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Effects of Ca^{2+}, microRNAs, and rosuvastatin on insulin-secreting beta cell function

VISHAL A. SALUNKHE
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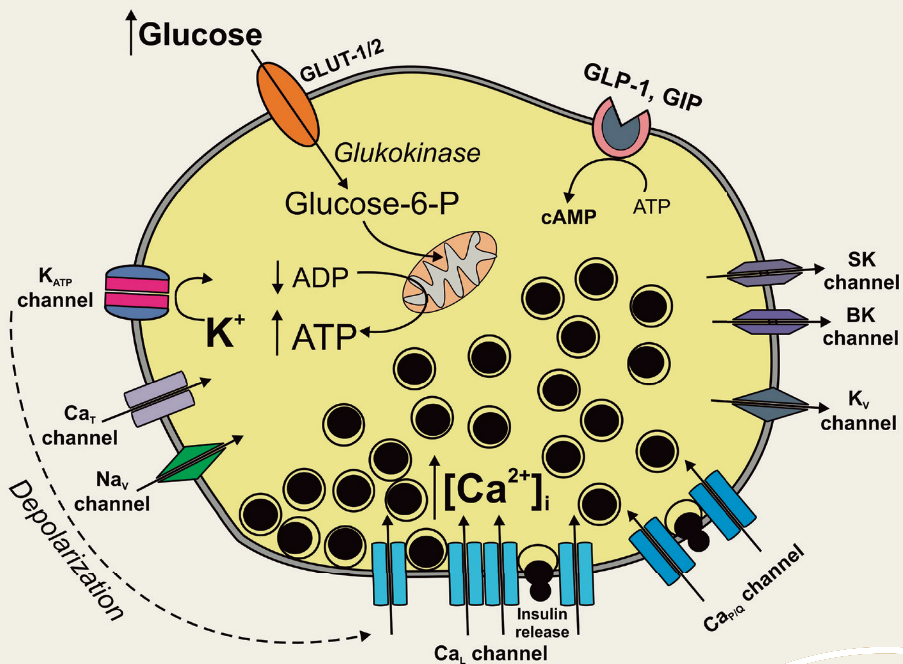

Effects of Ca^{2+}, microRNAs, and rosuvastatin on insulin-secreting beta cell function

Vishal A. Salunkhe

DOCTORAL DISSERTATION

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Abstract

Type 2 diabetes (T2D) is a condition of high blood glucose levels due to insulin resistance and defective insulin secretion. Impaired insulin secretion plays a major role in the pathophysiology of T2D, it is mainly attributed to beta cell function i.e. failure to secrete insulin or reduced beta cell mass. The exocytotic process is crucial for beta cell function and its dysregulation leads to impaired insulin secretion. Therefore, understanding the central mechanisms involved in the regulation of exocytosis is essential to recognize possible targets for therapeutic intervention and treatment of T2D. In this thesis I have investigated the role of Ca^{2+}, miRNAs and rosuvastatin in the regulation of ion channels, exocytosis and insulin secretion of beta cells. For this purpose, pancreatic rat INS-1 832/13 beta cells, rodent animal models, and islets from human cadaver donors has been used. Whole-cell patch clamp was used to study exocytosis measured as changes in cell membrane capacitance.

In beta cells, biphasic exocytotic pattern was previously mainly attributed to insulin granule pool depletion. In paper I, we used the pulse length protocol and mixed-effect modelling; the latter takes care of cellular heterogeneity, to study exocytosis as a function of Ca^{2+} influx (measured as Q). The data suggests that pool depletion plays a minor role in observed biphasic exocytotic pattern in INS-1 832/13 cells; instead exocytosis is mostly determined by the kinetics of Ca^{2+} current inactivation. In paper II and III, we have investigated the effects of miRNA modulation on insulin secretion and exocytosis. First we investigated miRNA-regulation of voltage-gated Na^{+} channels since their role in beta cell function is not yet clear. Down-regulation of miR-375 differentially affected Na^{+} channel inactivation properties in INS-1 832/13 cells and miR-375 knock-out mice beta cells, suggesting species differences. As steady-state inactivation determines the number of channels available for generation of action potential, this study is a proof of principle that mir-375 could be important in regulating electrical activity in human beta cells. Next, miRNA-regulation of the exocytotic process was investigated. Overexpression of miR-335 reduced exocytosis and thereby insulin secretion through decreased expression of the exocytotic proteins STXBP1, SNAP25 and SYT11. In this paper I also made the novel observation that SYT11 increase basal insulin secretion and decrease rapid exocytosis, two phenomenons associated with T2D. The work on miR-335 emphasizes the importance of miRNAs in the regulation of the exocytotic process. In paper IV and V the effects of the cholesterol-lowering drug rosuvastatin was investigated. Rosuvastatin treatment dose dependently affected Ca^{2+} influx, exocytosis, basal and glucose-induced insulin secretion in INS-1 832/13 cells. Interestingly, most of this effect was though mevalonate pathway and not from the cholesterol lowering ability of rosuvastatin. *In vivo* rosuvastatin had an overall positive effect on glucose homeostasis in mice but negative effects on beta cell function such as disturbed Ca^{2+}-signalling.

In conclusion, the data in my thesis demonstrate the need for investigations of the mechanisms behind defective insulin secretion and exocytosis in order to understand and treat T2D.

Key words: T2D, exocytosis, Ca^{2+}, miR-375, miR-335, rosuvastatin, voltage-gated Na^{+} channels, STXBP1, SNAP25, SYT11, glucose homeostasis, beta cell function

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Effects of Ca^{2+}, microRNAs, and rosuvastatin on insulin-secreting beta cell function

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ॐ असतो मा सद्गमय ।
तमसो मा ज्योतिर्गमय ।
मृत्योर्मा अमृतं गमय ।
ॐ शान्तिः शान्तिः शान्तिः ॥

Om Asato Maa Sad-Gamaya **/**
Tamaso Maa Jyotir-Gamaya **/**
Mrtyor-Maa Amrtam Gamaya **/**
Om Shaantih Shaantih Shaantih **//**

From ignorance lead me to truth
From darkness lead me to light
From death lead me to immortality
May there be peace, peace, peace.
-- Upanishad

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I. Morten Gram Pedersen, Vishal A Salunkhe, Emma Svedin, Anna Edlund, Lena Eliasson (2014) Calcium current inactivation rather than pool depletion explains reduced exocytotic rate with prolonged stimulation in insulin-secreting INS-1 832/13 cells. *PLoS One* 9: e103874.

