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# CFTR in pancreatic islets



# CFTR in pancreatic islets

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DOCTORAL DISSERTATION

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<b>Abstract</b>		
<p>Cystic fibrosis (CF) is caused by mutations in the anion channel and protein regulator CFTR. The most common co-morbidity in CF is CF-related diabetes (CFRD) affecting ~50% of adult patients. The etiopathology of CFRD is largely unknown but the destruction of the exocrine pancreas is thought to contribute. However, the hypothesis that CFTR has a direct role in the endocrine pancreas has not been explored. In this thesis I have investigated if CFTR have a direct function in insulin, glucagon and somatostatin secretion. Experiments were performed on islets and single cells from NMRI mice and the CFTR<sup>TM1Eur</sup> mouse model (F508del) that carries the most common human mutation in CFTR, the deletion of a phenylalanine at position 508.</p> <p>We found CFTR to be present in human and mouse alpha and beta cells but not in delta cells. A CFTR-dependent Cl<sup>-</sup> current was recorded in alpha- and beta-cells. In beta cells, a large part of the CFTR-dependent current was mediated by the Ca<sup>2+</sup>-activated chloride channel Anoctamin 1 (ANO1), and we suggested that CFTR most likely regulates ANO1 in beta cells. In human and mouse islets inhibition of CFTR or ANO1 reduced cAMP-enhanced insulin secretion by direct effects on exocytosis. In addition, ANO1 transcripts were found to be upregulated in islets from type 2 diabetic (T2D) donors. Analysis of transmission electron microscopy micrographs revealed that beta cells from the F508del CF mouse model or in NMRI mice after pharmacological inhibition of CFTR have reduced number of docked granules. CFTR also co-localizes to the SNARE protein Syntaxin 1A and places CFTR in the exocytotic machinery. Moreover, isolated islets from F508del mice have an increased proinsulin secretion and correspondingly decrease in release of c-peptide. This was especially evident during cAMP stimulation when CFTR is activated. Based on these findings we propose a model where CFTR is involved in granular priming and maturation of insulin. Cl<sup>-</sup> ions are believed to be necessary to lower the pH to levels needed for cleavage of proinsulin to insulin and c-peptide.</p> <p>Glucagon secretion is dysregulated in diabetes. We found that inhibition of CFTR increased glucagon secretion in isolated islets from human and mouse. Mathematical modelling of alpha cell physiology revealed that the CFTR-dependent current was involved in the regulation of alpha cell electrical activity. Moreover, the F508del mice had increased serum glucagon and isolated F508del islets had increased glucagon secretion. Inhibition of ANO1 in islets from T2D donors enhanced glucagon secretion while having no effect on islets from normal glucose tolerant donors, further supporting the regulatory role of Cl<sup>-</sup> in glucagon secretion.</p> <p>The novel data presented in this thesis suggests that CFTR regulates human and mouse beta cells by direct effects on exocytosis of the insulin granule. A defective CFTR increases secretion of immature proinsulin. Furthermore, CFTR negatively regulates glucagon secretion by direct effects on electrical activity and thereby CFTR works as a break in alpha cells.</p>		
Key words Cystic fibrosis, Cystic fibrosis-related diabetes, CFTR, ANO1, current, Type 2 diabetes, exocytosis, granular priming, patch-clamp, hormone secretion measurements, insulin, glucagon		
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# CFTR in pancreatic islets

Anna Edlund



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Cover picture: The cystic fibrosis transmembrane conductance regulator (CFTR) by  
Anna Edlund

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*To Olle and Betty*



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# List of publications

Papers included in this Thesis

1. **CFTR and Anoctamin 1 (ANO1) contribute to cAMP amplified exocytosis and insulin secretion in human and murine pancreatic beta cells.** Edlund A, Esguerra JL, Wendt A, Flodström-Tullberg M, Eliasson L. BMC Med. 2014 May 28;12:87. doi: 10.1186/1741-7015-12-87.
2. **CFTR is involved in the regulation of glucagon secretion in human and rodent alpha cells.** Edlund A, Pedersen MG, Lindqvist A, Wierup N, Flodström-Tullberg M, Eliasson L, *manuscript under revision*
3. **Defective insulin processing and reduced insulin secretion in islets from the F508del CFTR /Cftr<sup>tm1eur</sup> mouse model of cystic fibrosis** Edlund A., Hühn M., Abels M., Mollet I., Svedin E., Wierup N. Scholte BJ., Flodström-Tullberg M., Eliasson L. *manuscript under revision*
4. **Characterizing the role of Anoctamins in insulin secretion.** Edlund A, Ofori JK, Esguerra JLS and Eliasson L, *manuscript*

# Additional papers not included in this thesis

1. **Reduced insulin secretion correlates with decreased expression of exocytotic genes in pancreatic islets from patients with type 2 diabetes.** Andersson SA, Olsson AH, Esguerra JL, Heimann E, Ladenvall C, **Edlund A**, Salehi A, Taneera J, Degerman E, Groop L, Ling C, Eliasson L: *Mol Cell Endocrinol* 2012, 364(1-2):36-45.
2. **Reduced insulin exocytosis in human pancreatic beta cells with gene variants linked to type 2 diabetes.** Rosengren AH, Braun M, Mahdi T, Andersson SA, Travers ME, Shigeto M, Zhang E, Almgren P, Ladenvall C, Axelsson AS, **Edlund A**, Pedersen MG, Jonsson A, Ramracheya R, Tang Y, Walker JN, Barrett A, Johnson PR, Lyssenko V, McCarthy MI, Groop L, Salehi A, Gloyn AL, Renström E, Rorsman P, Eliasson L: *Diabetes* 2012, 61(7):1726-1733.
3. **Calcium current inactivation rather than pool depletion explains reduced exocytotic rate with prolonged stimulation in insulin-secreting INS-1 832/13 cells.** Pedersen MG, Salunkhe VA, Svedin E, **Edlund A**, Eliasson L: *PLoS One* 2014, 9(8):e103874.
4. **Beta cell specific deletion of Dicer1 leads to defective insulin secretion and diabetes mellitus.** Kalis M, Bolmeson C, Esguerra JL, Gupta S, **Edlund A**, Tormo-Badia N, Speidel D, Holmberg D, Mayans S, Khoo NK, Wendt A, Eliasson L, Cilio CM: *PLoS One* 2011, 6(12):e29166.
5. **Increased GABA(A) channel subunits expression in CD8(+) but not in CD4(+) T cells in BB rats developing diabetes compared to their congenic littermates.** Mendu SK, Akesson L, Jin Z, **Edlund A**, Cilio C, Lernmark A, Birnir B: *Mol Immunol* 2011, 48(4):399-407.

# Abbreviations

ANO	Anoctamin family of Ca <sup>2+</sup> -activated proteins
CaCC	Ca <sup>2+</sup> -activated chloride current
CaCCinh-AO1	6-tert-butyl-2-(furan-2-carboxamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid
cAMP	Cyclic adenosine monphosphate
CF	Cystic fibrosis
CFRD	Cystic fibrosis-related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
CFTRinh-172	3-[(3-Trifluoromethyl)phenyl]-5-[(4-carboxyphenyl methylene)-2-thioxo-4-thiazolidinone
ClC3	Chloride channel 3
DIDS	4,4'-Diisothiocyanostilbene-2,2'-disulfonic Acid
Epac	Exchange factor directly activated by cAMP
F508del	Deletion of phenylalanine at position 508 in CFTR
GABA	Gamma amino butyric acid
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GlyH-101	N-(2-Naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide
GΩ	Giga ohm
gSUR	Granular sulfonylurea receptor
K <sub>ATP</sub> channel	ATP-sensitive K <sup>+</sup> channel
KO mouse	Knock-out mouse
miRNA	Micro RNA
Munc	Mammalian homologue of unc-18 proteins

ORCC	Outward rectifying Cl <sup>-</sup> channel
PC1/3	Prohormone convertase
PC2	Prohormone convertase 2
PKA	Protein kinase A
SERCA	Sarco/endoplasmic reticulum Ca <sup>2+</sup> -ATPase
SNAP	Synaptosomal-associated protein, 25kDa
SNARE	Soluble NSF attachment protein receptor
Stx1a	Syntaxin 1A
Stxbp1	Syntaxin-binding protein 1 (also called Munc 18)
SUR1	Sulfonylurea receptor 1
TEM	Transmission electron microscopy
T16Ainh-AO1	2-(5-ethyl-4-hydroxy-6-methylpyrimidin-2-ylthio)-N-(4-(4-methoxyphenyl)thiazol-2-yl)acetamide
Vamp2	Vesicle-associated membrane protein 2
WT	Wild-type



# Introduction

## Type 2 Diabetes and Hormonal secretion

Type 2 diabetes (T2D) is believed to affect 9% of the adult population in the world and of them 90% have T2D [1]. This alarming prediction carries suffering to affected individuals and an enormous burden on the healthcare system.

T2D onset occurs when the pancreatic beta cell cannot secrete enough insulin to supply the need of the body. However, there is no clear consensus on the mechanism of insulin deficiency [2]. T2D have a genetic component but there is also a strong contribution of lifestyle factors such as age, obesity and pregnancy to the development of disease [3, 4]. Several risk genes have been identified by genome-wide association studies (GWAS) [5]. Epigenetic modifications of the genome [6] and aberrant expression of miRNAs has been associated with T2D [7, 8]. Moreover, several exocytotic proteins are downregulated in islets from T2D donors [9-11].

Loss of beta cell mass has been observed postmortem in individuals with T2D with a durational decline [12, 13]. In murine diabetes models dedifferentiation of existing beta cells rather than beta cell death has been observed [14]. However, it is unknown if this is applicable to human beta cells [15].

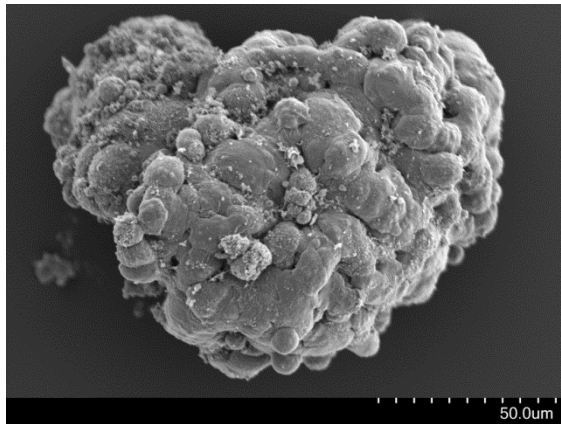
Insulin resistance is often present in T2D and is believed to put an extra burden on the beta cells since the target tissues e.g. the liver, adipose and muscle tissue fail to respond to insulin forcing the beta cell to secrete more and more insulin.

T2D is considered a bi-hormonal disease. There is an increasing awareness that an aberrant glucagon secretion from the pancreatic alpha cell worsens glycemic control in diabetes [16-18]. Glucagon is released in between meals or during exercise when blood glucose is low. Glucagon acts mainly on the liver to increase hepatic glucose production (gluconeogenesis) or breakdown of glycogen to increase blood glucose levels. In diabetes there is an insufficient release of glucagon at low glucose as well as a reduced capacity to suppress glucagon in response to elevated blood glucose levels [19, 20]. The role of glucagon release in the development of diabetes was recently highlighted by the discovery that the glucagon receptor KO mice remained normoglycemic after ablation of pancreatic beta cells using streptozotocin [21].



## The islet of Langerhans

Insulin and glucagon are released from the islets of Langerhans that constitute the endocrine part of the pancreas (below, Fig.1).



**Figure 1.** A scanning electron microscopy (SEM) image of a human islet of Langerhans (by the courtesy of Lena Eliasson).

