief, 1μL protein samples or serial BSA standards were added to 200 μL working solution and incubated in room temperature for 5 min. Color development was measured at OD595.

The proteins were diluted with 6 × buffers, denatured at 95°C for 5 min, then separated on 8% polyacrylamide gels. The CBS SG-125 (CBS Scientific, USA) apparatus was used and was run with a constant current of 25 mA per gel for 1h. The proteins were then transferred from the polyacrylamide gels to Hybond-ECL nitrocellulose membranes (Amersham, USA) using a Trans-Blot® SD semi-Dry Electrophoretic Transfer Cell (BIO-RAD, USA) with a constant current of 75mA per gel for 1hr. The membrane was blocked with 4% milk in TBST for 1h and then incubated with primary antibody (Table III.3) in 2% milk in TBST overnight at 4°C. After washing with TBST 4 times for 5 min each time, the membrane was incubated with secondary antibody for 1 hour at room temperature (Amersham Biosciences, USA). The membrane was washed with TBST 6 times for 5 min each time and the protein was detected using ECL Western Blot detection reagent (Amersham Biosciences, USA).

Table III.3. The antibody used in Western blot

NAME	SIZE(kDa)	TYPE	HOST	DILUTIONS	COMPANY	CROSS REACTIVIATY
SLC26A3	80	Anti-Serum	Rabbit	1:350	Gift from Prof.	Human,Murine
SLC26A6	85	Anti-Serum	Rabbit	1:250	Höglund P	Human, Murine
β-tubulin	55	Polyclonal	Rabbit	1:2000	Santa Cruz	Human, Murine

III.3.7 Immunofluorescent staining

For indirect immunofluorescence studies of SLC26A6 localization, GC-1 spg cells were passaged on 10 mm diameter cover glasses. After one day, the cells were washed with PBS three times for 5 min each time, and then fixed with 4% PFA for 10 min and washed with PBS three times for 5 min each time. The cells were then permeablized with 0.1% Triton X-100 for 30 min, washed, blocked with 5% normal goat serum for 1h and then incubated with SLC26A6 antibody (1: 100) at 4°C overnight. After further washing in PBS three times for 5min each, GC-1 spg cells were incubated with secondary antibody (Alexa-488 conjugated goat anti rabbit IgG, Invitrogen, A11070, 1:500) in a dark room for 1 hour at room temperature. Unbound antibody was removed by washing with PBS three times. The cells were counterstained with DAPI for 30 min and then washed with PBS three times and mounted on slides with anti-fade reagents. The slides were then stored in a dark box before visualization.

For sperm, details see II.3.9. primary antibody: SLC26A3 (1: 50)or SLC26A6 antibody (1: 50); secondary antibody: Alexa-488 conjugated goat anti rabbit IgG, Invitrogen, A11070, 1:500.

III.3.8 Statistical analysis

Results are means ± S.E.M. For statistical analyses, comparisons were made with student t test or one-way ANOVA followed with Tukey's or Dunnett's post-hoc tests. Values of $P<0.05$ were regarded as statistically significant.

III.4 RESULTS

III.4.1 The effect of anion exchanger inhibitors as well as SLC26A3 antibody on GC-1 spg cells

III.4.1.1Membrane potential

When bathing solution was changed from the normal solution to a HCO_3^--deficient solution, the intensity of the DiBAC4(3) in GC-1 spg cells was increased, indicating that the cells were depolarized. This depolarization could be blocked by pretreatment of cells with10 μM DIDS, but not 10 μM niflumate. Also, when the cells were first put into normal solution and then changed into Cl^--deficient solution, they were depolarized. However, this depolarization could not be blocked by DIDS or niflumate (Figure III.3).

When the cells were pretreated with SLC26A3 antibody (1:250) for 1 hour, the depolarization induced by HCO_3^-- or Cl^--deficient solution could not be inhibited compared to the normal goat IgG treated groups (Figure III.5).

III.4.1.2 Intracellular calcium ($[Ca^{2+}]i$) measurement

When GC-1 spg cells were challenged with 25 mM HCO_3^-, the intensity was increased, indicating the intracellular calcium was increased. The increased intracellular calcium level could not be inhibited by DIDS or niflumate (Figure III.7).

III.4.2 The effect of anion exchanger inhibitors as well as SLC26A3 antibody on sperm.

III.4.2.1Membrane potential

When bathing solution was changed from complete medium to HCO_3^--deficient medium, the intensity of the DiBAC4(3) in sperm was also increased, indicating depolarization of the sperm. This depolarization could be blocked by pretreatment with 10 μM DIDS, while 10 μM niflumate could not induce the blocking effect. The depolarization resulting from the lack of extracellular Cl^- could also be blocked by DIDS (Figure III.4).

When sperm were pretreated with SLC26A3 antibody (1:250) for 2 hour, the depolarization in Cl^- deficient but not HCO_3^- deficient condition could be blocked as compared to the normal goat IgG treated groups (Figure III.6).

III.4.2.2 Intracellular calcium ($[Ca^{2+}]i$) level

Sperm were challenged with 20 mM (final 25 mM) HCO_3^-, when $[Ca^{2+}]i$ level was measured. The intensity was increased indicating the increase in intracellular calcium level. The increased intensity could be inhibited by niflumate but not DIDS (Figure III.8).

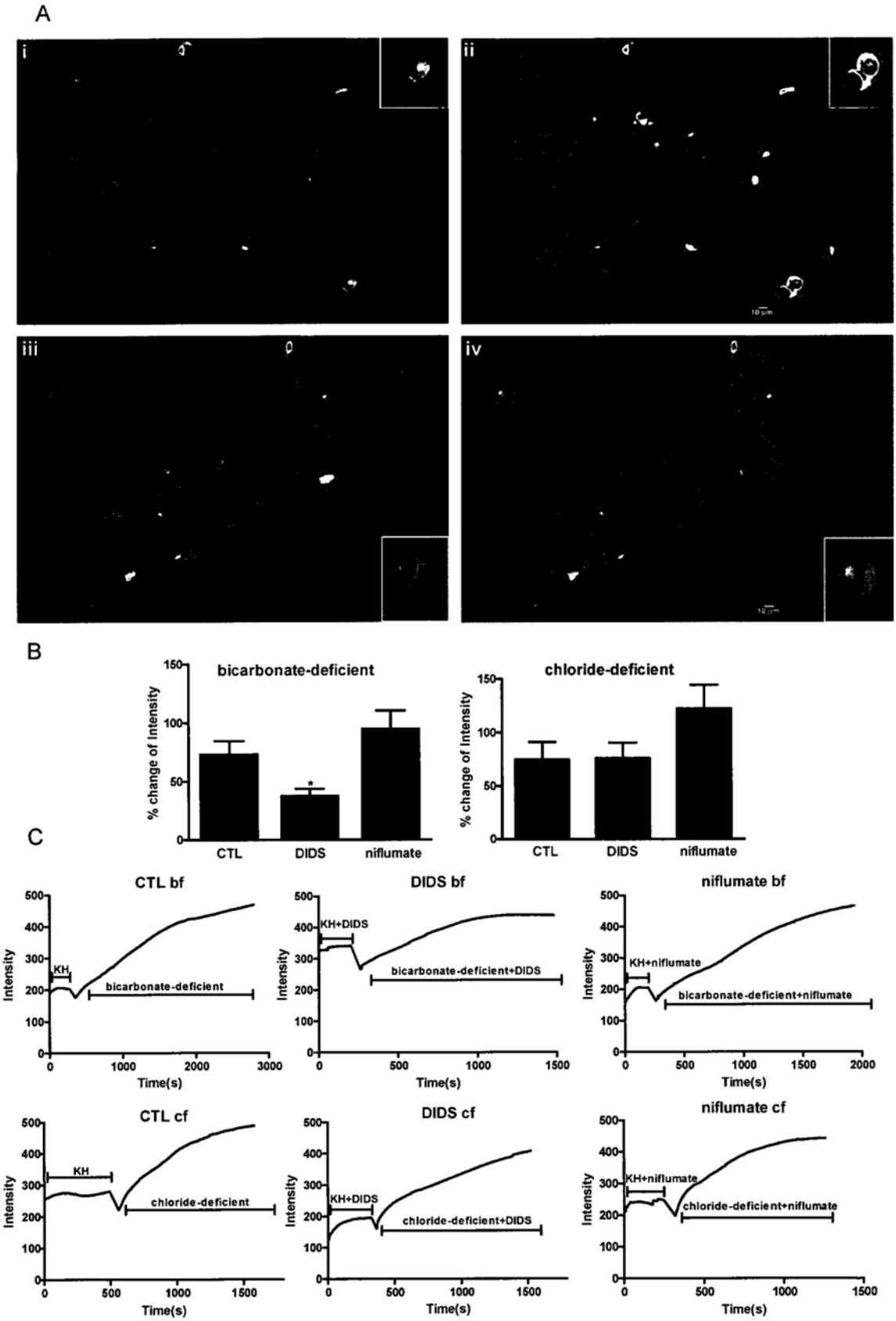

A
i
ii
iii
iv
B
bicarbonate-deficient
chloride-deficient
% change of Intensity
CTL
DIDS
niflumate
C
CTL bf
DIDS bf
niflumate bf
KH
bicarbonate-deficient
KH+DIDS
bicarbonate-deficient+DIDS
KH+niflumate
bicarbonate-deficient+niflumate
CTL cf
DIDS cf
niflumate cf
chloride-deficient
chloride-deficient+DIDS
chloride-deficient+niflumate
Intensity
Time(s)