II. Vishal A Salunkhe, Jonathan LS Esguerra, Jones K Ofori, Ines G Mollet, Matthias Braun, Markus Stoffel, Anna Wendt, Lena Eliasson (2015) Modulation of microRNA-375 expression alters voltage-gated Na^+ channel properties and exocytosis in insulin-secreting cells. *Acta physiologica*. Apr; 213(4):882-92.

III. Vishal A Salunkhe, Jones K Ofori, Nikhil R Gandasi, Sofia A Salö, Sofia Hansson, Markus E Andersson, Anna Wendt, Sebastian Barg, Jonathan LS Esguerra and Lena Eliasson (2015) MiR-335 over-expression impairs insulin secretion in β-cells through defective priming of insulin vesicles. *Manuscript*.

IV. Vishal A Salunkhe, Olof Elvstam, Lena Eliasson and Anna Wendt (2015) Rosuvastatin treatment affects both basal and glucose-induced insulin secretion in INS-1 832/13 cells. *Manuscript*.

V. Vishal A Salunkhe, Ines G Mollet, Helena A Malm, Jones K Ofori, Jonathan LS Esguerra, Thomas Reinbothe, Karin G Stenkula, Anna Wendt, Lena Eliasson and Jenny Vikman (2015) Dual effect of rosuvastatin on glucose homeostasis through improved insulin sensitivity and reduced insulin secretion. *Manuscript*.

Publications not included in the thesis

Original paper:

- Olatz Villate, Jean-Valery Turatsinze, Loriana G Mascali, Fabio A Grieco, Tatiane C Nogueira, Daniel A Cunha, Tarlliza R Nardelli, Michael Sammeth, Vishal A Salunkhe, Jonathan LS Esguerra, Lena Eliasson, Lorella Marselli, Piero Marchetti and Decio L Eizirik (2014) Nova1 is a master regulator of alternative splicing in pancreatic beta cells. *Nucleic acids research* 42: 11818-11830.

Review article:

- Jonathan LS Esguerra, Ines G Mollet, Vishal A Salunkhe, Anna Wendt, Lena Eliasson (2014) Regulation of Pancreatic Beta Cell Stimulus-Secretion Coupling by microRNAs. *Genes* 5: 1018-1031

Abbreviations

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
Ago2	Argonaute-2
ANDIS	All New Diabetics in Scania
cAMP	Cyclic AMP
cDNA	Complementary DNA
C_m	Membrane capacitance
CVD	Cardiovascular disease
DPP-4	Dipeptidyl peptidase 4
ER	Endoplasmic reticulum
F-actin	Filamentous actin
GK rat	Goto-Kakizaki rat
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GWAS	Genome-wide association studies
HbA1c	Glycated hemoglobin
HCSP	Highly Ca^{2+}-sensitive pool of granules.
HDL	High-density lipoprotein
HFD	High fat diet
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coA
HVA	High-voltage activated
IAPP	Islet amyloid polypeptide
IDL	Intermediate-density lipoprotein
I_p	Peak current
IRP	Immediately releasable pool
I_{sus}	Sustained current
K_{ATP} channels	ATP-sensitive potassium channels
Kir6.2	Inward rectifier K^+ channel
LADA	Latent autoimmune diabetes of adults
LDL	Low-density lipoprotein
LNA	Locked nucleic acid
LVA	Low-voltage activated
MCU	Mitochondrial Ca^{2+} uniporter
MGB	Minor groove binder
miRNAs	MicroRNAs
MODY	Maturity-onset diabetes of the young
NCLX	Na^+/ Ca^{2+} exchanger

ND	Neonatal diabetes
ND	Normal diet
NFQ	Nonfluorescent quencher
NPY	Neuropeptide-Y
NSF	N-ethylmaleimide Sensitive Factor
OATP	Organic Anion Transporting Polypeptides
OGTT	Oral glucose tolerance test
PACT	Protein activator of PKR
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
RER	Rough endoplasmic reticulum
RT-qPCR	Reverse transcription quantitative real-time PCR
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RNA_i	RNA interference
RP	Reserve pool
RRP	Readily releasable pool
SERCAs	Sarco-endoplasmic reticulum Ca^{2+}-atpases
SGLT2	Sodium-glucose co-transporter-2
SLMVs	Small GABA-containing synaptic-like microvesicles
SMGs	Small-molecular-mass (20-28 kda) GTP-binding proteins
SNAP-25	Synaptosomal-associated protein-25
SNAREs	Soluble N-ethylmaleimidesensitive factor attachment protein receptors
Stxbp1	Syntaxin-binding protein 1
SUR	Sulfonylurea receptor
Syts	Synaptotagmins
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TRBP	Tar RNA binding protein
TTX	Tetrodotoxin
VAMP-2	Vesicle-associated membrane protein-2
V_h	Half maximal inactivation
VLDL	Very-low-density lipoprotein
WHO	World Health Organization

Introduction

Diabetes mellitus

A great amount of work has been done by researchers worldwide in order to understand the metabolic disease diabetes. However, due to its heterogenetic nature a picture of this epidemic disease is not yet clear (World Health Organization, 2015, Wild et al., 2004). According to International Diabetes Federation in year 2013 diabetes caused 5.1 million deaths i.e. every six seconds one person dies from diabetes (International Diabetes Federation, 2013). Hence more aggressive efforts to understand this disease better are needed.

Normal fasting blood glucose levels are 3.5-5.5 mmol/l, these levels are maintained within this narrow range by factors which control glucose production and glucose utilisation (Guemes et al., 2015). The key hormones responsible for glucose homoeostasis include insulin, glucagon, epinephrine, norepinephrine, cortisol, growth hormone and incretins (Guemes et al., 2015, Kim and Egan, 2008). Insulin is the major glucose lowering hormone. It is secreted by pancreatic beta cells in response to nutrient stimuli (e.g. glucose), travels through the blood circulation, and binds to receptors on insulin-responsive tissues like liver, skeletal muscle and adipose tissue, to stimulate uptake of glucose (Kalwat and Thurmond, 2013).