The islets are clusters of cells encapsulated by a basement membrane. They are interspersed in the whole pancreas and are extensively vascularized to enable glucose sensing and distribution of the released hormones to target tissues. [22] There are at least 1-3 million islets of Langerhans in the human pancreas and each islet contains 1000-3000 islet cells [15, 23]. The main cell types in the islets are insulin secreting beta cell, glucagon secreting alpha cells and somatostatin secreting delta cells but also less well characterized pancreatic polypeptide secreting PP cells (F-cells in mouse) and ghrelin secreting ghrelin cells are present [24]. The islet architecture is species dependent and in the mouse, beta cells are in the center surrounded by a mantle of alpha-and delta cells. In addition, mouse islets are heavily innervated by the nervous system. In the human islets, alpha- beta- and delta cells are mixed [25, 26] and there is little evidence for innervation of the islets by the nervous system. Instead the smooth muscle cells of the capillaries have been shown to be innervated [27]. The lack of innervation of the human islets suggests that paracrine regulation is of special importance. In addition, beta cells are connected via gap-junctions to mediate cell to cell contact and regulation [28, 29].

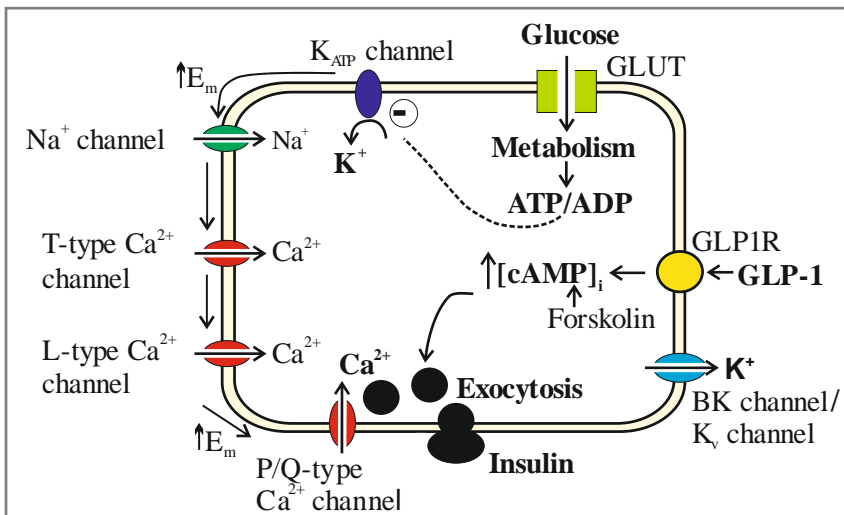
Also, the relative distribution of different islet cells differs between mouse and human. Beta cells constitute 50% of the cells in human islets and 60-80% in mouse, alpha cells constitute 20-40% of the cells in human islets and 10-20% in mouse, and delta cells constitute 10% of the cells in human islets and 5% in mouse. Finally PP cells (E-cells in mouse) constitute 2% and Ghrelin cells 1% of the islet cells [15, 30, 31].

## Regulation of insulin secretion

### *Glucose stimulus secretion coupling*

Glucose is taken up by the beta cell via glucose transporters (GLUT1-3 in human and GLUT2 in mouse) [32-34] and through a complex chain of enzymatic reactions metabolic substrates are generated that ultimately cause an increase in intracellular ATP [35] (Fig 2). An increase in the ATP:ADP ratio closes ATP-dependent K<sup>+</sup> channels (K<sub>ATP</sub> channel) that contributes to the depolarization of the plasma membrane [36]. An additional depolarizing current is needed, and which identity is debated [37, 38]. Membrane depolarization opens voltage-dependent Ca<sup>2+</sup> channels and influx of Ca<sup>2+</sup> triggers exocytosis of insulin-containing granules. Thereafter opening of voltage-gated K<sup>+</sup> channels (Kv2.1) and Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK channel) repolarize the cell membrane to resting membrane potential at -70 mV [39].

Beta cells fire action potentials that trigger oscillations in intracellular Ca<sup>2+</sup> and a pulsatile release of insulin [40, 41]. In mouse beta cells the upstroke (depolarization) of an action potential is mainly mediated via Cav1.2 L-type Ca<sup>2+</sup> channels and the down stroke (repolarization) is mainly mediated via Kv2.1 channels [42]. In the human beta cell T-type Ca<sup>2+</sup> channels mediate depolarization of the plasma membrane from ~-60 mV leading to opening of L-type Ca<sup>2+</sup> channels and TTX-sensitive Na<sup>+</sup> channels. From -20 mV and above P/Q-type Ca<sup>2+</sup> channels open and trigger exocytosis of insulin granules [39].



**Figure 2. Stimulus secretion coupling in the human beta cell.** Glucose is taken up by the beta cell and metabolized to generate ATP. The shift in ATP:ADP ratio closes the K<sub>ATP</sub> channel and depolarizes the cell membrane potential (E<sub>m</sub>) opening voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels. Influx of Ca<sup>2+</sup>, mainly through voltage-gated P/Q-type Ca<sup>2+</sup> channels, triggers exocytosis of insulin-containing granules. The cell membrane potential is repolarized by opening of the voltage-gated K<sup>+</sup> channel (K<sub>v</sub> channel) and the Ca<sup>2+</sup>-activated K<sup>+</sup> channel (BK channel). Binding of GLP-1 to GLP-1 receptor (GLP1R) or exogenous forskolin increase intracellular cAMP that amplifies insulin secretion.

### *The insulin granule and exocytosis*

Insulin is a 6 kDa hormone expressed from the *INS* gene at human chromosome 11 [43] and by two separate genes located at chromosome 7 and 19 in mouse [44]. It is transcribed as preproinsulin and processed to proinsulin in the endoplasmic reticulum (ER) and packed into intracellular vesicles (granules) in the Golgi apparatus [45, 46]. The immature insulin granule is transported to the plasma membrane via the cytoskeleton.

At the plasma membrane the granule have to dock and prime before its content can be released [47, 48]. Docking means that the granules attach to the plasma membrane. Priming is an ATP-,  $\text{Ca}^{2+}$  and temperature-dependent process [49-51] believed to take place in the close vicinity of the plasma membrane to make the granules ready for fusion. Several proteins have been suggested to be part of the priming process e.g. Epac2, SUR1 and the CIC3 chloride channel [52-54]. Another process believed to take place during priming is insulin maturation where proinsulin is cleaved into mature insulin and c-peptide by the prohormone convertases PC1/3 and PC2 [55]. The prohormone convertases require pH 5.5 and protons are pumped into the granule via granular V-type  $\text{H}^+$  ATPase. To prevent the formation of an electrostatically gradient,  $\text{Cl}^-$  ions are simultaneously pumped into the granule via CIC3. [54]

Assembly of the SNARE complex mediates docking and fusion of the insulin granule at the plasma membrane. Some of the central SNARE proteins are granular vamp2/synaptobrevin and the plasma membrane associated proteins Syntaxin 1a (Stx1a) and SNAP23/SNAP25 [56], as well as Munc18 (or Syntaxin binding protein; Stxbp1) and  $\text{Ca}^{2+}$ -sensitive synaptotagmins. Assembly of Stx1a and Munc18 at the plasma membrane has been shown to confer docking sites for insulin granules [57]. In that model, SNAP25 and Munc13 were recruited shortly after docking.

The assembly of the SNARE complex brings the granule closer to the plasma membrane, and Stx1a and SNAP25 have been demonstrated to be important for exocytosis in both alpha- and beta-cells [58, 59]. Voltage-gated  $\text{Ca}^{2+}$  channels are closely linked to the SNARE complex at the release sites [51]. Fusion of the insulin granule with the plasma membrane and release of insulin require  $\text{Ca}^{2+}$ -dependent conformational changes, which are achieved through the influx of  $\text{Ca}^{2+}$  through voltage-gated  $\text{Ca}^{2+}$  channels. In addition,  $\text{Ca}^{2+}$  is released from intracellular stores further enhancing exocytosis [60].

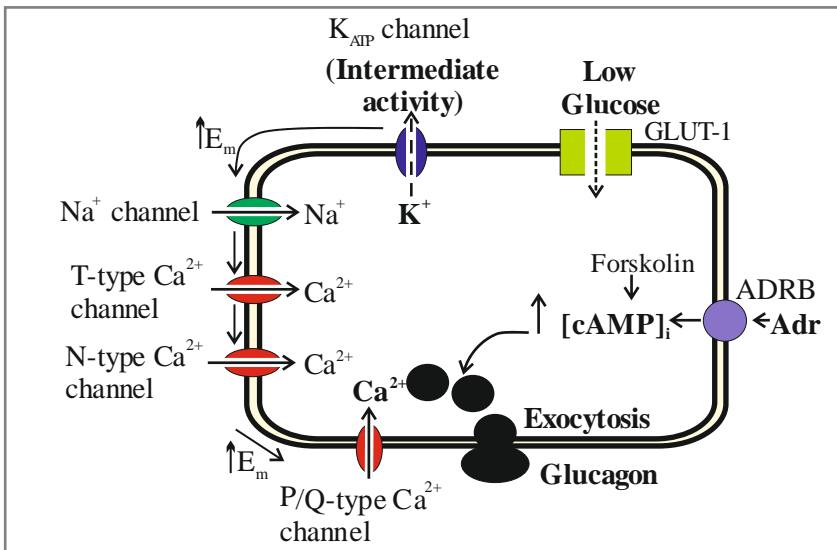
### *Amplification of insulin secretion by cAMP*

Insulin secretion is amplified by metabolites from glucose oxidation and external signaling via Gs- and Gq-coupled receptors; For example, the incretin GLP-1 released from intestinal L-cells binds to the GLP-1 receptor and activates adenylate cyclase that catalyzes the formation of cAMP. Adenylate cyclase can also be activated directly by the compound forskolin (Fig 2). Increased levels of intracellular

cAMP activate protein kinase A (PKA) and Epac2, that amplify glucose stimulated insulin secretion in both mouse and human islets [61, 62]. PKA enhances recruitment of granules and priming but also phosphorylates transcription factors and affects gene transcription. Epac2 have been shown to associate to the insulin granule protein gSUR and is believed to enhance priming by regulating CIC-3 [52]. In addition, Epac2 also directly binds to SUR1 subunit of the  $K_{ATP}$ -channel and allosterically inhibit the channel to increase beta cell electrical activity [63].

## Regulation of glucagon secretion

The regulation of glucagon secretion is as in the beta cell dependent on the  $K_{ATP}$  channel [64]. Alpha cells are electrically active at 1 mM glucose and are maximally inhibited at 6 mM glucose. This is explained by a lower density of  $K_{ATP}$  channels causing lower input resistance (3-5 G $\Omega$ ) [20, 65] therefore small changes in ion channel activity will affect the membrane potential dramatically.



**Figure 3. Stimulus secretion coupling in mouse alpha cells.** At low glucose concentrations the  $K_{ATP}$  channel is partially open leading to depolarization of the plasma membrane potential ( $E_m$ ) and opening of voltage-gated  $Na^+$  channel and voltage-gated T- and N-Type  $Ca^{2+}$  channels. Voltage-gated P/Q-type  $Ca^{2+}$  channels is believed to trigger exocytosis of glucagon containing granules. Glucagon secretion can be amplified by adrenalin (Adr) and forskolin that increase intracellular cAMP.