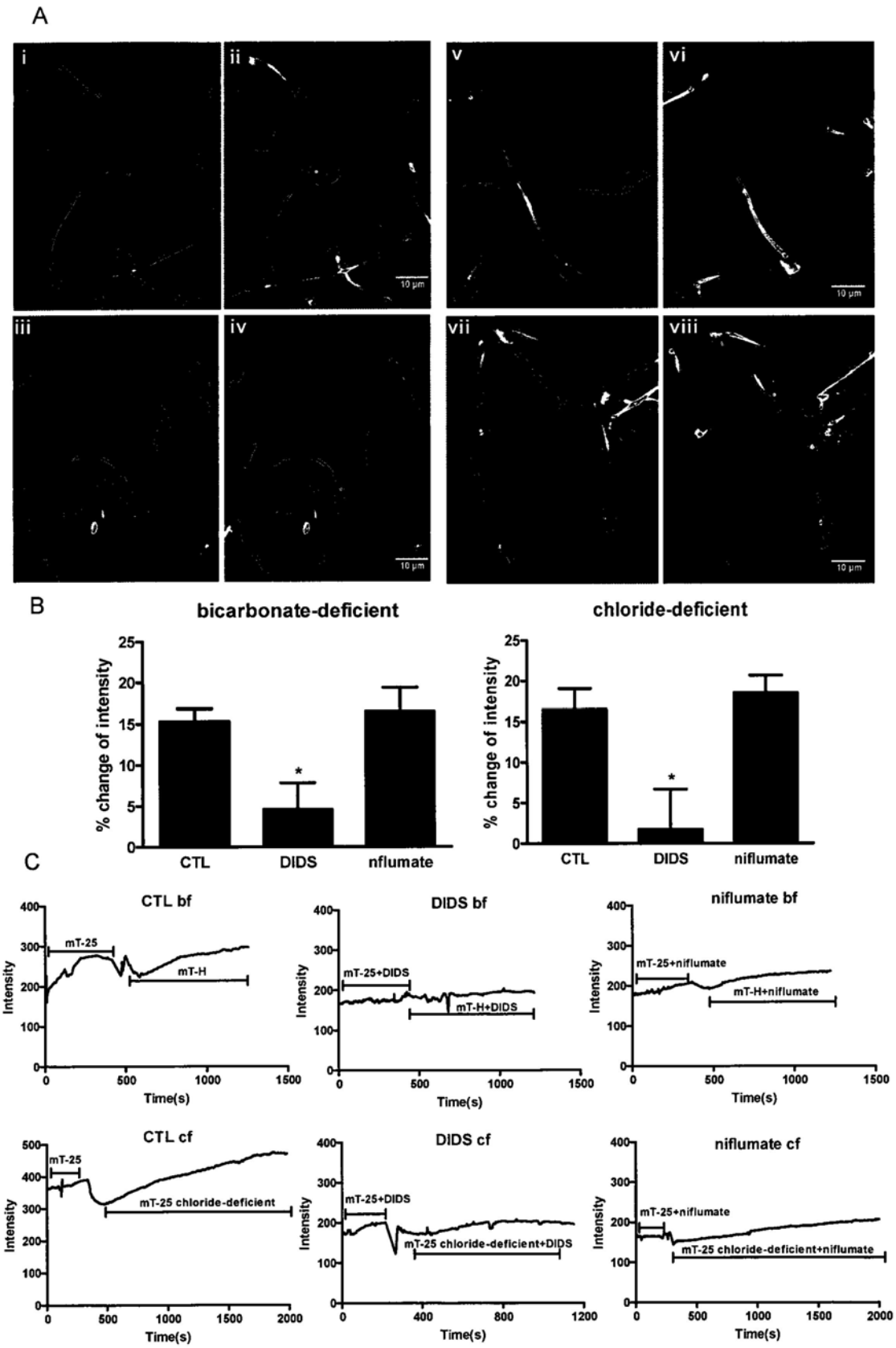
Figure III.3. The effects of anion blockers on membrane potential changes of GC-1spg cells

GC-1 spg cells were pretreated with 10 μM DIDS or niflumate for 30 min, followed by loading with 5 nM DiBAC4(3) in the normal (KH) solution, which was finally changed into HCO_3^-- or Cl^--, deficient solution. The cells with no pretreatment were as controls.

A. Photos of membrane potential changes of control and DIDS pretreated in HCO_3^--deficient conditions i. Cells in normal conditions; ii. Cells in HCO_3^--deficient conditions; iii. Cells pretreated with DIDS in normal conditions; iv. Cells pretreated with DIDS in HCO_3^--deficient conditions.

B. Diagram of intensity changes of GC-1 spg cells in Cl^-- or HCO_3^-- deficient conditions CTL:control; DIDS: DIDS pretreated; niflumate: niflumate pretreated. Results are means± SEM (n=7-10). *, compared to the DIDS pretreatment, $p<0.05$.

C. Intensity curve of GC-1 spg cells when pretreated with anion blockers. CTL bf: control in HCO_3^--deficient solution; DIDS bf: DIDS pretreatment in HCO_3^--deficient solution; niflumate bf: niflumate pretreatment in HCO_3^--deficient solution; CTL cf: control in Cl^--deficient solution; DIDS cf: DIDS pretreatment in Cl^--deficient solution; niflumate cf: niflumate pretreatment in Cl^--deficient solution.


A
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10 μm
iii
iv
vii
viii
B
bicarbonate-deficient
chloride-deficient
% change of intensity
CTL
DIDS
nflumate
niflumate
C
CTL bf
DIDS bf
niflumate bf
mT-25
mT-H
mT-25+DIDS
mT-H+DIDS
mT-25+niflumate
mT-H+niflumate
Intensity
Time(s)
CTL cf
DIDS cf
niflumate cf
mT-25 chloride-deficient
mT-25 chloride-deficient+DIDS
mT-25 chloride-deficient+niflumate

Figure III.4. The effects of anion blockers on membrane potential changes of sperm

Sperm were pretreated with 10 μM DIDS or niflumate for 30 min and then loaded with 5 mM DiBAC4(3) in the normal (mT-25) medium, which was then changed into HCO_3^-- (mT-H) or Cl^--deficient medium. The cells with no pretreatment were as controls.

A. Photos of membrane potential changes of sperm in HCO_3^-- and Cl^--deficient conditions. i. sperm in mT-25; ii. Sperm in HCO_3^--deficient conditions; iii. Sperm pretreated with DIDS in mT-25; iv. Sperm preteated with DIDS in HCO_3^--deficient conditions; v. sperm in mT-25; vi. Sperm in Cl^--deficient conditions; vii. Sperm pretreated with DIDS in mT-25; viii. Sperm preteated with DIDS in Cl^--deficient conditions.

B. The diagram of intensity changes of sperm in Cl^-- or HCO_3^-- deficient conditions. *, compared to the HCO_3^-- or Cl^--deficient CTL, $p<0.05$. CTL: control; DIDS: sperm pretreated with DIDS; niflumate: sperm pretreated with niflumate. Results are means± SEM (n=3-4).

C The intensity curve of sperm when pretreated with anion blockers.CTL bf: control in HCO_3^-- deficient conditions; DIDS bf: sperm pretreated with DIDS in HCO_3^--deficient conditions; niflumate bf: sperm pretreated with niflumate in HCO_3^--deficient conditions; ncf: control in Cl^--deficient conditions; DIDS cf: sperm pretreated with DIDS in Cl^--deficient conditions; niflumate cf: sperm pretreated with niflumate in Cl^--deficient conditions.