Diabetes is characterized by high blood glucose levels. World Health Organization (WHO) defines people with fasting plasma glucose level ≥7.0 mmol/l or plasma glucose ≥11.1 mmol/l two hours after oral glucose (75 g) tolerance test as diabetic (World Health Organization, 2006). Diabetes can also be diagnosed by measuring glycated hemoglobin (HbA1c) levels ≥6.5% (Diabetes Care, 2010). However, HbA1c measurements have limitations and may not be valid in certain subgroups, such as in children, in individuals with prediabetes or gestational diabetes, and in persons infected with human immunodeficiency virus (Juarez et al., 2014). Primarily metformin, sulfonylureas and insulin are used for diabetes treatment. In recent years many new medications have been introduced such as dipeptidyl peptidase 4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists and sodium-glucose co-transporter-2 (SGLT2) inhibitors (Tran et al., 2015a, Idris and Donnelly, 2009, Tran et al., 2015b). However, so far no treatment has succeeded to cure or to avoid the progression of the disease and its complications.

Traditionally diabetes has been categorized into two major types, type 1 diabetes (T1D) and type 2 diabetes (T2D) based on insulin dependency and age of onset of the disease (Pickup and Williams, 1991). But now with the advance experimental knowledge and large clinical studies like ANDIS (All New Diabetics in Scania) it is clear that there are many subtypes of diabetes (ANDIS, 2013). It is important to diagnose diabetes properly for more appropriate treatment.

T1D is an autoimmune condition which leads to the destruction of insulin producing beta cells. It usually occurs in children and is associated with the presence of islet-cell antibodies. Latent autoimmune diabetes of adults (LADA) is a type 1 diabetic-like autoimmune condition but as the name indicates it manifests in adults (Groop et al., 1986, Stenstrom et al., 2005). LADA patients could get misdiagnosed as type 2 diabetic patients due to their age. Therefore, for proper diagnosis it is necessary to check for the presence of islet-cell antibodies in diabetic patients irrespective of their age. Type 1 diabetes is considered irreversible due to the beta cell destruction. However, in a recent study using pancreatic tissue from living human subjects extracted shortly after their T1D diagnosis glucose-induced insulin secretion could be measured. Interestingly, biphasic insulin secretion could be normalized after a few days in a nondiabetogenic environment in vitro (Krogvold et al., 2015). This indicates that even in T1D there is a potential for endogenous insulin production, and dysregulation of mechanisms controlling insulin secretion such as the exocytotic process could potentially be involved in the disease process (Ohara-Imaizumi et al., 2004a).

T2D is a condition of insulin resistance and defective insulin secretion with strong association to lifestyle and genetic components (Groop, 2000, Marshall and Bessesen, 2002). T2D can be divided in to two subtypes, monogenic T2D and polygenic T2D (Ashcroft and Rorsman, 2012). Monogenic T2D is a rare form of diabetes that results from a mutation in a single gene which leads to reduced insulin secretion. The severity of the condition depends on the gene involved and treatment must be decided accordingly. Examples of monogenic T2D are neonatal diabetes (ND) and maturity-onset diabetes of the young (MODY). Monogenic T2D can get misdiagnosed as T1D due to its onset at an early age (Murphy et al., 2008). Polygenic T2D is the most common form of diabetes. For a long time it was considered mainly a problem of insulin resistance but now with novel discoveries during the recent years it is well accepted that beta cell dysfunction is actually a leading cause of T2D (Ashcroft and Rorsman, 2012). The possibility of using islets from T2D rodent models and human cadaver donors have to a great extent helped to understand beta cell dysfunction in diabetes.

Though there is a strong link between obesity and T2D (Golay and Ybarra, 2005) not all insulin resistance or obese people get diabetes (Golay et al., 1988, Meigs et al., 2006). This suggests that only those individuals who cannot compensate for their extra need of insulin are prone to get diabetes (Polonsky, 2000). Reduced

insulin secretion from islets is mainly attributed to beta cell failure or fewer beta cells (Pajvani and Accili, 2015).

There are studies indicating that reduced beta cell mass could be a major factor in T2D (Butler et al., 2003, Rahier et al., 2008) but it is unknown if this reduced mass is a cause or a consequence of diabetes (Pipeleers et al., 2008). However the fact that beta cell mass increase to overcome insulin resistance in conditions like obesity (Saisho et al., 2013) and pregnancy (Rieck and Kaestner, 2010, Sorenson and Brelje, 1997) indicates that beta cell mass turnover plays an important role in maintaining normoglycemia. Dedifferentiation of beta cells has also been proposed as a main cause of beta cell failure (Pajvani and Accili, 2015). On the other hand, it has been suggested that 40% of the original beta cell mass would be enough for normal functioning (Ashcroft and Rorsman, 2012) and the fact that removal of half of the pancreas has relatively small effect on blood glucose tolerance (Menge et al., 2008). This indicates that decreased beta cell mass is probably a less important etiological factor in T2D.

Interestingly people who undergo bariatric surgery get cured of T2D even before any considerable weight loss (Karra et al., 2010) and only 1 week of severe dietary energy restriction (600 kcal/day) was able to reverse T2D (Lim et al., 2011). This suggests that people with T2D independent of their beta cell mass have enough beta cell capacity to overcome disease condition and beta cell dysfunction is the main cause of the disease.

Understanding the central mechanisms involved in the control of beta cell function is essential to understand potential sites and cause of beta cell dysfunction. This knowledge is necessary to recognize possible targets for therapeutic intervention and treatment.

Pancreatic islets of Langerhans

In 1869, Paul Langerhans in his doctoral thesis reported a microscopic appearance of scattered cell clusters in the pancreas of the rabbit. Later these cell clusters were named 'islets of Langerhans'. The islets of Langerhans, or islets for short, are mini-organs important in maintaining blood glucose levels (Arrojo et al., 2015) (Fig.1). Islets of Langerhans are scattered throughout the pancreas. They constitute only about 1-2% of the total pancreas but they receive 5-10% of the total pancreatic blood flow (Lifson et al., 1985). This highly vascularized nature of islets enables efficient sensing of blood glucose, other nutrients (e.g free fatty acids) and secreted hormones (e.g. incretins) (Arrojo et al., 2015).

Pancreatic islets contains five different endocrine cell types insulin producing beta cells, glucagon producing alpha cells, somatostatin producing delta cells,

pancreatic polypeptide producing PP cells and a few ghrelin producing epsilon cells (Seino and Bell, 2008). There are some structural differences between rodent islets and human islets. Rodent islets have a beta cell core and non-beta cells in the mantle whereas beta cells of human islets are intermingled with other endocrine cell types. Moreover, the proportion of beta cells is lower in human as compared to rodent islets and the number of alpha cells is higher. In rodents the beta cells constitute ~80% and alpha cells ~15% of the total islet cells, whereas human islets contain ~65% beta cells and ~30% alpha cells (Brissova et al., 2005, Cabrera et al., 2006, Arrojo et al., 2015). Islet cells can influence each other by autocrine and paracrine effects or gap junctions and they are extensively innervated by cholinergic, adrenergic and peptidergic nerve endings (Ashcroft and Rorsman, 1989, Arrojo et al., 2015).