In mouse alpha cells (Fig 3), voltage-gated  $Na^+$  channels are active during action potential firing at low glucose concentrations. During the peak of the action potential voltage-gated  $Ca^{2+}$  channels, mainly P/Q type, open and trigger exocytosis of glucagon-containing granules. However, at 6 mM glucose the  $K_{ATP}$  channels are closed and partially depolarize the membrane leading to inactivation of voltage-gated

Na<sup>+</sup> channels and diminished glucagon secretion. This is explained by reduced amplitude of the action potentials and incomplete inter-spike repolarization of the alpha cell membrane. Voltage-gated Na<sup>+</sup> channels have to repolarize to re-activate and incomplete repolarization diminishes the number of active Na<sup>+</sup> channels to 30%, explaining the reduced amplitude. Since the amplitude of action potentials are reduced fewer Ca<sup>2+</sup> channels open and hence glucagon secretion is reduced. [20, 66]

K<sub>ATP</sub> channel independent release of glucagon has been demonstrated in the K<sub>ATP</sub> channel KO mouse, but the exact mechanism has not been elucidated [67]. One hypothesis is that glucagon secretion is regulated by the store-operated Ca<sup>2+</sup> channel Orai1. During high glucose stimulation Ca<sup>2+</sup> would be sequestered in the sarcoendoplasmic reticulum via the SERCA Ca<sup>2+</sup> channel causing hyperpolarization of the plasma membrane and reduction in glucagon secretion. [68]

Many of the components of the exocytotic machinery described above for the beta cell are similar in the pancreatic alpha cells [59, 69, 70]. It is worth mentioning that exocytosis of glucagon containing granules is amplified by PKA and Epac2 in a delicate balance where low levels of cAMP inhibits N-type Ca<sup>2+</sup> channels through a PKA-dependent mechanisms. High intracellular levels of cAMP amplifies fusion and mobilization through activation of Epac and PKA [69]. Adrenalin amplifies glucagon secretion by increasing intracellular cAMP through activation of this pathway and through mobilization of Ca<sup>2+</sup> from intracellular stores [69, 71].

## **Regulation of somatostatin secretion**

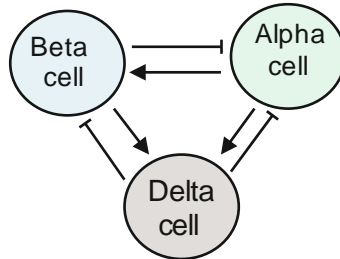
The mechanism of somatostatin secretion is obscure and not fully elucidated. In human delta cells somatostatin secretion is dependent on the K<sub>ATP</sub> channel and voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels contribute to action potential firing. Exocytosis of somatostatin-containing granules is dependent on the P/Q-type Ca<sup>2+</sup> channels. [72] In mouse delta cells glucose-stimulated (>10 mM) somatostatin secretion is independent of K<sub>ATP</sub> channels. Instead Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release via R-type Ca<sup>2+</sup> channels activate Ca<sup>2+</sup>-induced exocytosis [73].

Somatostatin secretion is enhanced by forskolin suggesting existence of an amplifying pathway in delta cells. However, there are few reports on the function of PKA/PKC and Epac2 in delta cells.

## **Paracrine regulation in islets**

The islet cells are under paracrine regulation by their neighboring cells. The currently envisioned network of paracrine feed-back within the islets of Langerhans is described in Fig 4.

Mouse and human alpha- and beta-cells express somatostatin receptors and somatostatin most likely confer fast (inwardly-rectifying  $K^+$  channels [74]) and tonic ( $G_i$ -pathway [75]) inhibition of both insulin and glucagon secretion. Somatostatin has a half-life  $<1$  min in circulation indicating that it exerts most of its action within the islet [76]. It has been postulated that somatostatin tightly regulates insulin secretion to avoid hypersecretion and hypoglycemia [76], since somatostatin and insulin is stimulated by increasing glucose concentrations [77].



**Figure 4. Intra-islet paracrine feed-back regulation network.** Glucagon from alpha cells stimulate insulin and somatostatin secretion. Beta cell confer stimulatory action on somatostatin secretion directly via insulin or via the co-secretagogue Ucn3. The beta cell inhibitory action on alpha cells might be mediated directly via insulin or by the co-secretagogue  $Zn^{2+}$  or GABA. Somatostatin from delta cells inhibit both insulin and glucagon secretion.

Glucagon directly stimulate both insulin and somatostatin secretion by binding to the glucagon receptor and increase the intracellular levels of cAMP [78-80]. Glucagon can also stimulate its own secretion in a feed-forward mechanism [81].

The role of insulin is more uncertain. Perfusion of human pancreas with an insulin antibody was shown to reduce somatostatin secretion, suggesting that insulin directly stimulate somatostatin secretion [82]. Recently the peptide, Urocortin 3 (Ucn3) was shown to be co-released with insulin and glucagon. Ucn3 stimulated somatostatin secretion and the Ucn3 KO mice hyper-secreted insulin. [83]

Insulin inhibits glucagon secretion directly [84] but also via the co-secretagogues  $Zn^{2+}$  [85] and GABA that is released from beta cells [86].

## Chloride channels in hormonal secretion

Closure of  $K_{ATP}$  channels is not sufficient to open voltage-gated  $Ca^{2+}$  channels and an alternative anionic mechanism has been suggested in mice [87, 88]. In human beta cells the need for such current is not as well established but it has been demonstrated that  $GABA_A$  channel activation contributes to depolarization [89].

Beta cells actively maintain a high intracellular  $Cl^-$  concentration ( $\sim 30$  mM) [90, 91]. Activation of  $Cl^-$  channels at resting membrane potential ( $-70$  mV) would lead to  $Cl^-$  efflux and depolarization up to  $Cl^-$  reversal potential at  $\sim -40$  mV, where  $Cl^-$  instead

begins to enter the beta cell. Several anion channels have been proposed to control electrical activity in beta cells: VRAC [87],  $I_{Cl, \text{islet}}$  [92], CFTR [93], ANO1 [94] and GABA<sub>A</sub> channels [89]. VRAC have been proposed to be activated by glucose [37].  $I_{Cl, \text{islet}}$  is activated by cAMP and contribute to cAMP-dependent depolarization [92]. CFTR is activated by cAMP and ATP [95]. ANO1 is activated by increased intracellular  $Ca^{2+}$  and membrane depolarization and has been demonstrated to be involved in insulin secretion through regulation of glucose-induced membrane potential oscillations [94]. So far, the majority of studies on the importance of Cl<sup>-</sup> channels in insulin secretion have been performed in rodent islets except for one study showing that activation of GABA<sub>A</sub> channels depolarizes the human beta cell [89].

Furthermore, insulin, glucagon and somatostatin are released in a pulsatile manner driven by  $Ca^{2+}$  oscillations [96, 97]. Insulin and somatostatin is released in antisynchrony from glucagon [98]. Cl<sup>-</sup> ions have been suggested to be important to control the oscillatory pattern in the islets. This was highlighted by the finding that application of the Cl<sup>-</sup> channel inhibitor DIDS to beta cells resulted in loss of  $Ca^{2+}$  oscillations and a sustained increase in intracellular  $Ca^{2+}$ . [90]

## Cystic Fibrosis

The observation that a child with cystic fibrosis (CF) tastes salty has been described since hundreds of years. Although, at that time they did not know it was CF and that the salty taste arises from the increased Cl<sup>-</sup> content in sweat. In 1606 Alonso y de Los Ruyzes de Fontecha a Spanish professor in medicine wrote;

The fingers would taste salty after rubbing the forehead of a bewitched child [99].

In the Almenac of Childrens songs and games from Switzerland from 1857 the following phrase could be read;

The child will soon die whose brow tastes salty when kissed.

The pathology of CF was first described in 1938 by Dr Dorothy Andersen at the New York babies hospital who published a landmark paper, in the American Journal of Diseases in Children “Cystic Fibrosis of the Pancreas and its Relation to Celiac Disease”, [100]. The primary symptoms described were fibrosis of the pancreas and chronic lung infections. At the same time in Europe, Fanconi and co-workers described a group of patients having celiac disease with fibrosis of the pancreas [101]. Even though the patients could now be diagnosed there was no treatment for the symptoms. It was not until the ending of World War 2 that doctors were able to treat the chronic lung infections characteristic for CF by administrating antibiotics. In

1953, Paul di Sant'Agnes showed that patients with CF have increased salt content in sweat [102] and the sweat test for diagnosis was developed. A retrospective survival study between the years 1944-1964 revealed that 80% of the patients died before 5 years of age, 90% of the patients were dead before reaching the age of 10 and there were no patient that reached adulthood [103]. Today in Sweden, life expectancy for individuals with CF is 50 years [104]. With increasing life expectancy co-morbidities develop and the most common being cystic fibrosis-related diabetes (CFRD) [105].

### *Identification of the CF gene*

In the 1980's, a region on chromosome 7q31 was pin-down and in 1989, the group of Lap-Chee Tsui identified the gene transcribing the defective protein responsible for CF. They named it the cystic fibrosis transmembrane conductance regulator (CFTR). [106]

### *CFTR in airways*

Over the years it was established that CF is a viscous disease that primarily affect mucus production in epithelial tissue [107]. CFTR contributes to produce liquid mucus that can be cleared by cilia. The mechanism is believed to involve secretion of  $\text{Cl}^-$  via CFTR and  $\text{Na}^+$  via the Epithelial  $\text{Na}^+$  channel (ENaC) that creates an ion gradient that attracts  $\text{H}_2\text{O}$ , to form liquid mucus. A defective CFTR results in thick viscous mucus that cannot be cleared by cilia and that entraps bacteria [107]. It is also suggested that CF patients have a defective immune response to infections that further exacerbates tissue damage [108, 109]. Repeated lung infections and colonization of *pseudomonas aeruginosa* and other opportunistic bacteria progressively diminish lung function in CF. The repeatedly occurring lung infections are treated with antibiotics frequently combined with cortisone [110]. Respiratory failure is the main cause of death in CF [111].

### *CFTR in pancreas*

Pancreatic insufficiency, characterized by obstruction of the pancreatic ducts, is seen in 80-90% of all CF patients [112, 113]. Mutations in CFTR that do not cause CF have been associated with pancreatitis [114]. Already as early as 17 weeks *in utero* pancreatic duct obstruction is visible [115]. It has been demonstrated that obstruction of the ducts in the pancreas and the biliary tree arise from a defective bicarbonate ( $\text{HCO}_3^-$ ) secretion. Digestive enzymes secreted from the acinar cells do not reach the small intestine but instead causes inflammation, destruction and fibrosis of the pancreas.[116, 117] CF patients with pancreatic insufficiency are administered digestive enzyme replacement treatment.

There is increasing evidence that CFTR is present and functional in the islet of Langerhans that constitute the endocrine part of the pancreas [118]. Several animal



studies have demonstrated that defective CFTR causes direct defects in glucose homeostasis and insulin release [93, 119-121].

#### *CFTR in the intestine*

The gastrointestinal tract can be affected already at birth with meconium ileus occurring in ~10% of CF infants [122]. Digestive problems are common including steatorrhea, excessive stool and constipation. Malabsorption of nutrients is an additional problem that leads to undernourishment and a low BMI.

Infections by *vibera cholera* and *enterobacteria coli* mainly target CFTR causing hypersecretion of Cl<sup>-</sup> and watery diarrhea. It has been suggested that heterozygous and homozygous CFTR mutation confer immunity to infection by enterobacteria [123-125].

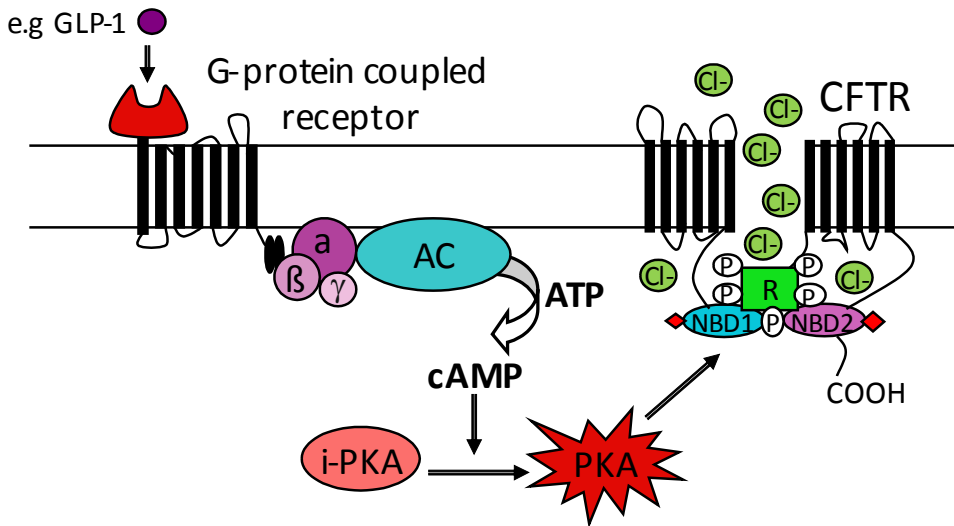
#### *CFTR in the reproductive organs*

Almost all men with CF are aspermic or hypospermic due to obstruction of vas deferens [126]. Also non-CF causing mutations in CFTR have been associated to male infertility [114]. The female genital tract is affected by thick mucus causing obstruction of the ducts and decreased fertility [127].