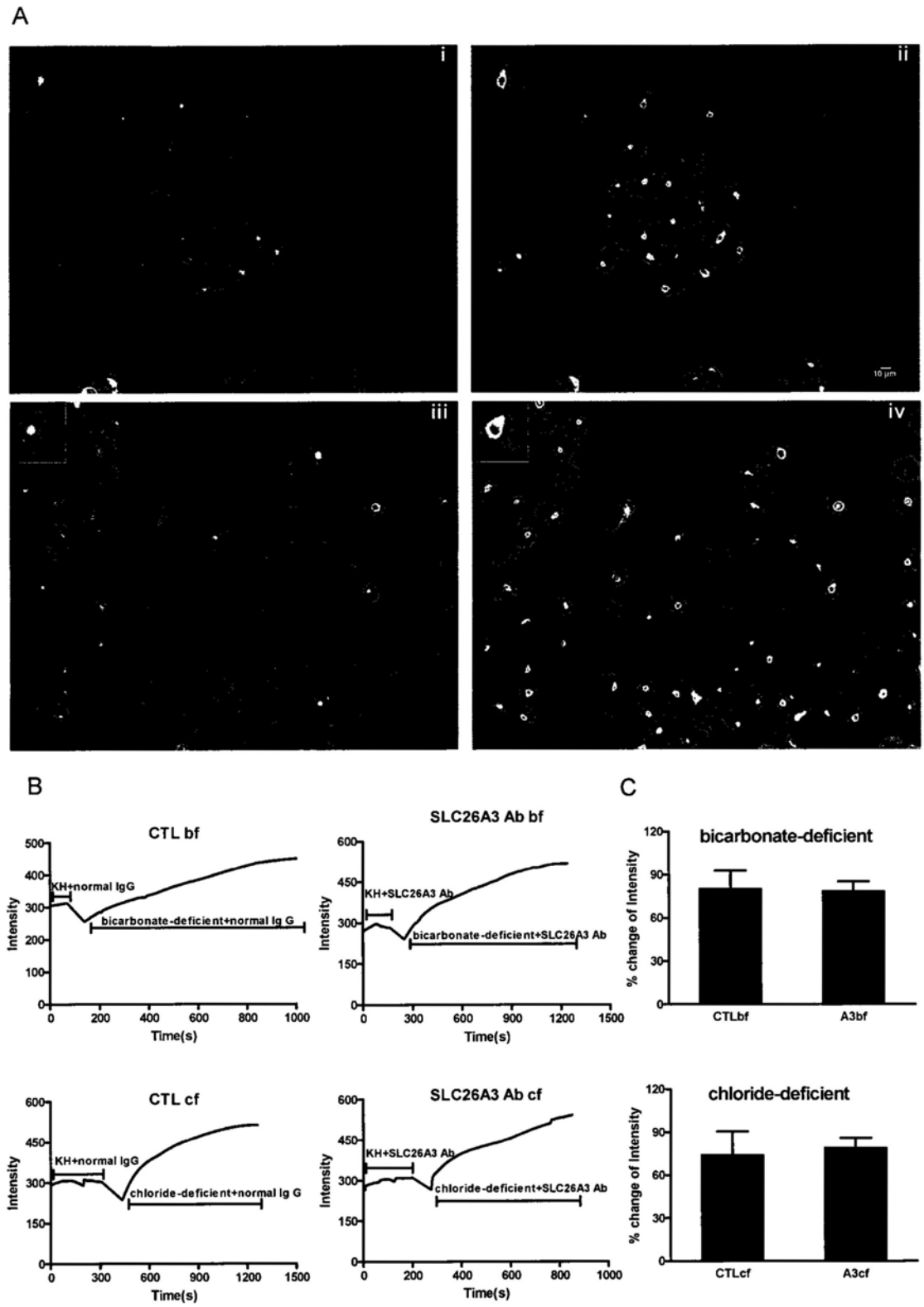
A
i
ii
iii
iv
10 μm
B
CTL bf
KH+normal IgG
bicarbonate-deficient+normal Ig G
Intensity
Time(s)
SLC26A3 Ab bf
KH+SLC26A3 Ab
bicarbonate-deficient+SLC26A3 Ab
Intensity
Time(s)
C
bicarbonate-deficient
% change of Intensity
CTLbf
A3bf
CTL cf
KH+normal IgG
chloride-deficient+normal Ig G
Intensity
Time(s)
SLC26A3 Ab cf
KH+SLC26A3 Ab
chloride-deficient+SLC26A3 Ab
Intensity
Time(s)
chloride-deficient
% change of Intensity
CTLcf
A3cf

Figure III.5. The effects of SLC26A3 antibody on membrane potential changes of GC-1 spg cells

The cells were pretreated with SLC26A3 antibody (1:250) for 1 hour, followed by loading with 5 nM DiBAC4(3) in the normal (KH) solution which was finally changed into HCO_3^-- or Cl^--, deficient solution. The cells pretreated with normal goat IgG were used as controls.

A. Photos of membrane potential changes of control and SLC26A3 antibody pretreated in HCO_3^- deficient conditions i. Cells in the normal conditions; ii. Cells in HCO_3^--deficient conditions; iii. Cells pretreated with SLC26A3 antibody in normal conditions; iv. Cells pretreated with SLC26A3 antibody in HCO_3^--deficient conditions.

B. Intensity curve of GC-1 spg cells when pretreated with SLC26A3 antibody. CTL bf: cells pretreated with normal goat IgG used as control in HCO_3^--deficient condition; SLC26A3 bf: cells pretreated with SLC26A3 antibody in HCO_3^--deficient conditions; CTL cf: cells pretreated with normal goat IgG used as control in Cl^-- deficient conditions; SLC26A3 cf: cells pretreated with SLC26A3 antibody in Cl^--deficient conditions.

C.Diagram of intensity changes of GC-1 spg cells in Cl^-- or HCO_3^-- deficient conditions. CTL bf: cells pretreated with normal goat IgG as control in HCO_3^--deficient condition; A3 bf: cells pretreated with SLC26A3 antibody in HCO_3^--deficient condition; CTL cf: cells pretreated with normal goat IgG in Cl^-- deficient condition; A3 cf: cells pretreated with SLC26A3 antibody in Cl^--deficient condition;. Results are means± SEM (n=5-6). There is no statistical significance between CTL bf and A3 bf or CTL cf and A3 cf.

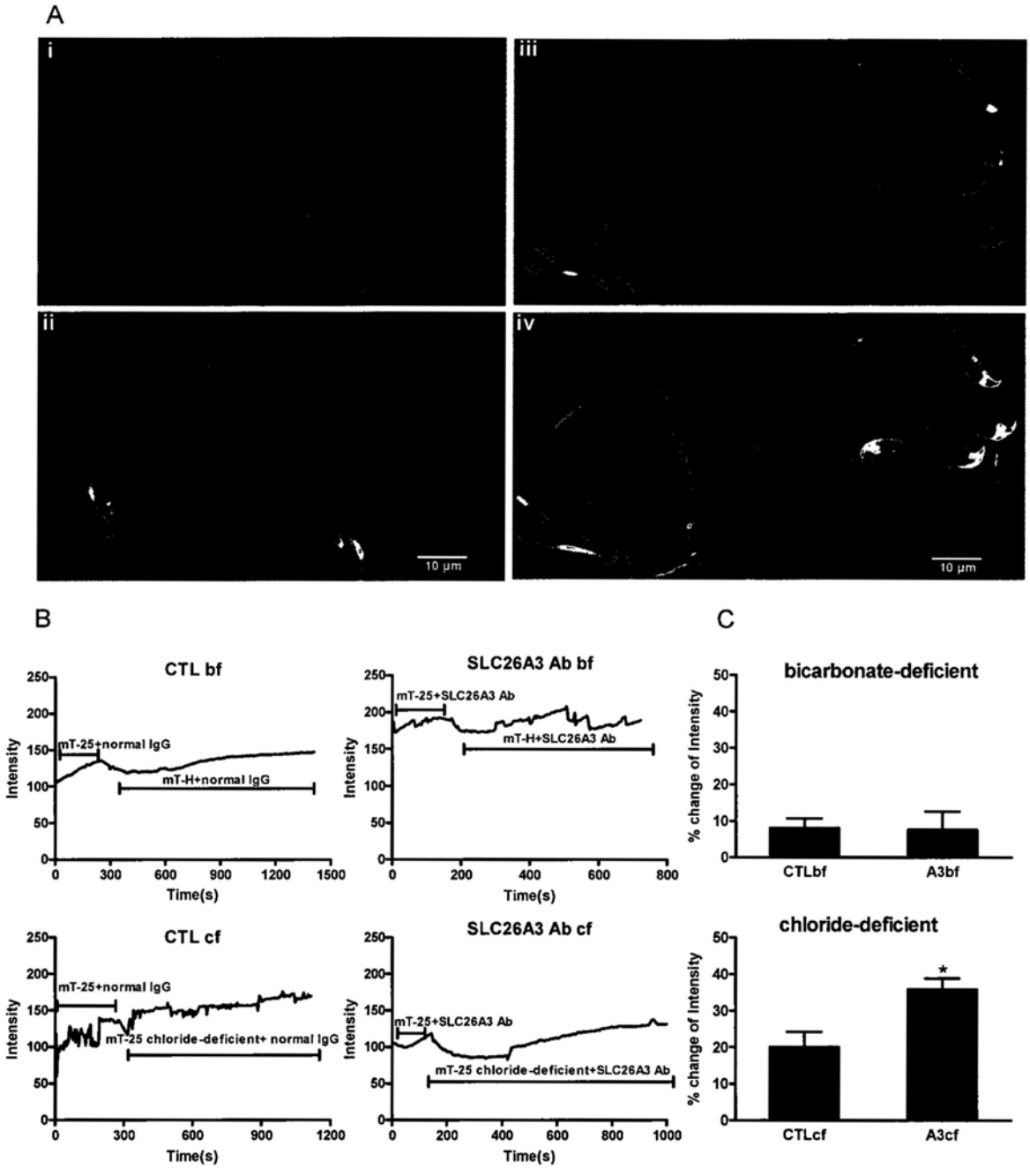
A
i
iii
ii
iv
10 μm
10 μm
B
CTL bf
Intensity
mT-25+normal IgG
mT-H+normal IgG
Time(s)
SLC26A3 Ab bf
mT-25+SLC26A3 Ab
mT-H+SLC26A3 Ab
Time(s)
CTL cf
mT-25+normal IgG
mT-25 chloride-deficient+ normal IgG
Time(s)
SLC26A3 Ab cf
mT-25+SLC26A3 Ab
mT-25 chloride-deficient+SLC26A3 Ab
Time(s)
C
bicarbonate-deficient
% change of Intensity
CTLbf
A3bf
chloride-deficient
% change of Intensity
*
CTLcf
A3cf

Figure III.6. The effects of SLC26A3 antibody on membrane potential changes of sperm

Sperm were pretreated with SLC26A3 antibody (1:250) for 2h and then loaded with 5 mM DiBAC4(3) in normal (mT-25) medium, which was then changed into HCO_3^--(mT-H) or Cl^--, deficient medium. The cells treated with normal goat IgG were used as controls.