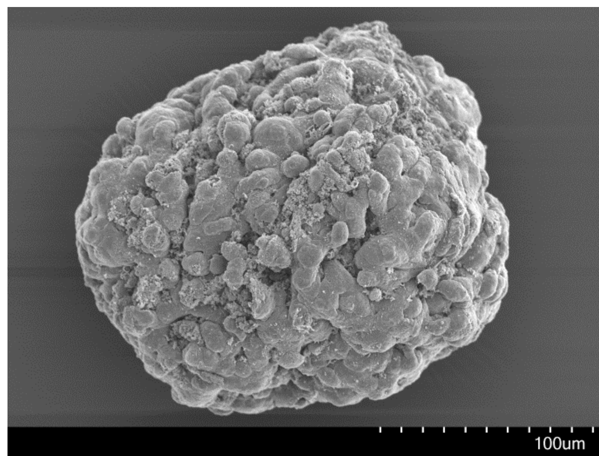

Figure 1. Electron microscopy image of pancreatic islets of Langerhans.

Role of Beta cells

In response to a glucose challenge beta cells in healthy subjects display a typical biphasic mode of insulin secretion with a rapid 1st phase which lasts for a few minutes followed by slow sustained 2nd phase (Curry et al., 1968). In T2D subjects there is complete loss of the 1st phase and strong reduction in 2nd phase of insulin secretion (Hosker et al., 1989). Hence, it is essential to study the molecular regulation of insulin secretion from the pancreatic beta cells.

Insulin biosynthesis

Frederick Banting and J.J.R. Macleod got the Nobel Prize in 1923 for the extraction of pure insulin (Nobelprize.org, 1923). Insulin was the first protein whose primary structure was elucidated; Frederick Sanger got the Nobel Prize for this in 1958 (Nobelprize.org, 1958).

Insulin is synthesized and stored exclusively in beta cells via a series of precursor proteins including preproinsulin and proinsulin. Preproinsulin is a single chain (110 amino acid) molecule. It contains an N-terminal 24 residue signal peptide which is cleaved off in the rough endoplasmic reticulum (RER) to form proinsulin. Proinsulin then undergoes folding and forms three disulfide bonds to get its native structure. It is then transported to the golgi apparatus for further processing and packaging (Halban, 1991, Huang and Arvan, 1995, Seino and Bell, 2008, Fu et al., 2013). In the golgi apparatus proinsulin is cleaved to yield insulin and C-peptide. Insulin and C-peptide are stored together in secretory granules along with small amounts of intact proinsulin and intermediate products. Mature secretory granules contain a central dense core of insulin molecules in their crystalline form (Zn_2-$Insulin_6$) (Fu et al., 2013). Contained in the secretory vesicles are also ~50 polypeptides e.g. islet amyloid polypeptide (IAPP) as well as compounds such as ATP and GABA (Eliasson et al., 2008, Seino and Bell, 2008).

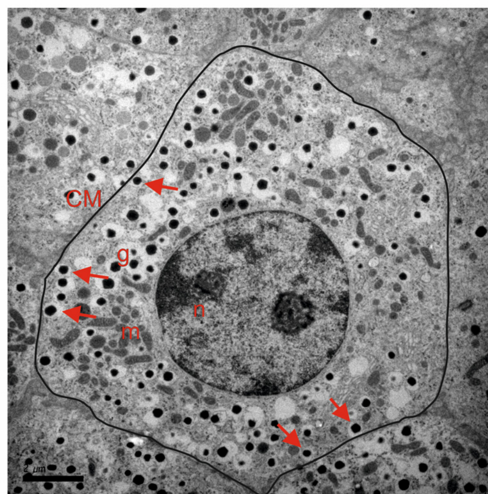

Figure 2. Electron microscopy image of beta cell showing docked granules (g) by arrow, cell membrane (CM), mitochondria (m) and nucleus (n).

There are about one million islets in a human pancreas and an average human islet is estimated to have ∼180 beta cells (Rorsman and Braun, 2013). Every beta cell has ~10000 insulin granules of which approximately ~5% are docked (Fig. 2) (Eliasson et al., 2008, Olofsson et al., 2002, Rorsman and Braun, 2013) each containing ~1.7 amol of insulin (Rorsman and Renstrom, 2003).

Glucose sensing

Insulin is the central hormone to lower blood glucose and hence the beta cells must sense and respond aptly to increased blood glucose. Failure to do so may lead to pathophysiological condition like diabetes. Diffusion of blood glucose into the beta cells is facilitated via high capacity low affinity glucose transporter 2 (GLUT2) in rodents (Newgard and McGarry, 1995) and GLUT1 in humans (De Vos et al., 1995). Glucose is phosphorylated to glucose-6-phospate by high K_M glucokinase (the glucose sensor of the beta cell) followed by glycolysis to yield pyruvate. Pyruvate is then metabolized in the mitochondria through the citric acid cycle which leads to the formation of many intermediate products and ATP (MacDonald et al., 2005). Generation of ATP at the expense of ADP is important to stimulate electrical activity in the beta cells.

Stimulus-secretion coupling

Electrical activity in beta cells plays a central role in coupling increased blood glucose concentration to insulin secretion. The concerted action of a large number of different types of ion channels, transporters and pumps leads to the generation of burst of action potentials and release of insulin (Fig. 3).