## **Cystic fibrosis transmembrane conductance regulator**

The cystic fibrosis transmembrane conductance regulator (CFTR) is encoded by the *CFTR/ABCC7* gene situated at chromosome 7q31. The sequence spans 6.1 kbp in the genome that encodes a 1480 amino acids membrane protein [106]. The gene sequence shares homology with the ATP-binding cassette (ABC) superfamily of proteins [95]. All ABC proteins are ATP-dependent transporters except CFTR that is an anion channel that conducts Cl<sup>-</sup> ions. CFTR have two membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs) and a regulatory domain (R-domain) (Fig, 5). The MSDs forms the ion selective pore. CFTR is activated by phosphorylation of the R-domain by cAMP-dependent PKA, and ATP binding and hydrolysis at the NBDs control channel gating [95].

Besides its function as an anion channel CFTR regulates other proteins and ion channels [128]. Numerous proteins and ion channels have been reported to be regulated by CFTR e.g. Stx1a [129], Stxbp1/Munc18A [129], SNAP-23 [130], ORCC [131, 132] and Anoctamin 1 (ANO1) [133]. In epithelial cells and in expression systems CFTR physically interacts with Stx1a, SNAP23 and Munc18 [129, 130]. SNAP23 and Stx1a bind to the N-terminal of the cystic fibrosis transmembrane conductance regulator (CFTR), an interaction that is disrupted by Munc18 [129].



**Figure 5. Activation of CFTR.** Activation of G-protein coupled receptor activates adenylate cyclase (AC) that converts ATP to cAMP that activates protein kinase A (PKA). Phosphorylation of the R-domain by PKA activates CFTR and binding of ATP (red squares) to the nucleotide binding domains (NBD1 and NBD2) control channel gating.

The most common mutation in CFTR is the deletion of a phenylalanine residue at position 508 (F508del) carried by 65-80% of the patients [134]. To date there are nearly 2000 known mutations in CFTR but not all are CF causing mutations [135]. CF is an autosomal recessive inherited disease. The mutations cluster to 5 different classes depending on how CFTR is affected [136]. The F508del mutation is a class 2 mutation and cause misprocessing of CFTR in the endoplasmic reticulum and subsequent degradation. However, a fraction of F508delCFTR escapes degradation and reaches the plasma membrane. In the membrane F508delCFTR has been shown to exhibit defective channel gating [137] and protein instability [138]. The G511DCFTR traffic to the plasma membrane but have reduced conductivity. A newly developed therapeutic drug Ivacaftor/VX-770 (Kalydeco®, Vertex pharmaceuticals) is a potentiator that restores channel gating [139]. The corrector Lumacaftor/VX-809 (Vertex pharmaceuticals) targets the trafficking defect in F508delCFTR. Combination treatment with Lumacaftor and Ivacaftor (Orkambi®, Vertex pharmaceuticals) partially rescue F508delCFTR trafficking and function [140, 141].

#### *Pharmacological inhibitors of CFTR*

At the moment there are two specific CFTR inhibitors CFTRinh-172 and GlyH-101 with different mechanism on the protein. CFTRinh-172 binds to the intracellular domain and inhibits the channel [142]. GlyH-101 is an open-channel blocker [143]. CFTR is insensitive to the general Cl<sup>-</sup> channel blocker DIDS [144].

## **Cystic fibrosis-related diabetes (CFRD)**

With increasing life-span in patients with CF, CF-related diabetes (CFRD) has emerged as a major clinical problem. Acquiring CFRD is associated with worsened prognosis and increased mortality [145]. CFRD affects 2% of children, 19 % of adolescents and 40-50% of adults [146]. The incidence increases with age at 5% /year in patients above the age of 10 and 9.3%/year in patients older than 20 years [147]. CFRD has its own etiology and do not fall into T1D or T2D [148].

CF patients without CFRD have preserved peripheral insulin sensitivity while modest insulin resistance is present in CFRD [149]. Insulin resistance can arise from decreased peripheral glucose uptake or increased hepatic glucose production [147, 150]. The latter have been shown in individuals with CF [145, 151]. Glucose intolerance is common in CF and almost all individuals with CF have a delayed insulin peak in response to an oral glucose challenge [148, 152]. Furthermore, in response to an intravenous glucose challenge individuals with CF have reduced first-phase insulin secretion [153, 154].

The etiopathology of CFRD is unclear but it is generally believed that the progressive destruction of the exocrine pancreas spreads to the islet of Langerhans causing diabetes with time [155]. However, recently this model has been questioned when it was reported that there is no correlation between pancreatic exocrine insufficiency and CFRD, since exocrine sufficient patients also have CFRD [112]. Moreover, there is no correlation between CFRD and loss of beta cell mass [148]. Recently, CFRD patients carrying gating mutations were treated with the newly developed CFTR potentiator Ivacaftor in two independent pilot studies [154, 156]. In both studies Ivacaftor restored first-phase insulin secretion and glucose tolerance improved in the majority of patients. Interestingly, CF patients with impaired glucose tolerance and CFRD have increased proinsulin levels in serum [157, 158].

Glucagon secretion is less well studied in CF but impaired glucagon secretion has been reported [153, 159]. The ability to suppress glucagon during a glucose challenge is increasingly impaired with decreased glucose tolerance [160, 161]. Glucagon increases hepatic glucose production. Individuals with CF regardless of the degree of glucose tolerance have increased hepatic glucose production [149, 151, 162].

### *The islet of Langerhans in CF animal models*

There are several animal models for CF. The CF ferret [119] and the CF pig [120] share features with CF in humans. In the CF ferret glucose intolerance was observed in kits before any signs of pancreatic insufficiency. The CF ferret kits have a reduced first-phase insulin secretion with an accentuated second phase. Isolated islets from CF ferret had a blunted response to glucose challenge [119]. The CF pig has alterations in exocrine pancreas already *in utero*. However, decline in glucose

tolerance and decreased insulin secretion starts before severe pancreatic disease develops [120]. The F508del mouse model on a FVB background develops insulin resistance with time (24 weeks) [163]. Beta cells in another strain of F508del mouse model (C57) had a more hyperpolarized resting membrane potential and an attenuated glucose-induced insulin secretion [93].

## Anoctamins

Anoctamin 1-10 (ANO1-10; TMEM16A-K) is a family of  $\text{Ca}^{2+}$ -activated proteins with various degree of known function. ANO1 and ANO2 have been identified as  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels. In epithelial cells the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current (CaCC) was identified to at least partly be mediated by ANO1 [164-166]. In human islets, *ANO1* expression is positively regulated by the insulin (*INS*) promoter and human islets treated with siRNA targeting ANO1 have reduced glucose-stimulated insulin secretion [167]. ANO1 KO mice die soon after birth due to tracheal malformations [168]. Heterozygous ANO1 mice fed a high fat diet have a reduced first-phase insulin secretion suggesting an intrinsic beta cell defect [167]. Moreover, in rodent beta cells, ANO1 regulates glucose-induced oscillations in the membrane potential. Inhibition of ANO1 using a chemical inhibitor or an ANO1 antibody was demonstrated to almost abolish glucose-stimulated insulin secretion in isolated rodent islets [94].

ANO3, ANO4, ANO6, ANO7 and ANO9 have been identified as  $\text{Ca}^{2+}$ -dependent lipid scramblases that transport phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma membrane [169]. Externalisation of PS is important for  $\text{Ca}^{2+}$ -dependent exocytosis in neurons and entero-chromaffin cells [170, 171]. In the inner leaflet PS are negatively charged and have been proposed to form lipid platforms that recruits signalling molecules involved in endocytosis [172].

Dominant mutations in ANO5 (GDD1) causes a rare skeletal disorder Gnathodiaphyseal dysplasia (GDD) [173] while recessive alleles have been associated with muscular dystrophy [174, 175]. The function of ANO5 *in vivo* is poorly understood. The ANO5 mutant mice have difficulties repairing muscular sarcolemma after injury [176]. Two KO strains have been characterized but failed to recapitulate the human phenotype [177, 178]. ANO5 do not seem to localize to the plasma membrane. One study on the role of ANO5 in development in mice found ANO5 to be predominantly expressed in intracellular membranes [179]. Overexpression of ANO5-GFP in HEK cells localized ANO5 to intracellular vesicles associated to Clathrin and Stx [180].

Mutations in ANO6 have been associated with a rare bleeding disorder, the Scotts syndrome, where platelets fail to expose lipids on the cell surface [181]. The exact

function of ANO6 is elusive. ANO6 has been reported to be a  $\text{Ca}^{2+}$ -activated scramblase [182] or a  $\text{Ca}^{2+}$ -activated cation channel [183, 184]. Yet another possibility is that ANO6 is a  $\text{Ca}^{2+}$ -activated ion channel that regulates a scramblase or vice versa [185].

# AIMS

The etiopathology of CFRD is largely unknown. The concept that the progressive inflammation of the exocrine pancreas eventually affects the endocrine part has been questioned. It has been proposed that an intrinsic islet cell defect contributes to the glucose intolerance seen in almost all affected by CF. Here we aim to investigate the presence and function of CFTR in pancreatic islet cells.

The specific Aims were:

- I. To investigate the presence and function of CFTR in human and mouse pancreatic beta cells. More specifically, examine if CFTR is involved in exocytosis and secretion of insulin granules in isolated human and mouse islets.
- II. To examine the presence and function of CFTR in human and mouse alpha- and delta-cells. Explore if CFTR affects glucagon and somatostatin release from human and mouse islets.
- III. To study glucose homeostasis in the F508del CFTR/*Cfr*<sup>TM1Eur</sup> mouse model of CF and to examine if the F508del mutation in CFTR affects hormonal secretion in isolated islets.
- IV. To investigate if the ANO1-10 family of Ca<sup>2+</sup>-activated proteins are differentially expressed in isolated islets from T2D human donors and if ANO1-10 expression correlates to HbA1c. Explore if ANO1 is involved in hormonal secretion and exocytosis in isolated islets from T2D and normal glucose tolerant donors.

# Material and Methods

The methods presented below are the main methods used in the work included in this thesis.

## The patch-clamp technique

In 1978 Erwin Neher and Bert Sakman published their ground breaking work on the patch-clamp technique of which they were rewarded the Nobel Prize in Physiology and Medicine 1991 [186]. The patch-clamp technique offers a tool to investigate ion channel activity and changes in membrane capacitance in a single cell.

A tight seal is established between the pipette tip and the cell membrane, referred to as a giga-seal with high electrical resistance ( $>1\text{G}\Omega$ ). When the giga-seal is established the cell-attached configuration enables measurements of single channel activity. By a pulse of negative pressure (suction) or pore-forming compounds (*e.g.* Gramicidin, Amphotericin B) the patch of membrane is ruptured or perforated and the whole-cell configuration or perforated-patch configuration, respectively is established. In the whole-cell configuration the cytosolic solution of the cell is replaced by the pipette solution. In perforated-patch the cell interior remains more intact and only ions and compounds  $<200$  Dalton can pass through the pores. The latter two configurations enable measurements of the summed ion-channel activity or changes in membrane capacitance in voltage-clamp mode. Using the perforated patch configuration, it is also possible to measure changes in membrane potential in current-clamp mode. From the cell-attached configuration the pipette can also be lifted up from the cell with the patch of membrane intact establishing inside-out configuration. Lifting the pipette in the whole-cell configuration establishes the outside-out configuration and enables measurements of single channel activity in the patch.