A. Photos of membrane potential changes of sperm in Cl^--deficient conditions. i. Sperm pretreated with normal goat IgG in mT-25; ii. Sperm pretreated with normal goat IgG in Cl^--deficient medium; iii. Sperm pretreated with SLC26A3 antibody in mT-25; iv. Sperm pretreated with SLC26A3 antibody in Cl^- deficient medium.
B. Intensity curve of sperm when pretreated with SLC26A3 antibody. The cells were pretreated with SLC26A3 antibody (1:250) for 1 hour and then loaded with 5 mM DiBAC4(3) in the normal (mT-25) solution, which was then changed into HCO_3^-- or Cl^--, deficient media. CTL bf: sperm pretreated with normal goat IgG as control in HCO_3^--deficient conditions; SLC26A3 bf: sperm pretreated with SLC26A3 antibody in HCO_3^--deficient conditions; CTL cf: sperm pretreated with normal goat IgG as the control in Cl^--deficient conditions; SLC26A3 cf: sperm pretreated with SLC26A3 antibody in Cl^--deficient conditions.
C. Diagram of intensity changes of sperm in Cl^-- or HCO_3^-- deficient conditions. CTL bf: sperm pretreated with normal goat IgG as the control in HCO_3^--deficient conditions; A3 bf: sperm pretreated with SLC26A3 antibody in HCO_3^--deficient conditions; CTL cf: sperm pretreated with normal goat IgG as the control in Cl^--deficient conditions; A3 cf: sperm pretreated with SLC26A3 antibody in chloride-deficient conditions. Results are means± SEM (n=5-7). *, compared to CTL cf, $p<0.05$.

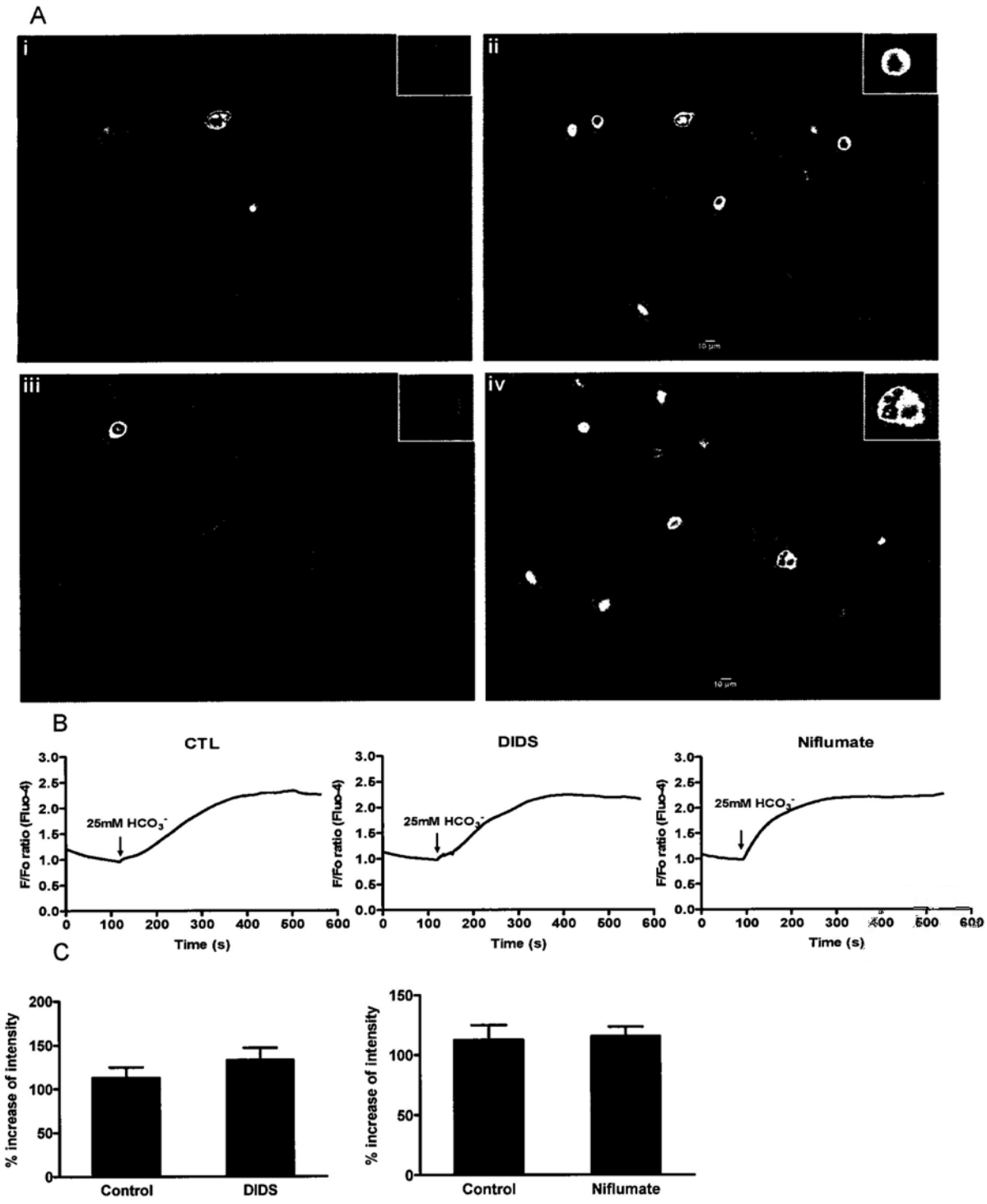
A
i
ii
iii
iv
B
CTL
DIDS
Niflumate
F/Fo ratio (Fluo-4)
25mM HCO3-
Time (s)
C
% increase of intensity
Control
DIDS
Niflumate

Figure III.7 The effect of anion blockers on intracellular calcium level of GC-1 spg cells

The cells were pretreated with 10μM DIDS or niflumate for 45min while loading with 3μM fluo-4 and 3μM F127. The cells were then centrifuged and changed into a HCO_3^- deficient solution and induced with 25 mM HCO_3^-.

A. Photos of calcium intensity when induced with 25 mM HCO_3^-. GC-1 spg cells in HCO_3^--deficient conditions, before(i) and after stimulation(ii) by 25 mM HCO_3^-; GC-1 spg cells pretreated with niflumate in HCO_3^--deficient conditions, before(iii) and after stimulation(iv) by 25 mM HCO_3^- .
B. Intensity curve of GC-1 spg cells when treated with anion blockers. Control: cells with no pretreatment; DIDS: cells pretreated with 10μM DIDS; niflumate: cells pretreated with 10μM niflumate.
C. Diagram of intensity changes of GC-1 spg cells pretreated with anion blockers. Results are means± SEM (n=4-6). There is no statistical significance between control and DIDS or niflumate group.

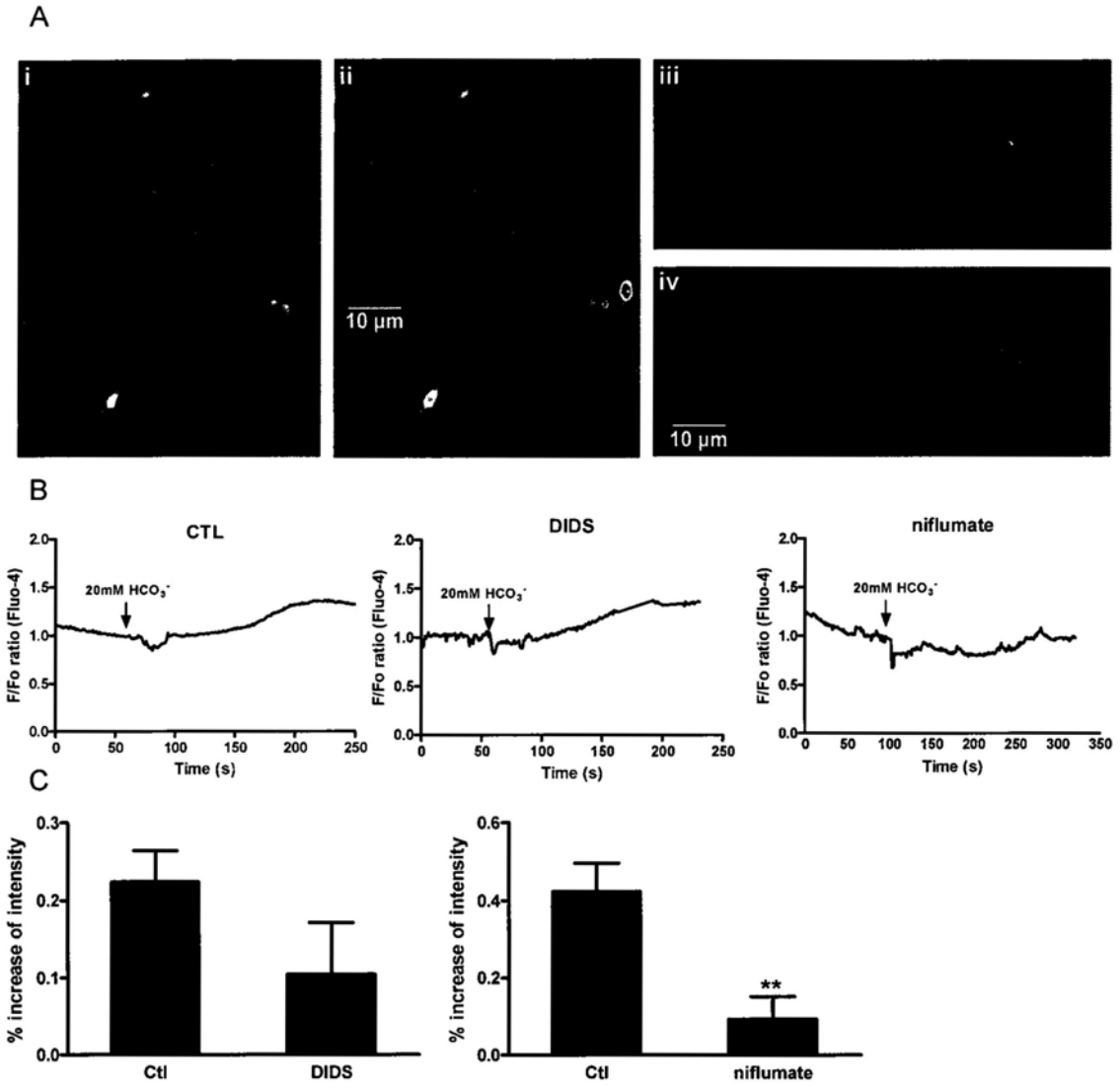

Figure III.8. The effect of anion blockers of intracellular calcium level on sperm

Sperm were pretreated with 10μM DIDS or niflumate for 30min while loading with 3μM fluo-4 and 3μM F127, and then centrifuged, resuspended in sperm washing medium, after that 20 mM HCO_3^- was added and the fluorescent intensity was measured.