Generation of electrical activity

Identification of ATP-sensitive potassium channels (K_{ATP} channels) in beta cells was a major breakthrough to understand their electrical activity (Ashcroft et al., 1984, Cook and Hales, 1984, Rorsman and Trube, 1985). K_{ATP} channels consist of two different subunits Kir6.2 (inward rectifier K^+ channel) and SUR (sulfonylurea receptor), that together form a functional hetero-octameric complex (Inagaki et al., 1997, Zerangue et al., 1999, Inagaki et al., 1995). ATP binds to the Kir6.2 to close the channel (Tucker et al., 1997), and the SUR subunit is the target for drugs which can inhibit (e.g. sulfonylurea) or stimulate (e.g. diazoxide) the opening of K_{ATP} channel (Tucker et al., 1997, Gribble and Reimann, 2003). The resting membrane potential of the beta cell is ~ -60 to -100mV (Gopel et al., 1999, Speier and Rupnik, 2003). Increased ATP and reduced ADP as a result of increased glucose metabolism leads to closure of K_{ATP} channels, causing an inhibition of K^+ efflux and membrane depolarization (Ashcroft et al., 1984, Cook and Hales, 1984,

Rorsman and Trube, 1985). In rodent beta cells the depolarization activates opening of high-voltage activated (HVA) Ca^{2+} channels (Ammala et al., 1993b). Interestingly, in human beta cells the opening of the HVA Ca^{2+} channels are preceded by activation of low-voltage activated (LVA) T-type Ca^{2+} channels and Na^{+} channels (Barnett et al., 1995). The overall depolarization and opening of voltage activated ion channels constitute the upstroke of action potentials. The resultant influx of Ca^{2+} and the increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) initiates exocytosis (Ammala et al., 1993b, Rorsman and Braun, 2013).

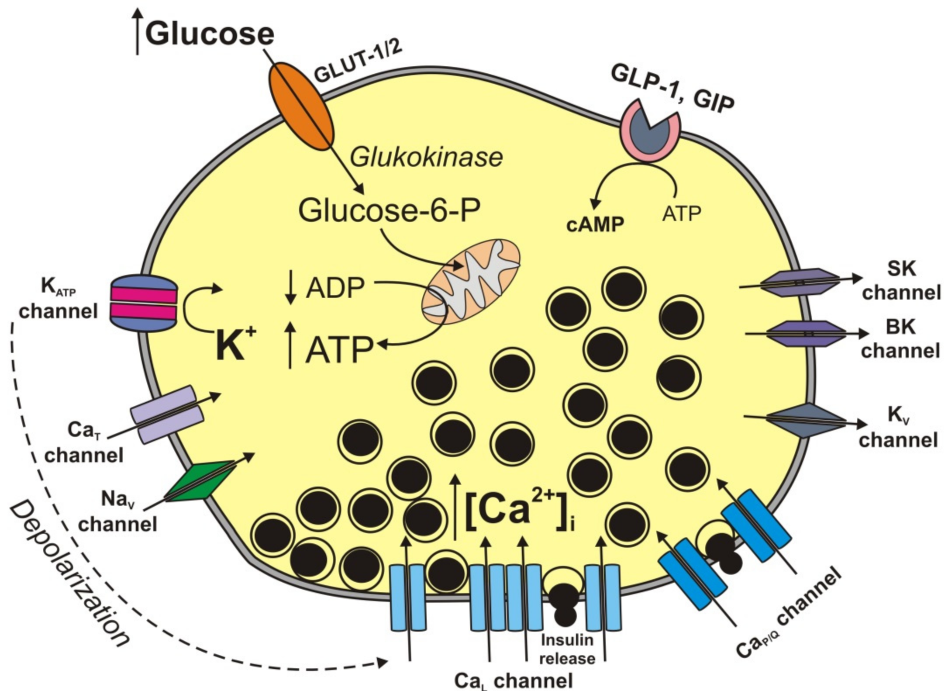

Figure 3. Stimulus-secretion coupling of the pancreatic beta cell.

The downstroke of the action potential possibly results from rapid voltage-dependent inactivation of the Na^{+} channels, Ca^{2+}-dependent inactivation of the L-type Ca^{2+} channels, activation of voltage-gated K^{+} channels and Ca^{2+}-activated K^{+} channels (e.g. SK channel, BK channel), which leads to membrane repolarization (Herrington et al., 2006, MacDonald et al., 2002, Seino and Bell, 2008, Rorsman and Braun, 2013).

Voltage-gated Na^{+} channel consist of pore-forming α subunit and auxiliary β subunits. So far nine mammalian α subunits and four auxiliary β subunits of sodium channels have been identified (Catterall et al., 2005). Voltage-gated Na^{+}

channels play a crucial role in the initiation and propagation of action potentials in neurons and endocrine cells (Catterall et al., 2005). Voltage-gated Na^+ channels can be present in three separate states; closed, open and inactivated (Fig. 4). The membrane depolarization leads to opening of the closed voltage-gated Na^+ channels, these channels will subsequently within ~1 ms go to an inactive state. Once inactivated the channel cannot switch to open state. The channel can only return to the open state via the closed state, which requires the resting membrane potential to be restored (Van Petegem et al., 2012, Guyton and Hall, 2000). Different types of Na^+ channels are present in mouse and human beta cells (Zhang et al., 2014, Rorsman and Braun, 2013). In human beta cells voltage-gated Na^+ channels shows half maximal inactivation at ~-40 mV and they are important in glucose stimulated insulin secretion (Braun et al., 2008). The role of voltage-gated Na^+ channels in rodent beta cells is not clear. Because of their more negative half maximal inactivation (at -70 mV or below) they are thought not to be important in generation of action potential (Hiriart & Matteson, 1988). However, in rat beta cells their inhibition using tetrodotoxin (TTX) reduces glucose induced insulin secretion (Vidaltamayo et al., 2002).

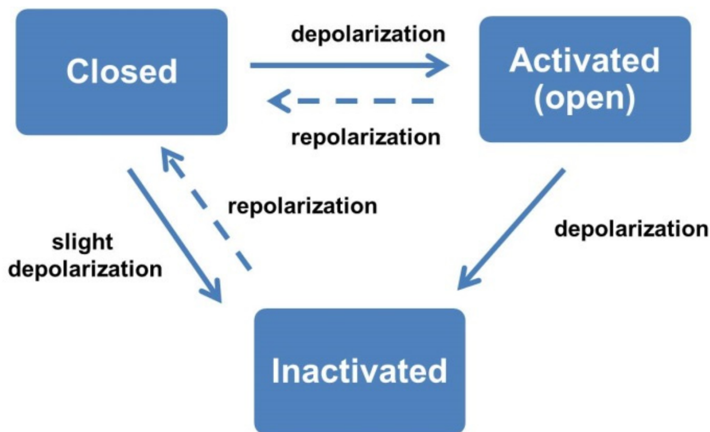

Figure 4. Different states of voltage-gated Na^+ channels.