Here, the electrophysiological measurements were made in voltage-clamp mode and whole-cell or cell-attached configuration. Membrane currents and changes in membrane capacitance were monitored with a software controlled amplifier (EPC9, HEKA, Lambrecht/Pfalz, Germany) connected to a recording probe to which an AgCl coated electrode was attached. The current injections or cell membrane potentials were manipulated by software based stimulation protocols (Pulse or

Patchmaster, both from HEKA). A ground electrode/reference electrode in the extracellular solution connects the outside of the cell to the inside of the cell.

The cell membrane is composed of a lipid bilayer that in electrical terms can be viewed as a capacitor. The membrane capacitance ( $C$ ) is given by the equation:

$$C = (\varepsilon * A) / d$$

Where  $A$  is the surface membrane area,  $\varepsilon$  is the specific membrane capacitance ( $9\text{fF}/\mu\text{m}^2$ ) and  $d$  is the distance between the two layers of phospholipids.  $\varepsilon$  and  $d$  is constants and therefore  $C$  is proportionally related to  $A$  [187].

In exocytosis the fusion of granules adds membrane to the plasma membrane and hence causes an increase in the surface area membrane. This increase in membrane area can be monitored as an increase in cell capacitance [188]. By ultrastructural determination it has been estimated that fusion of a single insulin granule contributes 3 fF and, a glucagon granule 2 fF, while somatostatin granule contribute 1 fF to the measured increase in membrane capacitance. The granules in alpha- and beta-cells are considered to have spheroid shape [189].

In my thesis work, the increase in membrane capacitance was stimulated by a train of ten membrane depolarizations from -70 mV to 0 mV for 500 ms applied at 1 kHz sine wave. The first two depolarizations trigger exocytosis independent of ATP and are referred to as the readily releasable pool (RRP) constituting granules that are already docked and primed. The following eight depolarizations reflects granules that are ATP-dependent and need to be docked and primed before fusion and are referred to as the reserve pool (RP). [49, 190]

To differentiate the cell types in dispersed mouse islet preparations we investigated  $\text{Na}^+$  channel inactivation properties. The  $\text{Na}^+$  current is half maximally inactivated at  $\sim -70$  mV in beta cells,  $\sim -40$  mV in alpha cells and  $\sim -20$  mV in delta cells [191]. The human islet cells cannot be distinguished based on their electrophysiological properties. Instead, the fraction of cells bigger than 9 pF is considered to be beta cells. Below 9 pF the identity is uncertain and needs to be confirmed by immunocytochemistry [192].

## Hormonal secretion measurements

The hormonal secretion measurements were performed on isolated human and mouse islets. The isolation procedure involves injection of collagenase to digest the exocrine part of the pancreas. The incubation time is tightly regulated since the basement membrane enclosing the islets partly consists of collagen. Even though the organ is intact the islet are deprived of neurological and vascular innervation. Due to that oxygen has to diffuse into the inner parts of the islets the center of the islet might



suffer of hypoxia resulting in apoptosis and necrosis. Whether this affects hormonal secretion is unknown. The organ is still resilient and under the right conditions isolated islets can be cultured for months with retained hormonal secretion [193].

The glucose concentrations used for stimulation 1, 2.8, 6, 16.7 mM can seem odd but corresponds to 18, 50, 108, and 300 mg/dl glucose. Traditionally the intermediate glucose concentrations 8.3 and 11.1 mM are used corresponding to 150 and 200 mg/dl glucose. When the islets are stimulated at 1 mM glucose the basal or glucose-independent insulin and somatostatin secretion are measured. The most physiological glucose concentration is 6 mM which is in the upper limit for fasting conditions in humans. However, 16.7 mM glucose is traditionally used in measurements of insulin and somatostatin secretion to ensure that beta- and delta-cells are maximally stimulated.

It is also possible to distinguish effects on membrane depolarization or direct effects on exocytosis by stimulating the islets with high  $K^+$ . Supplementing Krebs ringer solution with  $>50$  mM  $K^+$  depolarizes the cells. The resting membrane potential is determined by the  $K^+$  equilibrium potential. When extracellular  $K^+$  ions are added a new  $K^+$  equilibrium potential is established that shift the resting membrane potential to more positive potential. A resting membrane potential above the threshold for voltage-dependent  $Ca^{2+}$  channels causes influx of  $Ca^{2+}$ . Thereby the natural depolarization of the cell is by-passed and any direct effects of voltage-gated  $Ca^{2+}$  channels or exocytosis can be examined.

The islets are deprived from their normal distribution system i.e. blood vessels and all chemicals have to diffuse into the layers of cells that make up the islet. Therefore, a higher concentration of chemical compounds is traditionally used in whole islet stimulation than in single cell stimulations.

Hormonal content in the supernatant was analyzed with radioimmunoassay (RIA). In RIA analysis radiolabeled antigen is mixed with a known quantity of antibody. The unknown antigen/hormone content is competing with the radiolabeled antigen for binding to the antibody. The amount of unbound radiolabeled antigen is measured in a gammacounter to determine concentration in the unknown sample. A draw-back with this method is that it is based on competing binding and only the relative concentration of antigen can be determined and not absolute concentration. This contributes to experimental variations.

## Immunochemistry

Immunochemistry offers a tool to view proteins or peptides within a cell (immunocytochemistry) or tissue (immunohistochemistry). The technique is based on antibody-antigen binding where a primary antibody is raised in an animal against

the protein of interest (antigen). The secondary antibody recognizes the species in which the primary antibody was raised. In addition, the secondary antibody is conjugated to a fluorescent-tag that enables visualization.

### *Immunocytochemistry*

A confocal laser microscope was used to excite the secondary antibody fluorophore (Zeiss instruments, Germany) and the emitted wavelength was measured by Zen software. With immunocytochemistry it is possible to determine where in the cell a protein is localized. An antibody signal within 0.5  $\mu\text{m}$  in the outer borders of the cell is considered to be localized in the plasma membrane compartment. The nuclei can be visualized with DAPI staining. The compartment between nuclei and plasma membrane are considered to be cytosol. To calculate if the protein has a higher plasma membrane expression than in the cytosol the single cells were analyzed in Zen software and plasma membrane expression (*PI*) calculated by the equation:

$$PI = (((S1 - S2)/(a1 - a2)) - ((S2 - S3)/(a2 - a3)))$$

Where S1 is the total fluorescent intensity in the cell, S2 is the fluorescent intensity in the cytosol and nucleus, S3 is the fluorescent intensity in the nucleus, a1 the total area in the cell, a2 is the area of the cytosol and nucleus and a3 is the area of the nucleus.

Zen software was also used to analyze pixel by pixel co-localization in double- and triple staining.

### *Immunohistochemistry*

Whole pancreas embedded in 4% paraformaldehyde was sliced in 5  $\mu\text{m}$  sections in a microtome and mounted on cover slip. An epifluorescence microscope was used to detect immunofluorescence (Olympus BX60, Olympus, Japan).

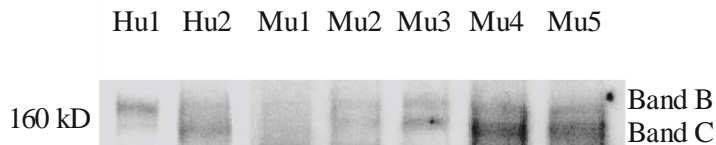
In three different parts of the pancreas (at least 200  $\mu\text{m}$  apart) all islets were quantified based on insulin, glucagon and/or somatostatin antibody signal as well as total section area. To determine beta- alpha- and delta-cell mass the antibody signal for each hormone was marked in NIS-Elements software and the area summed. Total area was divided by total section area and the mass expressed as a ratio between the two areas.

# Results paper I-IV

## Paper I

*CFTR is expressed in human and mouse pancreatic beta cells.*

We could detect CFTR protein in human and mouse beta cells using confocal immunocytochemistry. In addition, we found a higher CFTR expression in the plasma membrane compartment compared to the cytosol. Gene expression of *CFTR* was confirmed with qPCR. Further presence of CFTR in human and mouse islet of Langerhans was shown using western blot analysis (unpublished, Fig 6). The band is weak but CFTR is not easily detected on protein level in primary tissue as reflected by the lack of published blots. A major issue is to dissolve CFTR from the membranes and avoid that precipitation of CFTR that occurs  $\sim >60^{\circ}\text{C}$ . Therefore, to my knowledge, all published blots are from CFTR overexpressing cell-lines.



**Figure 6. Western blot of CFTR.** CFTR was detected in islets from two human donors and five mice using M3A7 Mab.

*A CFTR-dependent whole-cell current was evoked by forskolin in single human and mouse beta cells.*

CFTR whole-cell current was measured in human and mouse beta cells using the patch-clamp technique. A ramp protocol from -100 mV to +100 mV was applied on the cell and the evoked current measured. A small increase in current was measured in the absence of the cAMP-increasing agent forskolin. Addition of forskolin caused a large increase in current that was almost totally inhibited by the CFTR inhibitors CFTRinh-172 and GlyH-101. The GlyH-101 inhibitor could be washed out and the current rescued. However, the forskolin-evoked current had a strong outward

rectification not typical to CFTR whole-cell current. We investigated the identity of the rectifying Cl<sup>-</sup> current using the general chloride channel blocker, DIDS or an ANO1 inhibitor. Application of either DIDS or the ANO1 inhibitor reduced a large part of the cAMP-activated current and the small remaining part of the current was inhibited by CFTR inhibitors.

*Inhibition of CFTR decreases cAMP-amplified insulin secretion in human and mouse islets.*

We performed insulin secretion measurements in isolated human and mouse islets to investigate if CFTR affects insulin secretion. Forskolin or the more physiological cAMP increasing agent GLP-1 was added during islet stimulation to elevate intracellular cAMP and both compounds amplified insulin secretion. In both cases the increase was reduced by CFTR inhibition. Addition of DIDS or ANO1 inhibitor reduced GLP-1 stimulated insulin secretion. In mouse there was no additional effect by DIDS or ANO1 inhibitor after CFTR inhibition by GlyH-101. However, in the human islets simultaneous presence of DIDS or the ANO1 inhibitor and GlyH-101 produced a stronger reduction in GLP-1-stimulated insulin secretion compared to GlyH-101 alone.

*Inhibition of CFTR decrease exocytosis in human and mouse beta cells*

Depolarization-induced insulin secretion was initiated with high K<sup>+</sup> during 15 min to measure insulin secretion downstream of depolarization. In both human and mouse islets depolarization-induced and cAMP-enhanced insulin secretion was reduced in presence of the CFTR inhibitors GlyH-101 and CFTRinh-172.

In both mouse and human beta cells addition of CFTRinh-172 or GlyH-101 reduced the exocytotic response measured as a depolarization-induced increase in membrane capacitance. In the mouse beta cells both the RRP and RP was reduced while in the human beta cells the strongest effect of CFTR inhibition was on RRP.

*CFTR inhibition reduced the number of docked granules.*

To investigate if CFTR inhibition causes morphological changes in the beta cells, islets were collected for transmission electron microscopy (TEM) following secretion measurements. Analysis of the TEM micrographs revealed that the number of docked granules ( $N_s$ ; granules/ $\mu\text{m}^2$ ) was reduced in beta cells where CFTR had been inhibited. There were no differences in total number of granules ( $N_v$ ; granules/ $\mu\text{m}^3$ ).

## Paper II

*CFTR is expressed in human and mouse alpha cells.*

Confocal immunocytochemistry confirmed the presence of CFTR in single human and mouse alpha cells. A higher expression of CFTR was found in the plasma membrane compartment compared to the cytosol.

*A cAMP activated current in mouse alpha cells.*

Single mouse alpha cells were investigated using the standard whole-cell configuration of the patch-clamp technique. A ramp protocol from -100 mV to +100 mV evoked a measurable current that was significantly increased by forskolin. The induced increase in current was almost totally inhibited by CFTRinh-172.

We performed cell-attached single channel recordings on human dispersed islet cells. Due to the difficulties identifying cell type when performing NMDG-Cl recordings we aimed for small cells. Small channel openings of ~0.7 pA were recorded with increased opening frequencies when forskolin was added to the bath. The single-channel recordings measured on small islet cells shared similarities with CFTR single channel currents [194].