A. Photos of calcium intensity when induced with 20 mM HCO_3^-. Sperm in low HCO_3^- condition before (i) and after (ii) induced with 20 mM HCO_3^-; sperm pretreated with 10μM niflumate in low HCO_3^- conditions before (iii) and after (iv) induced with 20mM HCO_3^-.

B. The intensity curve of sperm treated with anion blockers. CTL: sperm with no pretreatment; DIDS: sperm pretreated with 10μM DIDS; niflumate: sperm pretreated with 10μM niflumate.

C. The diagram of intensity changes of sperm pretreated with anion blockers. **, compare to the Ctl, $p<0.01$; Ctl: control, DIDS: sperm pretreated with 10μM DIDS; niflumate: sperm pretreated with 10μM niflumate. Results are means± SEM (n=6-9).

III.5 DISCUSSION

Membrane potential (Em) arises from the action of ion channels, ion pumps, and ion transporters embedded in the membrane which maintain different ion concentrations inside and outside the cell. It governs the rates and direction of ion-flow through channels and exchangers. Its fluctuations modulate intracellular pH (pHi), intracellular calcium ($[Ca^{2+}]i$) and other second messengers that influence sperm maturation, sperm motility, sperm capacitation and sperm acrosome reaction (AR) (Darszon et al., 2006).

In the present study, when the bathing solution was switched from normal condition to HCO_3^--deficient condition, the HCO_3^- concentration gradient favors HCO_3^- transport out of the GC-1 spg cell membrane and Cl^- into the cell through SLC26A6. As SLC26A6 mediates $1Cl^-/2HCO_3^-$ exchanger (Shcheynikov et al., 2006), the net anion efflux led to depolarization. This depolarization could be inhibited by SLC26A6 sensitive anion exchanger blocker DIDS, consistent with the presence of SLC26A6 in GC-1 spg cells. Unpredictably, when sperm were at the same conditions, they showed the same properties as GC-1 cells. This result indicates that at least under the experimental HCO_3^--deficient condition, SLC26A6, but not SLC26A3 predominantly contribute to the Em in sperm. When GC-1 spg cells were first in normal KH solution and later switched to Cl^--deficient solution, to our surprise, cells were depolarized rather than hyperpolarized and this depolarization could not be inhibited by DIDS. This may indicate that there were other ion channels/transporters which were not sensitive to DIDS and niflumate rather than SLC26A6 involved in the Em change in Cl^--deficient condition. Surprisingly, the depolarization induced by Cl^--deficient condition in sperm can still be inhibited by DIDS but not niflumate. As we state above, since SLC26A6 does not significantly contribute to Em in Cl^--deficient condition, combine with the inhibition effect of DIDS but not niflumate, the major cause of depolarization maybe due to other ion channel/transporters rather than SLC26A6 and SLC26A3. The inhibition may partly due to the wide spectrum of anion exchangers/transporters that DIDS inhibits, which may also contribute to Em.

There was no effect of SLC26A3 antibody on Em changes of GC-1 spg cells in either Cl^-- or HCO_3^--deficient condition which further demonstrated the specificity of the SLC26A3 antibody. This antibody had no effect on HCO_3^--deficient induced

depolarization in sperm which was consistent with the notion that SLC26A3 does not predominantly contribute to Em, inhibition of SLC26A3 had no significant effect in HCO_3^--deficient condition. When pretreated with SLC26A3 antibody, we have observed that the antibody treatment increased rather decreased the depolarization in Cl^-- deficient conditions. The possible explanation is that although SLC26A3 did not contribute significantly to Em, the inhibition of SLC26A3 might also lead to some other ion channels/transporters to open, resulting in depolarization of sperm. The depolarization induced by Cl^--, HCO_3^--deficient condition or inhibition of SLC26A3 antibody were also consistent with the former results that under those conditions sperm can not capacitated since capacitation needs the hyperpolarization of Em.

sAC activation depends on HCO_3^- and Ca^{2+}. The Ca^{2+} channel activity has been found to increase after conditioning of sperm with HCO_3^- (Wennemuth et al., 2000). The elevation of intracellular calcium caused by HCO_3^- stimulation of GC-1 spg cells could not be inhibited by DIDS and niflumate; while this elevation in mature sperm, which expressed SLC26A3, could be inhibited by niflumate. This result shows that SLC26A3 rather than SLC26A6 is involved in the HCO_3^- stimulated sAC-cAMP-PKA pathway, which is consistent with our previous conclusions that SLC26A3 is important in sperm capacitation.

Taken together, these results indicate that SLC26A3 in sperm plays a role in HCO_3^--induced $[Ca^{2+}]i$ but does not a dominant role in Em.

Chapter IV

Possible role of SLC26A3 in spermatogenesis

IV.1 SUMMARY

Although we have demonstrated the involvement of SLC26A3 in transporting HCO_3^- important for sperm capacitation, the involvement of SLC26A3 in spermatogenesis and the other post-testicular maturation processes are still unknown. It has been shown that SLC26A3 is also expressed in human and mouse testes. This part of study was designed to investigate the role of SLC26A3 in sperm spermatogenesis and the other post-testicular maturation processes *in vivo*. The SLC26A3 antibody was injected into the BALB/C mice seminiferous tubules using micropipette. The animals were sacrificed three days after injection; computer-assisted sperm analysis (CASA) and daily sperm production (DSP) were used to evaluate sperm motility and spermatogenesis. The results showed that sperm velocity average path (VAP), velocity straight line (VSL), velocity curvilinear (VCL), motile percentage (MOT), progressive percentage (PROG) and rapid percentage (RAP) were increased while there was no significant difference between DSP. In vitro studies to evaluate the function of SLC26A3 on sperm motility were also performed. Sperm from cauda epididymis and vas deferens were suspended and incubated at 37°C for 2h with 1:250 SLC26A3 antiserum or antibody, respectively; normal rabbit serum or normal goat IgG were used as controls. At 0, 30, 60, 90,120 min after the start of experiments, subsamples were taken out for observation of motility under phase contrast microscopy. There was no significant difference between antibody-treated group and control group at all.

Taken together, these results indicate that SLC26A3 in sperm does not seem to play a significant role in spermatogenesis, epididymal maturation and sperm motility; but rather, as demonstrated in the previous study, it plays a significant role in sperm capacitation.

IV.2 INTRODUCTION

Our previous work (chapter II) has demonstrated the involvement of SLC26A3 in transporting HCO_3^- important for sperm capacitation (Chen et al., 2009). However, the role of SLC26A3 in other sperm functions is unknown. Evidence has shown that elongating human spermatids (of stages III-VI in human testis) express SLC26A3 (Hihnala et al., 2006). SLC26A3 is also expressed in mice spermatoyctes, spermatids and mature sperm (Figure III .1 & Figure IV. 1). Therefore, SLC26A3 may also play a role in spermatogenesis.

Congenital chloride–losing diarrhea (CLD), which is due to SLC26A3 mutations, has been shown to accompanied with male subfertility but normal spermatogenesis (Hoglund et al., 2006)in homozygous V317del mutation. Remarkably, there are 30 mutations of SLC26A3 have been shown to be associated with CLD (Makela et al., 2002). Therefore it is possible that the other CLD related mutations or heterozygous mutations of *SLC26A3* may affect male fertility including spermatogenesis.

After spermatogenesis in the testis, spermatozoa also require post-testicular maturation in male and female reproductive tract, which need to undergo a series of events before reaching the oocyte (Yanagimachi, 1994a). It is widely accepted that HCO_3^- plays an important role in these processes, including sperm maturation in epididymis, sperm motility, sperm capacitation, sperm hyperactivated motility. When sperm transit through the duct in the epididymis, low HCO_3^- in the duct lumen is required to avoid premature capacitation that might lead to impaired fertility(Litvin et al., 2003;Gadella and Van Gestel, 2004). Therefore, abnormal transport of HCO_3^- in the epididymis may lead to impaired fertility.

The most important role of HCO_3^- is the stimulation of sAC and its downstream signaling pathways. The HCO_3^- dependent sAC/cAMP/PKA is one of the central regulation mechanism for sperm motility (Okamura et al., 1985;Yanagimachi, 1994a;Wennemuth et al., 2003). While the role of HCO_3^- has been demonstrated in sperm motility, the transport of HCO_3^- remains poorly understood.

However, whether SLC26A3 plays a role in the other processes in sperm post-testicular maturation as well as in capacitation remains unclear as HCO_3^- is also

involved in these processes and the transport of HCO_3^- is far from understood. Male CLD patients accompanied with poor sperm motility (Hoglund et al., 2006) which indicates the possibility that SLC26A3 may also play a role in sperm motility.