The timing of action potential firing is regulated by depolarizing versus repolarizing ion fluxes. In mouse islets, glucose concentration of ~6 mM leads to a generation of electrical activity whereas in human islets as low as 3 mM glucose can be enough (Ashcroft and Rorsman, 1989, Braun et al., 2008, Rorsman and Braun, 2013). These differences may be because of the fact that the nonfasting plasma glucose concentration is lower in humans (~5 mM) than in mice (7–10 mM) (Rorsman and Braun, 2013). At glucose concentration >20 mM uninterrupted action potential firing occurs. This ability of glucose to elicit

electrical activity and the underlying chain of events is called the triggering pathway of insulin secretion (Henquin, 2000).

Modulation of insulin secretion

If beta cell depolarization and resultant increased $[Ca^{2+}]_i$ is induced experimentally by using sulfonylurea or high concentrations of extracellular K^+ when holding K_{ATP} channels open with diazoxide, glucose still increases insulin secretion in a concentration-dependent manner (Gembal et al., 1992, Henquin, 2000). This effect is attributed to the K_{ATP}-independent or amplifying pathway of insulin secretion (Henquin, 2000). The amplifying pathway and its role in exocytosis can also be studied by using depolarization induced exocytosis measurements as a function of change in cell membrane capacitance (Eliasson et al., 1997, Renstrom et al., 1997). The molecular basis of this pathway is still not fully understood but some glucose metabolites such as GTP, ATP, NADH, NADHP have been suggested to be involved (Maechler and Wollheim, 1999, Ivarsson et al., 2005b, Wollheim and Maechler, 2002, Eliasson et al., 1997). However, glucose is unable to increase insulin secretion in the absence of increased $[Ca^{2+}]_i$ suggesting that the triggering pathway predominates over the amplifying pathway (Henquin, 2000).

Several hormones and neurotransmitters can affect insulin secretion. Based on their effect they are called either potentiators or inhibitors of insulin secretion. Gut hormones such as gastric inhibitory polypetides (GIP) and GLP-1 can potentiates insulin secretion in the presence of glucose by elevating cyclic AMP (cAMP) (Fehmann and Goke, 1995, Gromada et al., 1995b, Kim and Egan, 2008). This phenomenon is known as the incretin effect and it is reduced in T2D (Nauck et al., 1986, Kim and Egan, 2008). Insulin secretion is potentiated by cAMP through several mechanisms including increased Ca^{2+}-influx through voltage gated Ca^{2+} channels, activating Ca^{2+} release from intracellular stores and accelerating the process of exocytosis (Gromada et al., 1995a, Gromada et al., 1998b, Gromada et al., 1997, Gromada et al., 1998a, Ammala et al., 1993a). In the process involved in exocytosis of insulin granules cAMP acts both in a PKA-dependent and a PKA-*in*dependent manner (MacDonald et al., 2003, Ozaki et al., 2000, Eliasson et al., 2003, Seino and Shibasaki, 2005, Renstrom et al., 1997).

Process of exocytosis

After having been processed in the endoplasmic reticulum and golgi apparatus insulin is stored in mature secretory granules, waiting to be secreted by the regulated exocytosis process. Exocytosis is a process where insulin granules fuse with the cell membrane to release its content into extracellular space. The exocytosis of insulin containing vesicles is tightly regulated and involves 1)

translocation of insulin granules to plasma membrane (mobilization) 2) docking of the insulin granules at plasma membrane 3) priming of the insulin granules i.e. making them release-compatible and 4) actual fusion with the plasma membrane and release of insulin into extracellular space (Gerber and Sudhof, 2002, Eliasson et al., 2008).

Distinct pools of insulin granules and biphasic exocytosis

Exocytosis measured as capacitance increase in response to photorelease of caged Ca^{2+} in isolated beta cell has a biphasic pattern and consist of a rapid initial component followed by sustained late component (Olofsson et al., 2002). This type of electrophysiological studies together with ultrastructural data from islet beta cells have led to an idea that insulin granules exits in distinct functional pools (Fig. 5) i.e. readily releasable pool (RRP) and reserve pool (RP), which are responsible for kinetically separable components of exocytosis (Bratanova-Tochkova et al., 2002, Straub and Sharp, 2004). The RRP consist of ~1-5% of the total granules in the beta cells which are immediately available for release upon an increase in Ca^{2+} and their release is reflected in the rapid component of the capacitance increase. A subset of RRP situated in a close vicinity of the Ca^{2+} channels is called immediately releasable pool (IRP) (Barg et al., 2001). As RRP is depleted it gets refilled by granules from RP which represents 95-99% of the granules in the beta cell (Rorsman and Renstrom, 2003).

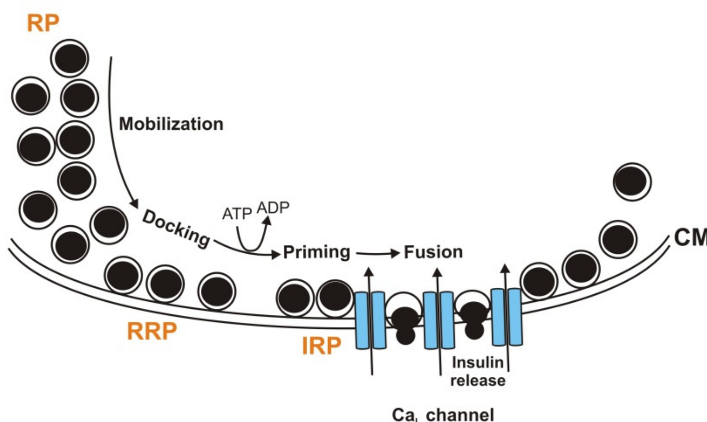

Figure 5. Different functional pools of granules and process of exocytosis.

Refilling of RRP which involves both mobilization and priming of granules is a rate limiting step of exocytosis, probably represented by later slower but sustained component of capacitance increase (Olofsson et al., 2002). Mobilization is the process by which granules is moved towards the plasma membrane to become docked. Priming is a Ca^{2+}, temperature and ATP-dependent process that make the granules release-ready (Eliasson et al., 1997, Proks et al., 1996, Renstrom et al., 1996).