*Inhibition of CFTR enhances glucagon secretion in human and mouse islets.*

In human islets forskolin amplified glucagon secretion at 2.8, 6 and 16.7 mM glucose an effect that was further enhanced by the CFTR inhibitor GlyH-101. Likewise, glucagon secretion at 1 mM glucose in mouse islets was amplified in presence of forskolin and further enhanced by GlyH-101. In mouse islets cAMP-enhanced glucagon secretion at 16.7 mM glucose was unaffected by CFTR inhibition.

*CFTR have different effect on depolarization induced secretion in human and mouse alpha cells*

To investigate if CFTR affects exocytosis downstream of depolarization, human and mouse islets were subjected to 50 mM K<sup>+</sup> for 15 min. In human islets the CFTR inhibitor GlyH-101 or ANO1 inhibitor T16Ainh-AO1 had no effect on cAMP-amplified and depolarization-induced glucagon secretion. However, under these conditions glucagon secretion was reduced in mouse islets in presence of ANO1, CFTRinh-172 and GlyH-101. Addition of the ANO1 inhibitor reduced cAMP-amplified and depolarization-induced glucagon secretion and the simultaneous presence of GlyH-101 had no further effect. These results suggest that CFTR have a direct effect on exocytosis in mouse alpha cells while in the human alpha cells CFTR mainly affect electrical activity.

To investigate if inhibition of CFTR has direct effects on exocytosis in mouse alpha cells capacitance measurements were performed on single alpha cells. Alpha cells

pre-incubated with CFTRinh-172 had a reduced exocytotic response and both RRP and RP were reduced compared to untreated alpha cells.

### *Mathematical modeling of alpha cell electrical activity*

Based on a published mathematical model of alpha cell electrical activity [195] we tested the hypothesis that CFTR control electrical activity in the alpha cell. We inserted the cAMP-dependent  $\text{Cl}^-$  conductance and simulated different  $\text{K}_{\text{ATP}}$  channel conductance [195]. Simulating the situation corresponding to the presence of 6 mM glucose with low  $\text{K}_{\text{ATP}}$  conductance ( $g_{\text{KATP}} = 0.200 \text{ nS}$ ), the electrical activity was silent and inhibition of CFTR led to a slight increase in action potential firing. Simulating the presence of 1 mM glucose ( $g_{\text{KATP}}$  was = 0.270-0.300 nS) when glucagon secretion is normally stimulated, removal of the cAMP-dependent  $\text{Cl}^-$  conductance increased action potential firing. At a  $g_{\text{KATP}}=0.300 \text{ nS}$  (still responding to 1 mM glucose) the alpha cell was silent but inhibition of CFTR activated the alpha cell to fire action potentials.

### *Somatostatin secretion is reduced by CFTR inhibition*

In human islets somatostatin secretion in 2.8, 6 and 16.7 mM glucose was enhanced by forskolin. Inhibition of CFTR increased the cAMP-stimulated somatostatin secretion further at the two lower glucose concentrations. In mouse islets neither forskolin nor the CFTR inhibitors had any effects on somatostatin secretion at low glucose. At high glucose (16.7 mM) somatostatin secretion was increased in presence of forskolin and the increase was reduced when CFTR was inhibited. Interestingly, depolarization induced and cAMP-enhanced somatostatin secretion was reduced in the presence of CFTR inhibitors in both human and mouse islets. Notably, inhibition of ANO1 did not differ from that of the CFTR inhibitors and the simultaneous presence of the ANO1 inhibitor and CFTRinh-172 or GlyH-101 had no further effect.

## Paper III

The CFTR<sup>TM1Eur</sup> mouse model carries the most common human mutation in *CFTR*, the deletion of a phenylalanine at position 508 (F508del). The strain used here was on a C57bl/6HJ background [196].

### *In vivo glucose tolerance in the F508del mouse model of CF*

Glucose tolerance was assessed *in vivo* by an intra peritoneal glucose tolerance test (IPGTT) for 120 min and blood glucose levels were measured. At time-point 0 F508del mice had lower fasting blood glucose levels. During the IPGTT the F508del mice had a lower increase in blood glucose compared to WT mice when calculated as

area under the curve (AUC). In the WT mice blood glucose levels peaked after 10 min but in F508del mice 6 out of 10 animals had a blood glucose peak after 30 min.

Blood glucose was normalized to time-point 0 to measure glucose clearance. At time-point 10 min both F508del mice and WT mice had doubled their blood glucose. In the WT mice this was followed by a continuous lowering of the blood glucose which returned to the fasted state after 120 min. However, in the F508del mice glucose clearance was delayed and did not return to fasted state within the sampling period (120 min).

Furthermore, serum glucagon was three-fold higher in F508del mice compared to WT mice, while, there was no difference in serum insulin levels between WT and F508del mice.

#### *Increased proinsulin and decreased c-peptide secretion in F508del islets*

To investigate hormonal secretion in detail isolated islets from F508del and WT mice were subjected to hormonal secretion measurements. Basal insulin secretion was lower in F508del islets compared to WT islets. In response to glucose and in the presence or absence of forskolin, there was no major difference in the insulin secretion response between WT and F508del islets. The CFTR inhibitors reduced cAMP-enhanced insulin secretion in both WT and F508del islets. However, in paper I, we proposed a model where CFTR is involved in priming of the insulin granule. We hypothesized that CFTR is needed for changes in granular pH. As cleavage of proinsulin to insulin is a pH-dependent process we wanted to investigate if CFTR might impact this process. The insulin RIA cannot distinguish between proinsulin and insulin. We therefore specifically measured proinsulin and c-peptide secretion in the same samples as insulin. The F508del islets released more proinsulin during cAMP-stimulation and correspondingly less c-peptide compared to WT islets.

#### *F508del islets have reduced insulin content and beta cell mass*

Insulin content in F508del islets were reduced compared to WT islets. Further immunohistological examination of the F508del pancreas revealed a decrease in beta cell mass. Interestingly, the number of nuclei in the insulin stained area was similar between WT and F508del pancreatic sections. To assess if the F508del beta cells contain less granules per cell we combined beta cell area measurements (from the confocal images) and granular density (from TEM analysis). From these data we estimated that F508del beta cells have ~10,500 granules as opposed to WT beta cells ~13,300 granules, an ~20% reduction. Interestingly, insulin content was reduced by ~20%. Moreover, analysis of the TEM images revealed reduced number of docked granules in the F508del beta cells.

### *F508del islets have elevated glucagon secretion*

Isolated islets from F508del mice had increased glucagon secretion in response to 1 mM glucose. The cAMP-enhanced glucagon secretion was blunted compared to islets from WT mice. Moreover, F508del islets failed to decrease glucagon secretion when glucose was elevated to 16.7 mM.

### *Somatostatin secretion is dysregulated in F508del islets*

Somatostatin secretion was elevated already at 1 mM glucose in F508del islets as compared to WT islets. Secretion was further enhanced by increasing glucose concentration to 16.7 mM in both WT and F508del islets. As expected, forskolin-enhanced the glucose-stimulated somatostatin secretion in WT islets. In contrast, in F508del islets secretion was reduced by forskolin. Inhibition of CFTR reduced cAMP-stimulated somatostatin secretion in the WT islets while in the F508del islets somatostatin secretion was elevated.

## Paper IV

### *Anoctamin 1-10 expression in RNA seq data*

Anoctamin 1-10 (ANO1-10) gene expression was investigated in islets from human cadaver donors with normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and type 2 diabetes (T2D) using post-processed normalized expression levels from published RNAseq data [197]. We found expression of *ANO1*, *ANO6*, *ANO8* and *ANO9* to be upregulated in islets from IGT/T2D donors and to be positively correlated to HbA1c. Gene expression of *ANO5* was downregulated in IGT/T2D islets with negative correlation to HbA1c. The gene expression of *ANO1*, *ANO7*, *ANO8*, and *ANO9* positively correlated while *ANO5* and *ANO10* expression negatively correlated to *INS* gene expression.

### *Inhibition of ANO1 diminish insulin secretion*

We found that islets from T2D donors secrete only 60% of the insulin secretion levels detected in islets from NGT donors at 16.7 mM glucose. Next, the effects of the Ca<sup>2+</sup> activated Cl<sup>-</sup> channel (CaCC) inhibitors CaCCinh-AO1 and T16inh-AO1 on insulin secretion was investigated using isolated islets from NGT and T2D donors. The CaCCinh-AO1 did not affect insulin secretion in islets from NGT donors but restored insulin secretion in islets from T2D donors to the same levels as measured in the islets from the NGT donors. In contrast, T16inh-AO1 severely diminished insulin secretion in presence or absence of cAMP in islets from both NGT and T2D donors, while basal insulin secretion was unaffected.



*T16Ainh-AO1 decrease exocytosis partly by reducing Ca<sup>2+</sup> influx*

Depolarization-induced and cAMP-enhanced insulin secretion was reduced by T16Ainh-AO1 in islets from NGT donors. Therefore we investigated if T16Ainh-AO1 affects exocytosis by capacitance measurements using the patch-clamp technique. T16Ainh-AO1 reduced the total capacitance increase in beta cells from both NGT and T2D donors, both RRP and RP was reduced. The inward voltage-dependent currents evoked from -70 mV to depolarizations from -40 to +40 mV were measured in presence of T16Ainh-AO1. The Na<sup>+</sup> current was unchanged by T16Ainh-AO1. In contrast, both the Ca<sup>2+</sup> current and Ca<sup>2+</sup> charge were reduced by T16Ainh-AO1.

*T16Ainh-AO1 enhances glucagon secretion in T2D islets*

Glucagon secretion was increased in islets from T2D donors and the islets were unresponsive to elevation of glucose and cAMP. When the alpha cells from NGT donors were subjected to the CaCC inhibitors there was no change in glucagon secretion. In contrast, T16Ainh-AO1 further enhanced glucagon secretion in islets from T2D donors.

# Discussion paper I-IV

## CFTR and ANO1 in beta cells

*CFTR interacts with the  $Ca^{2+}$ -activated  $Cl^-$  channel ANO1.*

We made the novel finding that CFTR is present and have a function in human pancreatic beta cells. We noticed that the cAMP-dependent  $Cl^-$  current evoked in beta cells was strongly outward rectifying (i.e. the current increase is bigger the more positive the membrane potential) and not a typical CFTR whole-cell current (Fig 2A, Paper I), which might suggest that other  $Cl^-$  channels are involved. CFTR has been suggested to regulate an unidentified  $Cl^-$  conductance, the outward rectifying  $Cl^-$  channel (ORCC) [131, 132]. Lately, it has been suggested that the  $Ca^{2+}$ -activated  $Cl^-$  channel anoctamin 1 (ANO1) is a part of ORCC in epithelial cells [133, 198-200]. Indeed, in paper I we show that CFTR regulates another  $Cl^-$  conductance (Fig 2E-F) and that this conductance is, at least partly, mediated by ANO1 in beta cells (Fig 3F, Paper I).

*Insulin secretion is dependent on CFTR and ANO1.*

In paper I, mouse islet insulin secretion enhanced by GLP-1 was reduced when CFTR was inhibited (Fig 1D). Moreover, there was no additional effect by the combined effect of DIDS or ANO1 inhibitor and GlyH-101 in mouse islets (Fig 3A and C, Paper I). This suggests that CFTR act up-stream of ANO1 consistent with the current measurements.

In the human islets insulin secretion was almost completely abolished by ANO1 inhibition (Fig 2A, paper IV). The reduction caused by ANO1 inhibition was more potent than inhibition of CFTR alone. However, when ANO1 and CFTR were simultaneously inhibited there was no additional reduction compared to ANO1 inhibition alone (Fig 3D, Paper I). Hence, CFTR is not the only factor that influences the function of ANO1 in human islets and the activation of ANO1 and role in insulin secretion is most likely affected by additional factors. Indeed, in paper IV show that ANO1 is also activated by glucose alone.