Therefore, this part of study was designed to study the role of SLC26A3 in spermatogenesis and the other post-testicular maturation processes apart from capacitation using the *in vivo* seminiferous tubules injection method. Daily sperm production (DSP), which is a common parameter for evaluation of spermatogenesis (Ashby et al., 2003), was evaluated. The motility of sperm was also analyzed using CASA.

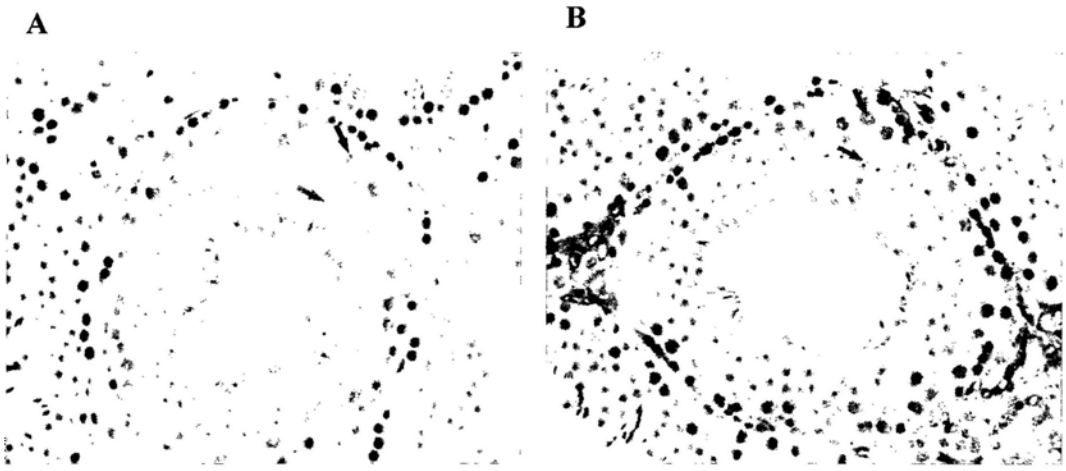

Figure IV.1 The DAB staining of SLC26A3 expression in ICR mice testes.

Formalin-fixed normal ICR mice testis were embedded in paraffin, cut into 5-μm sections, and mounted onto slides. Paraffin sections were dewaxed in xylene and rehydrated in descending concentrations of alcohol, then immerged into citrate buffer (pH 6.0) for 20 min at 98~99°C followed by cooling down for 20 min at room temperature. The SLC26A3 was detected using UltraVision ONE Detection System HRP Polymer & DAB Plus Chromogen (Thermo Fisher Scientific Inc.) was used for staining according to the manufacturer's instructions. Mayer's hematoxylin was used for counterstaining. The results were observed under microscope(Zeiss, Germany). nonspecific background staining was blocked by using normal goat serum (Santa Cruz) for 30 min, followed by overnight incubation at 4°C with SLC26A3 antibody (1:200). As a control, the primary antibody was omitted. A.The negtive control; B. The DAB staining of SLC26A3. The SLC26A3 is expressed on the membrane of the spermatocytes (black arrow) and spermatid (red arrow).

IV.3 MATERIAL AND METHODS

IV.3.1 Reagents and medium

Trypan blue solution (0.4%) was obtained from Sigma Aldrich (St. Louis, MO, USA). Modified sperm washing medium was purchased from Irvine Scientific (Santa Ana CA). Percoll, dNTPs, The SLC26A3 antibodies were from Santa Cruz (USA). The SLC26A3 antiserum was a gift from Dr. Höglund P(Haila et al., 2000). The normal goat IgG was from Santa Cruz (USA). The normal rabbit serum was from the laboratory animal service center (LASEC), The Chinese University of Hong Kong (CUHK).

IV.3.2 Animals

Sexually mature (about 10-12 weeks old) BALB/C male mice were obtained from LASEC, CUHK and kept on a schedule of 12h light/dark and had success to chow and water *ad labium*. All experiments were conducted in accordance with the guidelines on the use of laboratory animals laid down by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (Ref 07/062/MIS).

Iv.3.3 In vivo seminiferous tubules injection

The method used was a modified version of that described by Ogawa T. et al (Figure III.2) (Ogawa et al., 1997). The BALB/C mouse was anesthetized and the testis was exteriorized through a midline abdominal incision. Under a dissecting microscope (Nikon, Japan), the epididymis was pulled away from the testis and the fatty tissue overlying the efferent duct was carefully dissected to exposure the duct. A glass micropipette (~40μm) filled with antibody suspension (40μg/mL, prepared with 0.04% trypan blue) is inserted into the efferent duct and a fine forceps was used to grasp the duct and associated fibrous tissue surrounding the ducts to facilitate the pipette entry. The flow was monitored by observing the color change. About 70% of the surface tubules were routinely filled with the cell solution. Good filling of the tubules resulted in the appearance of dye-colored solution in the epididymis. The sperm motility and DSP were analysis 3days after injection. The mice with no treatment were used as blank controls. The mice injected with 0.04% Trypan blue represented sham treatment. The mice injected with normal goat IgG (40μg/mL) were the controls.

Figure IV.2 Schematic diagram of testis injection.
The micropipette filled with antibody is inserted into the efferent duct. The flow first enters the rete and fills most of the seminiferous tubules.

IV.3.4 Computer-assisted sperm analysis (CASA)

Sexually mature BALB/C mice (10-week old), which were injected with SLC26A3 antibody in seminiferous tubules for three days, were used. The mice were sacrificed by cervical dislocation. The procedures for mice caudal epididymal sperm collection were based on the method described by the Hamilton Thorne Research (Beverly, MA). In brief, the cauda was dissected from the mice and was immediately placed into a 35 mm Petri dish containing 1ml modified sperm washing medium (Irving Scientific, USA) and chopped with scissors to release spermatozoa. Sperm were kept in a 37°C incubator for 15 min until analysis. An HTM-IVOS system (version 10.8, Hamilton-Thorn Research) with the following settings was used: objective, ×4; minimum cell size, five pixels; minimum contrast, 56; low VAP cut-off, 5.4; low VSL cut-off, 6.2; threshold straightness, 80%; static size limits, 1.00-1.68; static head intensity, 0.41-0.93; magnification, 2.21. Sixty frames were acquired at a frame rate of 60Hz. At least 200 tracks were analyzed for each specimen at 37°C. The playback function of the system was used to check its accuracy.

IV.3.5 Daily sperm production (DSP)

Daily sperm production was evaluated using the method described by Robb et al. (Robb et al., 1978). In brief, the testes were homogenized in SMT buffer (0.15 M NaCl, 0.1 mM NaN_3, 0.05% Triton X-100). The buffer would lyses all cells except the condensed spermatid nuclei. First, the number of elongated spermatids per testis was determined using a hemocytometer with trypan blue staining and appropriate dilution,

and the DSP was then estimated by dividing the total elongated spermatid number per testis by 4.84, the number of days an elongated spermatid is resident within the testis.

IV.3.6 In vitro analysis of sperm motility

Sperm from cauda epididymis and vas deferens were suspended in modified sperm washing medium and adjusted to 2-3x10^6 cells/ml and incubated at 37慢 for 2h with 1:250 SLC26A3 antiserum or SLC26A3 antibody, respectively; normal rabbit serum or normal goat IgG are as control. At 0, 30, 60, 90,120 min after the start of experiments, subsamples were taken out for observation of motility in phase contrast microscopy.

IV.3.7 Statistical analysis

Results are means ? S.E.M. For statistical analyses, comparisons were made using students t test. Values of $P<0.05$ were regarded as statistically significant.

IV.4 RESULTS

IV.4.1 Effect of SLC26A3 antibody on BALB/C mouse daily sperm production

To determine whether spermatogenesis is affected by SLC26A3, we performed SLC26A3 antibody (40μg/mL) injection into the seminiferous tubules in BALB/C mice. The mice injected with 0.04% Trypan blue represented sham treatment. The mice injected with normal goat IgG (40μg/mL) were the treatment controls. The mice with no treatment were used as blank controls. There was no significant difference among the four treatment groups in DSP (Figure IV.3).

IV.4.2 *In vivo* study of effect of SLC26A3 antibody on BALB/C mice sperm motility

CASA was used to analyze sperm motility after SLC26A3 antibody injection into seminiferous tubules. The mice injected with 0.04% Trypan blue represented sham treatment. The mice injected with normal goat IgG (40μg/mL) were the treatment controls. The mice with no treatment were used as blank control. There were increases in VAP, VCL, VSL, percentage of motile spermatozoa (MOT), percentage of progressive spermatozoa (PROG) and percentage of rapid spermatozoa (RAP) parameters (Figure IV.4).

IV.4.3 *In vitro* study of effect of SLC26A3 antibody on BALB/C mice sperm motility

When sperm suspension subsamples were taken out for observation of motility under phase contrast microscopy at points of 0, 30, 60, 90, 120 minutes, there was no

statistical difference in motility between Control and SLC26A3 antibody or SLC26A3 antiserum treatment group (Figure IV. 5).