The estimated size of the RRP (20 to 100 granules) equates to the calculated number of granules secreted in 1st phase of insulin secretion in beta cells (Rorsman and Renstrom, 2003). Based on their similarity in appearance it is possible that biphasic insulin secretion and exocytosis have the same mechanistic background where distinct functional pools of granule play an important role. It is however important to bear in mind that the kinetics of capacitance increase and insulin secretion are different. Also, recently it was suggested that first phase secretion comprises not only pre-docked granules but also newly recruited granules, so-called 'restless newcomers' (Ohara-Imaizumi et al., 2004b, Kalwat and Thurmond, 2013, Shibasaki et al., 2007). Moreover, RRP is not homogenous and whether or not the size of the RRP is kept constant is not yet clear. Release probability of a granule depends on its distance from voltage-gated Ca^{2+} channels clusters and on the intrinsic state of the release apparatus (Wang and Thurmond, 2009, Neher, 2015). It is difficult to estimate the exact RRP size because results can vary depending on the strength of the stimuli used, $[Ca^{2+}]_i$ and the speed of recruitment of new vesicles. A method that takes into account heterogeneity among cells is the mixed-effect modelling (Pinheiro and Bates, 2000) used in paper I of this thesis.

In several studies where capacitance measurements has been used to study pool depletion exocytosis has been studied as a function of time (Gopel et al., 2004, Olofsson et al., 2002, Rorsman et al., 2011). However, since exocytosis is highly dependent on Ca^{2+} influx some studies have proposed that instead of pool depletion, changes in Ca^{2+} influx could be responsible for the observed biphasic exocytotic pattern (Ammala et al., 1993b, Engisch and Nowycky, 1996, Barg et al., 2001, Pedersen, 2011, Pedersen et al., 2011). Therefore to study whether depolarization-induced pool depletion occurs, exocytosis should be studied as a function of Ca^{2+}influx (measured as Q) rather than of time (Pedersen, 2011).

Molecular machinery for exocytosis

As mentioned above exocytosis is a multistep process. A plethora of proteins are necessary in this process. The complete molecular picture is not yet fully elucidated but some of the proteins which play key role in granule trafficking and membrane fusion have been identified. Key protein families which are involved in the exocytotic process are 1) Soluble N-ethylmaleimide-sensitive factor

attachment protein receptors (SNAREs) 2) SM proteins (sec1/munc 18-like proteins) 3) Rab proteins and 4) Synaptotagmins (Syts) (Gerber and Sudhof, 2002, Sudhof and Rizo, 2011, Rorsman and Renstrom, 2003).

The family of SNARE proteins shares a signature sequence called the SNARE motif. The SNARE motifs can bind to each other to form an extremely stable complex called core complex (coiled-coil motif) (Ernst and Brunger, 2003). These proteins are present on opposing membranes before fusion. There are two plasma membrane SNAREs (also called t-SNAREs), synaptosomal-associated protein-25 (SNAP-25) and syntaxin 1, and one vesicular SNARE (also called v-SNAREs), vesicle-associated membrane protein-2 (VAMP-2). Both VAMP-2 and syntaxin 1 possesses a transmembrane region, whereas SNAP-25 is instead bound to the plasma membrane through palmitoylation of a cysteine rich domain (Veit et al., 1996). Syntaxin 1 when not within the SNARE complex is in a closed conformation. For the formation of the SNARE complex (Fig. 6) in time of fusion, syntaxin 1 needs to be in an open state to expose its SNARE motif where SNAP-25 and VAMP-2 can bind (Burkhardt et al., 2008). Membrane fusion needs a lot of energy and according to the zippering model the force needed to bring the two membranes together comes from trans to cis conformational changes in the SNARE complexes (Chen and Scheller, 2001). Other important isoforms of SNARE proteins are SNAP23, Syntaxin 3 and Syntaxin 4. Syntaxin 1 is found to be important in first phase of insulin secretion whereas Syntaxin 3 and 4 are considered important for both phases of insulin secretion (Kalwat and Thurmond, 2013).

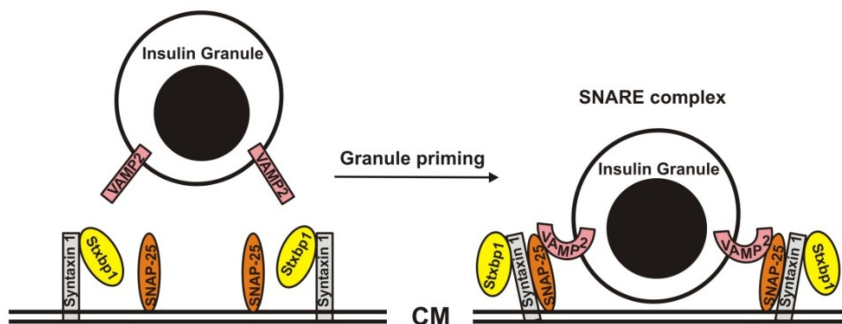

Figure 6. Process of granule priming and formation of the SNARE complex.

Among the SM proteins, syntaxin-binding protein 1, Stxbp1 (or Munc18-1) is one of the more important proteins in regulated Ca^{2+}-dependent exocytosis. The expression of Stxbp1 is positively associated with glucose-stimulated insulin secretion in islets from human donors (Andersson et al., 2012). Moreover, protein

level of Stxbp1 is significantly reduced in islets from the diabetic GK (Goto-Kakizaki) rat (Gaisano et al., 2002). Together with granuphilin, Stxbp1 is necessary for docking of insulin granules to the plasma membrane (Tomas et al., 2008). Moreover, Stxbp1 also binds to the closed conformation of syntaxin 1 (Fig. 6) to prevent core complex formation with other SNARE proteins under non-stimulatory conditions. When exocytosis is stimulated Stxbp1 facilitates the core complex formation during the priming step (Burkhardt et al., 2008, Tomas et al., 2008, Gulyas-Kovacs et al., 2007).

Other interesting proteins in exocytosis are the Rab proteins. They belong to a family of GTPases also known as SMGs (small-molecular-mass (20-28 kDa) GTP-binding proteins). Rab3A is one of the best studied in insulin secretion, and Rab3A null mice show glucose intolerance due to insufficient insulin secretion (Yaekura et al., 2003). Interestingly, activity of Rab proteins is suggested to be affected by statin treatment (Li et al., 1993). Rab proteins are essential in granule trafficking, docking and fusion of the granules (Balch, 1990, Lang, 1999, Takai et al., 2001). The small Rho-family GTPases are important in filamentous actin (F-actin) remodelling (Wang and Thurmond, 2009).