Interestingly, in paper III the F508del islets were found to secrete increased levels of proinsulin and decreased levels of c-peptide (Fig 4E-F, Paper III). It was especially pronounced during cAMP stimulation when the demand on the secretory capacity of the beta cells is increased. Furthermore, when insulin secretion was expressed in

relation to islet insulin content F508del islets had a two-fold increase in insulin secretion, again especially evident during cAMP-stimulation (Fig 4D, Paper III). Taken together, these findings suggest that the F508del beta cells are dysfunctional. The F508del mice on FVB background are insulin resistant at 24 weeks of age (Fontes 2016). It is tempting to speculate that this dysfunction stresses the beta cells and with time, as the age-dependent insulin resistance increases, this accounts for the diminished insulin secretion in CFRD [148].

#### *Inhibition of CFTR decrease exocytosis in human and mouse beta cells*

CF patients often lack first-phase insulin secretion [152], a defect reported to be rescued by Ivacaftor treatment [154]. In paper III we found that 6 out of 10 F508del mice had higher blood glucose levels after 30 min during the IPGTT suggesting that the F508del beta cells have a delayed glucose response (Fig 1D, Paper III). Moreover, our data in in paper I is in agreement with a reduced first-phase insulin secretion in CF. Previous data have suggested that first-phase insulin secretion can be coupled to the exocytotic fusion of RRP granules on a cellular level [49, 190, 201]. Therefore, the measured reduced exocytotic response in beta cells after CFTR inhibition (Fig 4C and E, paper I) is indicative of a functional role of CFTR in first-phase insulin secretion.

Exocytosis of insulin-containing granules is highly dependent on  $\text{Ca}^{2+}$  influx through voltage-gated  $\text{Ca}^{2+}$  channels [202]. The CFTR inhibitors GlyH-101 or CFTRinh-172 did not affect  $\text{Ca}^{2+}$  current or charge (Fig F-G, Paper I). In contrast, the ANO1 inhibitor T16Ainh-AO1 reduced both  $\text{Ca}^{2+}$  current and charge (Fig 3G-I, Paper IV). There are two plausible explanations for these results 1) T16Ainh-AO1 have unspecific effects causing direct inhibition of  $\text{Ca}^{2+}$  channels, 2) Inhibition of ANO1 indirectly affects voltage-gated  $\text{Ca}^{2+}$  channels due to protein interaction or loss of  $\text{Cl}^-$  conductance. An argument in support of the latter is that T16Ainh-AO1 did not affect glucagon secretion in islets from NGT donors (Fig 4A, Paper IV). In islets from T2D donors the inhibitor actually enhanced glucagon secretion (Fig 4B, Paper IV), similar to the results obtained with the CFTR inhibitors in islets from NGT donors (Fig 1A-C, Paper II). Exocytosis in alpha cells is highly dependent on influx of  $\text{Ca}^{2+}$  through voltage-dependent  $\text{Ca}^{2+}$  channels [65]. If T16Ainh-AO1 had unspecific effects on voltage-gated  $\text{Ca}^{2+}$  channels also glucagon secretion would have been reduced.

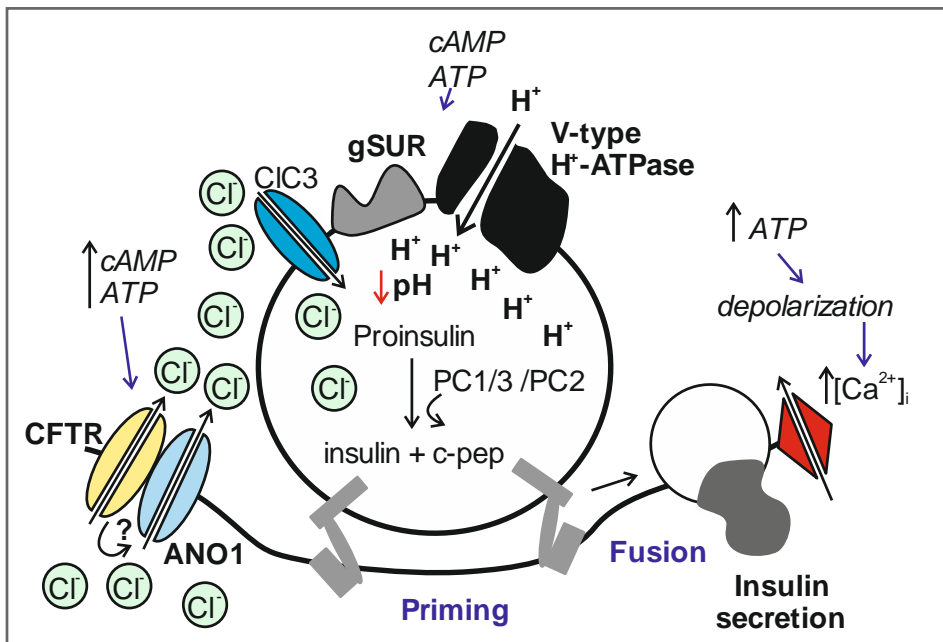
CFTR has been shown to be regulated by Stx1a and indeed, we found that CFTR and Stx1a co-localizes in human beta cells (Fig 5E, Paper I). Moreover, the number of docked granules was reduced in F508del beta cells (Fig 3F, Paper III) as well as after CFTR inhibition (Fig 5C, Paper I) measured by TEM. These results places CFTR in the exocytotic protein complex and supports the idea that in beta cells CFTR have a direct role in exocytosis.

*The beta cell mass is decreased in F508del mouse due to reduced beta cell size.*

The F508del mouse has decreased beta cell mass (Fig 3A, Paper III) in line with previous observations in the F508del mouse on a FVB background [163]. Fontes and colleagues did not investigate if the reduced beta cell mass is due to a reduction in size or number of beta cells. We found that F508del beta cells are smaller than WT beta cells and both the number of granules and islet insulin content was reduced by 20%, although volume density of granules was unchanged. The reduced beta cell size in F508del islets might therefore lead to a reduced number of available insulin granules. This needs further investigation and might arise from defects during beta cell development or changes in cellular osmosis due to defects in anionic homeostasis.

*A model of CFTR and ANO1 in beta cells.*

It is well-established that  $\text{Cl}^-$  ions are crucial for granular priming and that application of DIDS inhibit exocytosis and insulin secretion [54, 203]. Our data support a role for CFTR in priming and based on these findings we suggest a model where CFTR is involved in priming of the insulin granule (Fig 7).



**Figure 7. Proposed model on CFTR function in CFTR.** Priming prepare granules for exocytosis by lowering the pH. During priming the prohormone convertases (PC1/3 and PC2) cleaves proinsulin to insulin and c-peptide a process that needs a pH of 5.5. To acidify the granule protons are pumped into the granule via a V-type  $\text{H}^+$ -ATPase.  $\text{Cl}^-$  ions are simultaneously pumped into the granule via the chloride channel 3 (CIC3) to avoid the build-up of an electrostatic gradient. In this model CFTR and ANO1 provide CIC3 with  $\text{Cl}^-$  ions during membrane depolarization.

Acidification of the granule to a pH ~5.5 are necessary for optimal enzymatic activity of prohormone convertases PC1/3 and PC2 that catalyzes the cleavage of proinsulin to insulin and c-peptide. This is obtained by an ATP-dependent entry of protons via the V-type H<sup>+</sup>-ATPase. CFTR is associated with the exocytotic protein complex where it regulates ANO1. During membrane depolarization chloride ion enter the beta cell above a membrane potential of ~-40 mV. Cl<sup>-</sup> influx via ANO1 and to a lesser extent CFTR provides the proton counter-ion Cl<sup>-</sup> that enters the granule via ClC3. The driving force on Cl<sup>-</sup> ions is maintained by ATP-dependent entry of protons. This is a crucial step in priming to mature the insulin granule and prime it for release.

## **CFTR in alpha cells**

*In human islets CFTR negatively regulates glucagon secretion by direct effects on electrical activity.*

Glucagon secretion was enhanced when CFTR was inhibited in human islets (Fig 2A-C, paper II). Opposed to the human beta cell, ANO1 did not affect glucagon secretion in islets from NGT donors. In human islets, depolarization-induced glucagon secretion was unaffected by CFTR inhibition (Fig 5B, Paper III), suggesting that CFTR most likely do not affect exocytosis through regulation of ANO1 in alpha cells. We measured a higher CFTR-dependent conductance in alpha cells than in beta cells (Fig 4C-E, Paper II). Hence, it is more likely that CFTR influence electrical activity in alpha cells. Indeed, using mathematical simulations of the alpha cell confirmed that CFTR regulates electrical activity by two mechanisms. 1) Inhibition of CFTR increases alpha cell action potential firing frequency (7B, Paper II). In our measurements the cAMP-dependent current had a reversal potential at ~-60 mV which is close to the resting membrane potential of the alpha cell ~-60 mV [20] therefore activation of CFTR causes influx of Cl<sup>-</sup>. The alpha cell has a very high input resistance and small changes in membrane potential have big impact on electrical activity [20, 64]. A small Cl<sup>-</sup> current could therefore slow down action potential firing in these cells. 2) Alpha cells that are hyperpolarized and normally silent were activated (Fig 7C, Paper II) when CFTR was inhibited suggesting that the Cl<sup>-</sup> conductance counteract depolarization.

Interestingly, in islets from T2D donors the ANO1 inhibitor further enhanced the already elevated glucagon secretion (Fig 3B, paper IV). ANO1 is overexpressed in human T2D islets but it is not known if the overexpression applies to all cell types. However, it is clear that ANO1, similar to CFTR have an inhibitory action in T2D alpha cells.

### *CFTR have direct effects on exocytosis in mouse islets*

F508del mice had a three-fold increase in serum glucagon compared to WT mice (Fig 1C, Paper III). Isolated islets from F508del mice had increased glucagon secretion and a blunted response to cAMP compared to WT islets (Fig 5A, Paper III). Moreover, the F508del alpha cells were unresponsive to elevation of glucose (Fig 5B, Paper III). Furthermore, glucagon secretion measured in isolated mouse islets was increased when CFTR was inhibited (Fig 3A, Paper II).

In contrary to the human islets, depolarization-induced and cAMP-amplified glucagon secretion in mouse islets was reduced when CFTR was inhibited suggesting that CFTR have additional direct effects on exocytosis in mouse islets (Fig 5C, Paper II). Indeed, when exocytosis was measured as an increase in membrane capacitance during a train of ten depolarizations, the inhibition of CFTR reduced the exocytotic response (Fig 5F, Paper II). Both the RRP and recruitment of granules from the reserve pool was affected. Thus, in mice alpha cells CFTR have two functions; 1) as one of the regulators of electrical activity (as in the human alpha cells) and, 2) as part of the exocytotic machinery. These results show species differences which are not surprising considering the different organization and morphology of human and murine islets.

### **CFTR in delta cells**

Immunohistochemical investigation of human islets revealed that CFTR is not expressed in delta cells (Fig 1A, paper II). In isolated human islets cAMP-stimulated somatostatin secretion at 1 mM and 6 mM glucose was slightly increased when CFTR was inhibited (Fig 2 D-E, paper II). Glucagon was simultaneously increased at these glucose concentrations (Fig 2 A-B, paper II). Basal (1 mM glucose) insulin secretion was not affected by CFTR inhibition (Fig 1A, paper I). With increasing glucose concentration, the inhibition of CFTR increasingly impaired insulin secretion with the major effect at 16.7 mM glucose (Fig 1A, paper I), whereas somatostatin secretion was unchanged when CFTR was inhibited (Fig 2F, paper II). Glucagon has been demonstrated to directly stimulate somatostatin secretion and our data therefore suggest that glucagon exert paracrine stimulation of the human delta cells.