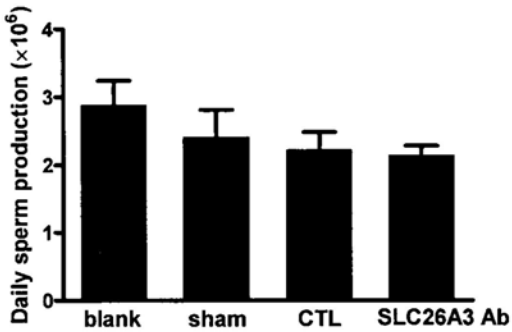

Figure IV.3. The effect of SLC26A3 antiserum on BALB/C mice daily sperm production

The SLC26A3 antiserum (1:100) was injected into the seminiferous tubules of mature male BALB/C mice testes. After three days, the mice were sacrificed and the testes were taken out for DSP analysis. Results are means? SEM (n=4-6). Blank: group with no treatment; sham: group injected with 0.04% trypan blue; CTL: group injected with normal rabbit serum; SLC26A3 Ab: group injected with SLC26A3 antiserum. There was no statistical difference among the four groups.

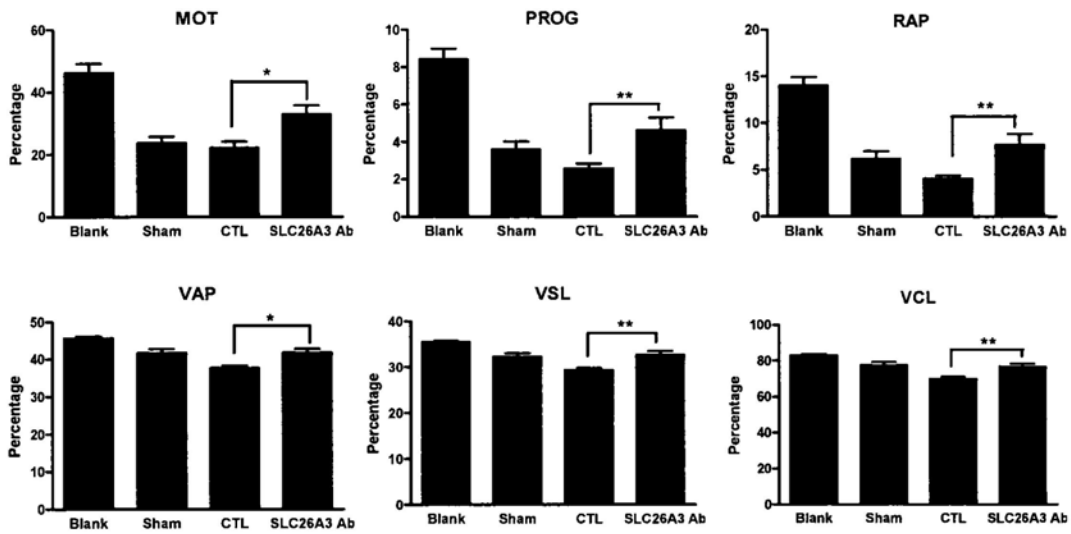

Figure IV.4. *In vivo* study of the effect of SLC26A3 antibody on BALB/C mice motility

SLC26A3 antibody (40μg/mL) was injected into the seminiferous tubules of mature male BALB/C mice testis. After 3days, the mice were sacrificed and the epididymis and vas deferens were taken out for CASA analysis. Results are means? SEM (n=13-19). Blank: group with no treatment; sham: group injected with 0.04% trypan blue; CTL: group injected with normal goat IgG; SLC26A3 Ab: group injected with SLC26A3 antibody. *, as compared to SLC26A3 antibody treatment, $P<0.05$.VAP: velocity average path; VSL: velocity straight line VCL: velocity curvilinear; MOT: motile spermatozoa; PROG: percentage of progressive spermatozoa; and RAP: percentage of rapid spermatozoa.

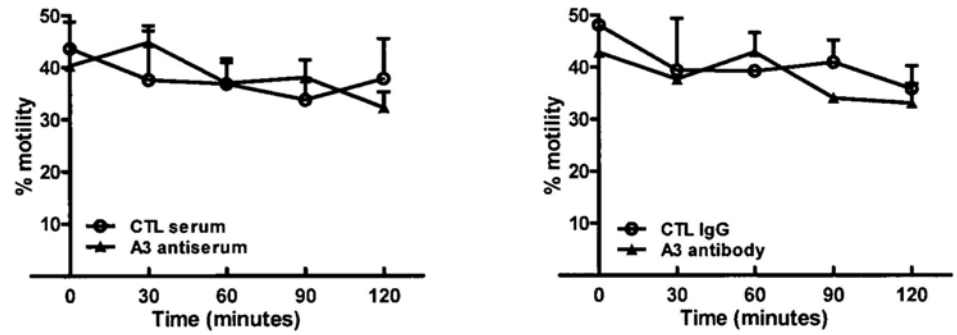

Figure IV.5. *In vitro* study of the effect of SLC26A3 antibody on BALB/C mice motility

Sperm from the cauda epididymis and vas deferens were suspended in modified sperm washing medium and adjusted to 2-3x10^6 cells/ml and incubated at 37慢 for 2h with 1:250 SLC26A3 antiserum or SLC26A3 antibody, respectively; normal rabbit serum or normal goat IgG were used as controls. At 0, 30, 60, 90,120 min after the start of the experiments, subsamples were taken out for observation of motility in phase contrast microscopy. CTL serum: treatment with normal rabbit serum; A3 antiserum: treatment with SLC26A3 antiserum; CTL IgG: treatment with normal goat IgG; A3 antibody: treatment with SLC26A3 antibody. There was no significant difference between control and SLC26A3 antibody or antiserum group.

IV.5 DISCUSSION

Our previous work has demonstrated that SLC26A3 is involved in transporting HCO_3^- necessary for sperm capacitation. The present work is to further extend to investigate whether SLC26A3 plays a role in spermatogenesis or other post-testicular maturation processes.

The injection of SLC26A3 antibody in seminiferous tubules had no effect on daily sperm production (DSP), which is a simple, quantitative and sensitive way to evaluate spermatogenesis (Blazak et al., 1993). This is in agreement with previous report that CLD male patient is subfertile with normal spermatogenesis (Hoglund et al., 2006;Hihnala et al., 2006). However, we can't exclude the possibility that the observation may probably due to insufficient treatment time (72h), considering the time needed for a full cycle of spermatogenesis in mice (35d). In fact, it is a dilemma to ascertain the time needed for antibody treatment. On the one hand, long treatment may be in line with the spermatogenesis cycle time; on the other hand, the longer the time duration the higher the possibility of antibody degradation and antigen antibody reaction.

CASA analysis shows that injection of the SLC26A3 antibody in seminiferous tubules resulted in VAP, VCL, VSL, percentage of motile spermatozoa (MOT), percentage of progressive spermatozoa (PROG) and percentage of rapid spermatozoa (RAP) parameters increases, showing an increase of the sperm motility; some of which are important predictive index for fertility and pregnancy (Larsen et al., 2000). Sperm released from the testis would be further mature when transit through the epididymis lumen and gradually gain the ability to swim and be prepared for capacitation and fertilization. Spermatozoa from mouse proximal epididymis have a lower *in vitro* fertilization rate than those from distal epididymis (Pavlok, 1974). The relative low pH (6.5-7.3) and HCO_3^- concentration (2.7-6.7mM) in the epididymis (Levine and Marsh, 1971;Levine and Kelly, 1978) maintain sperm at a quiescent state. Since HCO_3^- is also a major pH buffer, therefore, low HCO_3^- in the duct lumen is needed to avoid premature capacitation that might lead to impaired fertility(Litvin et al., 2003;Gadella and Van Gestel, 2004).The high pressure during the injection process may lead the testicular fluid into major epididymis region and may have effects on the epithelial cells of the

epididymis and their interaction with spermatozoa during their passage through the epididymis. SLC26A3 has been reported to express on the apical edge of the nonciliated cells in efferent ducts in human(Hihnala et al., 2006;Kujala et al., 2007), which are responsible for the major fluid reabsorption (Hess RA, 2002). Studies have shown significant water reabsorption, coupled to Na^+ and HCO_3^- reabsorption, in the efferent ducts and in the initial segments. NHE3, NBC, AE2 are reported to involved in this HCO_3^- reabsorption (Pastor-Soler et al., 2005). SLC26A3 may also involve in the reabsorption of the HCO_3^- in epididymis. We have observed that SLC26A3 is expressed in the apical membrane of principal cells in mouse initial segment and caput epididymis (Xu WM, et al, unpublished data). Therefore, the injection of SLC26A3 antibody may also lead to interference with SLC26A3 function in epididymal epithelial cells resulting in defect in HCO_3^- reabsorption and thus a higher luminal HCO_3^- concentration. This high HCO_3^- concentration may activate the sAC/cAMP/PKA signaling pathway in sperm, leading to premature increase in sperm motility.

However, when sperm are treated with specific SLC26A3 antibody *in vitro*, with the HCO_3^- concentration at 5mM in order to mimicking the epididymis condition, they do not show any significant difference in the motility when compared with the control. This is not consisted with our previous result that seminiferous tubule SLC26A3 antibody injection led to increased sperm motility. It can be explained by: 1) different treatment time, for *in vivo* (72h) is longer than that *in vitro* (2h); 2) the state of sperm is different. *In vivo*, sperm are in a relative quiescent state whereas sperm are in a constantly moving state *in vitro*; 3) the *in vivo* situation can be influenced by various unknown factors which lead to a more complicated circumstance than that of *in vitro*. Apart from all these possibilities, still, we can not exclude the possibility that SLC26A3 may also play a role in sperm motility.