In the F508del islets somatostatin and glucagon secretion was simultaneously increased at all glucose concentrations compared to WT. Furthermore, in the presence of forskolin glucose-stimulated somatostatin secretion was inhibited at the same time as proinsulin secretion was elevated. It is possible that reduced secretion of active insulin directly affects the delta cells and reduces somatostatin secretion due to loss of insulin stimulation. Indeed, pharmacological inhibition of CFTR reduced insulin and somatostatin secretion when measured from the same samples i.e. the beta- and delta-cells where under equal stimulatory conditions. These data suggest that insulin can exert feed-back stimulation on somatostatin secretion. Interestingly,

somatostatin has been suggested to confer tonic inhibition on insulin secretion to prevent over-secretion and hypoglycemia [76].

There seem to be species differences regarding paracrine regulation, which is not surprising since the architecture and morphology is very different between species. In both human and mouse islets glucagon stimulate somatostatin secretion at low and medium glucose concentration. In the mouse islets insulin has a feed-back stimulation on delta cells which is evident during glucose- and cAMP-stimulated conditions. In human islets, this effect is weaker if even existing.

# Conclusion

In this thesis I have investigated the novel function of CFTR in human and mouse pancreatic islets. Our findings contribute to a plausible explanation for the pancreatic endocrine pathology observed in CF.

The following conclusions were achieved:

- I. CFTR is present in human and mouse pancreatic beta cells. CFTR regulates another  $\text{Cl}^-$  channel, most likely ANO1. Pharmacological inhibition of CFTR reduced cAMP-enhanced insulin secretion by direct effects on exocytosis. Based on our results we suggest a model where CFTR is involved in priming of insulin granules by providing the necessary  $\text{Cl}^-$  ions.
- II. CFTR is present in human and mouse alpha cells but not in delta cells. Pharmacological inhibition of CFTR enhanced glucagon secretion in both human and mouse islets. By mathematical modelling of the alpha cell electrophysiology the CFTR-dependent current was found to negatively regulate electrical activity. In the mouse alpha cells CFTR have additional direct effects on exocytosis.
- III. The F508del/CFTR<sup>TM1Eur</sup> mouse has a delayed response to a glucose challenge and reduced glucose clearance during the IPGTT. Isolated F508del islets secreted increased levels of proinsulin with a corresponding decrease in c-peptide secretion during cAMP-stimulation. Together with a reduced number of docked granules these results support that CFTR is involved in granule priming and is necessary for cleavage of proinsulin to insulin and c-peptide. The F508del mouse had increased levels of serum glucagon and isolated F508del islets had increased glucagon secretion supporting the results in paper II.
- IV. ANO1, ANO5, ANO6, ANO8 and ANO9 are differentially expressed in islets from T2D islets compared to islets from NGT donors. Pharmacological inhibition of ANO1 severely diminishes insulin secretion by direct effects on  $\text{Ca}^{2+}$  influx and exocytosis. In NGT islets glucagon secretion is unaffected by ANO1 inhibition while the inhibitor further increases the already enhanced glucagon secretion in T2D islets.



# Future perspectives

The contribution of this thesis to recognize and bring attention to the presence of an intrinsic alpha and beta cell defect in CF is of special importance and raises the question to who might benefit from insulin treatment. CF is signified by catabolism where a decline in BMI often precedes worsened pulmonary function [204]. Insulin is an anabolic hormone that promotes growth and energy storage. Therefore, it might be beneficial to introduce insulin treatment before actual onset of CFRD.

Individualized treatment in CF has drawn attention in recent years. It is well-established that two patients with the exact same CFTR mutation have different disease and outcomes. The development of the CFTR gating corrector Ivacaftor was a great success and has opened the venue for research on other small molecules correctors targeting mutations in CFTR. Two small pilot studies on Ivacaftor treatment showed improvements in glucose tolerance mainly by improving first-phase insulin secretion in patients with gating mutations [154, 156]. Our results presented in this thesis support a function for CFTR in first-phase insulin secretion. The need to find personalized treatment is the future in CF care in which insulin treatment should be considered and explored.

Excessive glucagon secretion exacerbates glucose intolerance and is therefore also a concern in CF. We have demonstrated that the F508del CF mouse model hypersecrete glucagon and that pharmacological inhibition of CFTR enhances glucagon secretion. There is no consensus on if exogenous insulin directly targets glucagon secretion. Therefore, future developments of CFTR correctors that target all types of defects are of special importance and are likely to also correct the alpha cell defect.

Taken together I think that there is an urgent need in the field to establish proper treatment for glucose intolerance in CF. The notion that CFRD onset is preceded a decline in pulmonary function and a decline in BMI point to a potential benefit from early insulin treatment. Few CF patients have true normal glucose tolerance and although having normal fasting glycaemia as well as normal 2h OGTT their glucose levels are often increased in the middle of the OGTT (indeterminant glycemia). Fasting hyper glycaemia may also be present. [205] These glucose abnormalities are considered pre-diabetic in the general population [206]. Interestingly, one study showed that administration of pre-meal insulin improved weight in patients with CFRD without fasting hyperglycemia. [207]. Pre-diabetic CF-patients that are at the

course to develop CFRD are those who might benefit the most from insulin treatment. Another controversial question is if not most of the glucose intolerant patients, which are the majority of the CF patients, would actually gain from low dosage insulin. A study on pre-meal insulin treatment in IGT patients showed great variability in weight gain in the study group [207]. Again showing that CF is not a homogenic disease and some patients might actually benefit from early insulin treatment while others do not. To whom, when and at which dose insulin should be administered are questions that need to be solved. The potentially inevitable progression towards CFRD onset after the onset of insulin treatment needs to be considered. Another important notion is that these patients already have a burdensome medication program and insulin treatment would add to that burden.

We also found differential expression of five members of the Anoctamin (ANO) family of  $\text{Ca}^{2+}$ -activated proteins in islets from type 2 diabetic donors. ANO1 is the most well studied of the members. The expression of ANO1 was upregulated in islets from T2D donors. ANO1 is upregulated by the inflammatory marker IL-4 [164]. In islets from T2D donors there might be an ongoing continuous inflammation. It needs to be explored if IL-4 contributes to the upregulation of ANO1 in T2D. The other four members of the ANO family are less well studied and ANO8 is the least studied with unknown function. The putative involvement of ANO5 and ANO6 in exocytosis and endocytosis are intriguing and needs special attention.

# Populärvetenskaplig sammanfattning

Cystisk fibros är en ärftlig sjukdom som drabbar 1/2500 nyfödda i Sverige och orsakas av mutationer i proteinet CFTR. CFTR är en jonkanal som transporterar negativt laddade kloridjoner in och ut ur cellen. CFTR reglerar också andra proteiner. Patienter med cystisk fibros har primärt för mycket slem i lungorna som leder till att lungorna koloniserar av bakterier och på sikt skadas. Många patienter saknar matsmältningsenzym till följd av att bukspottkörtelns gångar till tarmen är tilltäppta. I bukspottkörteln orsakar matsmältningsenzymerna inflammation och nedbrytning av vävnaden. Glädjande nog har livslängden för personer drabbade av CF ökat till 50 år men baksidan är att fler drabbas av följsjukdomar. Den vanligaste följsjukdomen är cystisk fibros-relaterad diabetes (CFRD) som drabbar 50 % av de vuxna patienterna. Orsaken till CFRD är okänt men inflammationen och nedbrytning av bukspottkörteln tros bidra. I den här avhandlingen har jag utforskat möjligheten att CFTR är direkt involverad i frisättningen av insulin och glukagon.

Insulin är ett hormon som insöndras till blodet från beta-celler i bukspottkörtelns Langerhanska öar. Det är det hormon i kroppen som ger signal till målvävnaderna lever, fett och muskler att ta upp socker (glukos) från blodet och in i cellen efter en måltid. Vid diabetes fallerar detta på grund av att målvävnaderna inte svarar på insulinet (insulin resistans) i kombination med att det är minskad förmåga hos beta-cellerna att frisätta insulin till följd av defekt funktion eller brist i produktionen av insulin. Insulinet är packat i små membranomslutna blåsor som kallas vesiklar eller granula. Insulinvesikeln frisätts genom exocytos, en kalciumberoende process där vesikelmembranet smälter samman med cellmembranet och frisätter insulinet. Innan exocytosen kan ske måste insulinvesikeln mogna. Denna process kallas priming där vesikeln görs färdig för att kunna smälta samman med membranet och där omoget proinsulin klyvs till moget insulin och c-peptid. I den processen måste vesikelns pH vara 5 och för att det ska uppnås krävs kloridjoner.

I mitt arbete fann jag att beta-celler har en CFTR-beroende kloridström och att CFTR reglerar en annan kloridkanal, ANO1. Vi fann att farmakologisk blockering av CFTR och ANO1 minskade frisättningen av insulin från isolerade Langerhanska öar. Minskningen i insulinfrisättning var orsakad av direkt påverkan på den exocytotiska processen. Vidare fann vi att om CFTR blockeras med farmakologiska inhibitorer så minskade antalet insulinvesiklar som är dockade vid cellmembranet och redo att genomgå snabb exocytos. Baserat på våra resultat framför vi en modell där CFTR är viktig för priming.

För att studera hur muterat CFTR påverkar insulinfrisättningen undersökte vi en musmodell för cystisk fibros som bär den vanligaste förekommande mutationen i CFTR, där en fenylalanin saknas på position 508 (F508del). Till stöd för vår modell fann vi att isolerade Langerhanska öar från F508del-möss hade ökad frisättning av omoget proinsulin och minskad frisättning av c-peptid. Detta var speciellt tydligt under förhållanden då CFTR är aktivt. Dessutom hade F508del-mössen minskat insulininnehåll i de Langerhanska öarna och minskad beta-cellsmassa i bukspottkörteln till följd av att beta-cellerna hade reducerad storlek. Om storleksminskningen beror på defekter under utvecklingen eller om den defekta kloridströmmen leder till förändrat osmotiskt tryck och därmed minskad storlek är en framtida fråga.

I de Langerhanska öarna finns också alfa-celler som producerar hormonet glukagon. Glukagon frisätts när blodsockret är lågt och stimulerar levern att frisätta lagrat glukos (glykogen) eller producera nytt glukos. I diabetes är ofta alfa-cellerna också satta ur spel och fallerar att frisätta glukagon när blodsockret är lågt eller frisätter glukagon trots att blodsockret är högt och därmed försämras blodglukos kontrollen ytterligare. Detta ökar risken för kärlkomplikationer hos diabetikern. Ett okontrollerat blodglukos skadar de små kärlen som försörjer våra organ som njurar, ögon och nerver. Även de stora kärlen skadas och diabetiker har ökad risk för hjärt- och kärlsjukdomar.

Vi fann att CFTR finns i alfa-cellerna och kunde mäta en CFTR-beroende elektrisk ström i alfa-cellerna. F508del mössen hade förhöjda nivåer av glukagon i blodet och ökad frisättning av glukagon från isolerade Langerhanska öar jämfört med friska möss. Även farmakologisk blockering av CFTR i de Langerhanska öarna från människa och mus ökade frisättningen av glukagon. Jag fann att hos människa påverkar CFTR den elektriska aktiviteten i alfa-cellen. Blockering av CFTR ökade frekvensen av aktionspotentialer samt att vilande alfa-celler aktiverades. Därmed fungerar CFTR som en broms av glukagon-frisättningen i alfa-cellerna

Inhibitorer av kloridkanalen ANO1 påverkade inte frisättningen av glukagon i Langerhanska öar från friska donatorer men ökade frisättningen i öar från typ 2 diabetiker. Detta behöver ytterligare undersökas, men visar vikten av kloridkanaler i reglering av glukagon-frisättning inte bara för patienter med CF utan också vid uppkomst av typ 2 diabetes.

Sammanfattningsvis har jag i min avhandling visat att CFTR har en viktig direkt roll i bukspottkörtelns alfa- och beta-celler. Defekt funktion i CFTR leder till minskad insulinfrisättning genom direkt påverkan på exocytosen, mest troligt till följd av defekt priming och mognadsprocess av insulin. I alfa-cellen reglerar CFTR den elektriska aktiviteten och defekt CFTR ökar glukagon-frisättningen eftersom bromsen på glukagon-frisättningen inte fungerar.

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