Patients with congenital chloride diarrhea (CLD) with mutations in SLC26A3 exhibit reduced fertility (Hoglund et al., 2006;Hihnala et al., 2006) and our previous results have also shown that specific SLC26A3 antibody inhibited sperm hyperactivation as well as capacitation. However, these results do not conflict with the present result showing that *in vivo* seminiferous tubule injection of SLC26A3 antibody leads to increased sperm motility. It should be noted that up to now, the reasons why those CLD

patients exhibit reduced fertility are not thoroughly understood, although we give a possible reason relating the defective SLC26A3 with impaired HCO_3^- entry in sperm capacitation in the previous study. The treatment period in the experiment is relatively short which may not accurately mimick the situations in the CLD patients whose SLC26A3 function is defective since embryonic development. On the other hand, increased motility is not the prerequisite of successful capacitation and AR, not to mention successful fertilization.

Taken together, based on the present results, it appears that SLC26A3 does not play a predominant role in spermatogenesis but may affect other post-testicular maturation processes.

Chapter V

General Discussion

It is believed that HCO_3^- plays a central role in sperm function (Litvin et al., 2003;Gadella and Van Gestel, 2004), especially in sperm capacitation(Shi and Roldan, 1995;Boatman, 1997;Liu et al., 2005;Harrison and Gadella, 2005) by directly activating the soluble adenylyl cyclase (sAC) (Chen et al., 2000b;Litvin et al., 2003;Livera et al., 2005). Although its role in mammalian fertilization has been known for decades, the transportation of this ion in sperm is far from understood. Which ion channel/transporter in sperm is responsible for HCO_3^- entry? Do their channels/transporters interact with each other? Undoubtedly, the understanding of HCO_3^- transport mechanisms would provide potential targets for control and regulate the fertilizing event.

Although our previous work has demonstrated that CFTR is involved in the transport of HCO_3^- in sperm capacitation, and defect of which leads to impairment of fertilizing capacity of sperm (Xu et al., 2007); whether CFTR conducts HCO_3^- directly or indirectly remains unclear. The present study is extended further to understand the detailed mechanisms underlying HCO_3^- transport in sperm, particularly, the involvement of a Cl^-/HCO_3^- exchanger, SLC26A3.

The mechanisms of SLC26A3 in HCO_3^- transport during sperm capacitation

HCO_3^- plays an essential role in sperm capacitation (Shi and Roldan, 1995;Boatman, 1997;Visconti et al., 1999;Gadella and Harrison, 2000;Liu et al., 2005;Chan et al., 2006). It stimulates the sAC(Chen et al., 2000b;Cann, 2004), which is distinct from the G-protein-regulated, transmembrane adenylyl cyclases (tmACs). As sAC is inside the membrane, HCO_3^- should be transported into sperm through membrane via ion channels/transporters, since the membrane permeability to HCO_3^- is low in various types of cells (Murdoch and Davis, 1978;Okamura et al., 1988). However, the transport mechanisms underlying the entry of HCO_3^- into sperm membrane remain largely unknown. It has been suggested that a Cl^-/HCO_3^- exchanger exists in sperm (Spira and Breitbart, 1992;Zeng et al., 1996;Kaupp and Weyand, 2000), yet its exact role

in sperm function and exact molecular identity are unknown.

In the present study, we have demonstrated the Cl^- dependence, in addition to HCO_3^-, of sperm capacitation but not AR, which is sequential but separate process. Since some of the key events in the HCO_3^-/sAC cascade such as increased pHi, cAMP production and protein tyrosine phosphorylation, which have been previously shown to be dependent on HCO_3^-, are also found to be dependent on Cl^-. Functional studies also observed more potent effect of niflumate over DIDS in sperm capacitation and hyperactivated motility, suggesting that this Cl^-/HCO_3^- exchanger is probably the SLC26A3, which appears to play a more primary role in sperm capacitation. This is further confirmed by the inhibitory effect of its specific antibody on sperm capacitation , together with the results of immunostaining and western blot showing the localization and expression of SLC26A3 in sperm. The fertilizing ability is almost abolished in Cl^--deficient medium due to reduced capacity of sperm penetration of oocytes. Based on these studies, it is highly possible that defect in SLC26A3 may impair HCO_3^- entry, affecting sperm capacitation and therefore reducing their fertilizing ability. We, therefore, propose that SLC26A3 is involved in sperm HCO_3^- entry with CFTR providing a recycling pathway of Cl^- (chapter II, figure II.10), taken into consideration of the inhibition of sperm capacitation by specific CFTR inhibitor and the expression of CFTR in sperm.

DIDS inhibits a wide spectrum of anion exchangers and cotransporters, including SLC4A2 (also known as AE2), which is an predominant electroneutral Na^+ dependent Cl^-/HCO_3^- exchanger(Shmukler et al., 2005). Although SLC4A2 is also expressed on mature sperm (Parkkila et al., 1993), but it has been shown to play a role in spermatogenesis other than capacitation(Medina et al., 2003). On the other hand, the more potent inhibition of niflumate than DIDS also suggests SLC26A3 plays a more primary role in sperm capacitation, since SLC26A3 is more sensitive to niflumate (Ko et al., 2002;Chernova et al., 2003).

Recently, Demarco et al has demonstrated that a sodium HCO_3^- cotransporter is present in mouse sperm and is coupled to events regulating capacitation, indicating another possible HCO_3^- transport pathway(Demarco et al., 2003). Although this cotransporter may play a role in the HCO_3^- entry into the sperm, it is unlikely that it

plays a dominant role in this since deficiency in Cl^- almost completely abolishes sperm capacitation.

Although we have provided strong evidences demonstrating the involvement of SLC26A3 in sperm capacitation, the present functional studies using inhibitors or antibody may have limitation regarding specificity. Other approach for loss of functional studies such as knockout mice is needed to further elaborate the role of SLC26A3 in sperm capacitation.

Possible role of SLC26A3 in post-testicular sperm maturation and spermatogenesis

It is well known that SLC26A3 mutations are related to the congenital chloride diarrhea (CLD), whereas the other phenotypes related to SLC26A3 gene mutations are not well understood. Unlike CLD women who have normal fertility, CLD men show subfertility characterized by oligoasthenozoospermia with normal concentrations of sex hormones and normal testicular histology (Hoglund et al., 2006;Hihnala et al., 2006). However, the exact role and possible mechanisms of SLC26A3 in male fertility is not clear. Others have shown that SLC26A3 is expressed in human elongating spermatids (of stages III-VI in human testis) (Hihnala et al., 2006), and mouse spermatoyctes, spermatids and mature sperm cells. We postulated that SPC26A3 might be involved in spermatogenesis and other post-testicular sperm maturation processes such as epididymis maturation and sperm motility.

SLC26A3 and SLC26A6 are both Cl^-/HCO_3^- anion exchangers they have different stoichiometries with the former mediates a coupled 2 Cl^-/1 HCO_3^- and the latter 1 Cl^-/2 HCO_3^- exchanger. Together with CFTR, SLC26A6 has been proposed to be an important contributor to apical $HCO3^-$ secretion in the pancreatic duct (Steward et al., 2005). When it comes to the male reproduction system, SLC26A6 is coexpressed with CFTR and NHE3 in epididymal ducts (Kujala et al., 2007). The strict control of luminal HCO_3^- concentrations by epididymal epithelium is essential to sperm motility, membrane stability and fertility (Litvin et al., 2003;Gadella and Van Gestel, 2004). These three transporters may work together and are responsible for HCO_3^- transport to maintain luminal HCO_3^- concentration.

In the present study, by using GC-1 spg cell line, which only expresses SLC26A6,

as the negative control in comparison to sperm which expresses both SLC26A3 and SLC26A6. We have demonstrated that it is SLC26A3 but not SLC26A6 which involved in HCO_3^--stimulated $[Ca^{2+}]i$ change and that SLC26A3 does not contribute significantly to the alteration of membrane potential in sperm.

Interestingly, our experiments show that sperm motility is increased after injection of SLC26A3 antibody in seminiferous tubules *in vivo*. The interpretation is that blocking SC26A3 in epididymal epithelial cells cause impaired HCO_3^- reabsorption which may lead to higher HCO_3^- concentration and consequently results to premature increase in sperm motility. However, when sperm are treated with specific SLC26A3 antibody *in vitro*, there is no significant change in motility, suggesting that SLC26A3 does not play a direct role in sperm motility.

The observed more potent effect of niflumate (more sensitive to SLC26A3) over that of DIDS (more sensitive to SLC26A6) and the different results obtained from GC-1 spg cells (express SLC26A6) and sperm (express SLC26A3 and SLC26A6) on HCO_3^-–induced $[Ca^{2+}]i$ in this study strongly support that SLC26A3 plays a primary role in sperm capacitation. This notion is further supported by the *in vivo* studies showing insignificant effect of SLC26A3 antibody on DSP under current experimental condition.

As mentioned in the discussion, SLC26A3 in epididymal epithelial cells may exert its effect on sperm. Other experiments should be done to show the effect of SLC26A3 on epididymal epithelium during epididymal sperm maturation, such as co-culture with normal testicular sperm and SLC26A3-defective epididymal epithelial cells. The use of antibody has its limitation, thus better techniques, such as RNA interference and knockout mice need to perform in future to further demonstrate the role of SLC26A3 in sperm function and other post-testicular sperm maturation processes.

Conclusion

Taken together, these results indicate that SLC26A3 is predominantly involved in sperm capacitation but not spermatogenesis. SLC26A3 may also play some role in epididymis maturation and sperm motility through its action on epididymal epithelium rather than sperm directly. The present results may shed new light on the understanding of molecular mechanisms regulating male fertility and provide possible explanation on

some cases of male infertility. Furthermore, SLC26A3 may serve as a potential molecular target for diagnosis of male infertility.

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