The influx of calcium through the Ca^{2+} channels is sensed by Syts. These proteins possess two C2 domains (C2A and C2B), which make them either Ca^{2+}-sensitive or -insensitive according to their capacity to bind Ca^{2+}. There are 17 known isoforms of Syts. Syt1 to 3, 5 to 7, 9 and 10 are known to bind Ca^{2+} whereas syt4, 8 and 11 to 15 do not bind Ca^{2+} (Milochau et al., 2014). Various Ca^{2+} sensitive Syts (Syt 5,7,9) play an important role in insulin release (Gauthier et al., 2008, Grise et al., 2007, Gustavsson et al., 2008, Iezzi et al., 2004, Iezzi et al., 2005, Eliasson et al., 2008) but the role of the Ca^{2+}-insensitive forms is still largely unknown.

The disassembly of the SNARE complex after the fusion event involves the ATPase N-ethylmaleimide Sensitive Factor (NSF). NSF binds to the SNARE complex via the adaptor protein α-SNAP and dissociates the complex by hydrolysis of ATP which separates syntaxin, Snap-25 and VAMP (Sollner et al., 1993, Morgan et al., 1994). In beta cells antibodies against NSF reduce the refilling of the granules from RP to RRP (Vikman et al., 2003).

Multidirectional movement of the granules occur in the beta cells prior to docking, priming and fusion. The beta cell cytoskeleton (microtubule and actin network) plays an important role in these movements (Seino and Bell, 2008). F-actin cytoskeletal network is crucial in second-phase insulin secretion which requires recruitment of granules to the plasma membrane from intracellular stores, F-actin remodelling is a requisite for the normal biphasic insulin secretion (Kalwat and Thurmond, 2013). Also, different motor proteins (kinesins, dyneins and myosins) are involved in the granule mobilization process (Ivarsson et al., 2005a, Vitale et al., 1995, Varadi et al., 2003).

Role of Ca^{2+}

The Ca^{2+} ion is very important in cellular processes like neurotransmission and hormone secretion. It was understood long back that extracellular Ca^{2+} is a prerequisite for glucose-stimulated insulin secretion (Grodsky and Bennett, 1966, Milner and Hales, 1967). The $[Ca^{2+}]_i$ plays a central role in insulin secretion and a direct correlation between increased $[Ca^{2+}]_i$ and exocytosis was demonstrated in the early 1980s (Wollheim and Pozzan, 1984, Rorsman et al., 1984). Later, by monitoring exocytosis using patch-clamp technique in single beta cell, it was confirmed that exocytosis is dependent on the rise of $[Ca^{2+}]_i$ (Ammala et al., 1993b). Exocytosis evoked by voltage-clamp depolarizations in beta cells echoes the Ca^{2+} current and halts almost immediately upon repolarization or closure of the Ca^{2+} channels (Ammala et al., 1993b, Barg et al., 2001). Different types (L-,N-,P/Q, R- and T-type) of Ca^{2+} channels are expressed in beta cells with different biophysical properties (Yang and Berggren, 2006). In rodent beta cells L- and R-type Ca^{2+} channels play very important role in biphasic insulin secretion (Schulla et al., 2003, Jing et al., 2005). The L-type Ca^{2+} channels are organized in clusters and forms a tight complex with secretory granules which ensures that the exocytotic machinery is exposed to the very high $[Ca^{2+}]_i$ at the mouth of the channels (Wiser et al., 1999, Barg et al., 2001). However, beta cells also contain a highly Ca^{2+}-sensitive pool of granules (HCSP). In this pool exocytosis occurs at $[Ca^{2+}]_i$ almost close to basal levels (Barg and Rorsman, 2004, Yang and Gillis, 2004). Human beta cells are equipped with LVA T-type Ca^{2+} channels which give rise to transient Ca^{2+} currents and HVA L-type and P/Q-type Ca^{2+} channels. In humans, P/Q-type Ca^{2+} channels shows tight coupling to exocytosis (Rorsman and Braun, 2013, Barnett et al., 1995).

In addition to the influx of Ca^{2+} through LVA and HVA Ca^{2+} channels, the $[Ca^{2+}]$ in various intracellular organelles (endoplasmic reticulum (ER), mitochondria, the Golgi apparatus, secretory granules and lysosomes) play an important role in insulin secretion. Because most of these organelles have specific Ca^{2+} influx and efflux pathways, they mutually influence free $[Ca^{2+}]$ in the other organelles e.g. sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), mitochondrial Ca^{2+} uniporter (MCU), Na^+/ Ca^{2+} exchanger (NCLX) etc. (Gilon et al., 2014). Changes in $[Ca^{2+}]$ in these subcellular compartments can affect many processes such as exocytosis, cell metabolism, apoptosis, gene expression, ER stress, etc. (Gilon et al., 2014, Gromada et al., 1999, Schonthal, 2012)

It was found in 1988 that beta cells show pronounced $[Ca^{2+}]_i$ oscillations with a periodicity of 2–6 min after glucose-stimulation (Grapengiesser et al., 1988). This inherent ability to generate oscillatory signals and coordination of the secretory activity among beta cells results in pulsatile insulin secretion from the pancreas, which is important for the actions of insulin on its target tissues (Tengholm and

Gylfe, 2009). Intracellular sequestration and release of Ca^{2+} from the ER contribute to shaping these oscillations (Fridlyand et al., 2003). Oscillations of the $[Ca^{2+}]_i$ are synchronized with oscillations in beta cell metabolism, intracellular cAMP concentration, phospholipase C activity and plasma membrane phosphoinositide lipids (e.g. PIP3) concentrations (Tengholm and Gylfe, 2009).

MicroRNAs

MicroRNAs (miRNAs) are single-stranded, short (20-23 nucleotide), non-coding RNAs that negatively regulate their target proteins. MiRNAs are considered important in expanding cell diversity and maintaining cellular phenotype (Kosik, 2010). In beta-cells, several miRNAs have been suggested to modulate cellular processes, this include effects on beta-cell development and physiology. Studies of miRNAs have proposed that they can emerge as novel biomarkers (Guay and Regazzi, 2013) and therapeutic targets (Eliasson and Esguerra, 2014) for diabetes. However, detailed knowledge about the function of miRNAs in insulin secreting cells is required.

The production and maturation of most miRNAs occurs via the canonical pathway of miRNA processing (Fang and Bartel, 2015, Winter et al., 2009). After transcription in the nucleus, primary miRNA (pri-miRNA) is cleaved by microprocessor complex (Drosha–DGCR8 (Pasha)) to get precursor miRNA (pre-miRNA) (Denzler and Stoffel, 2015). The resultant pre-miRNA is exported into the cytoplasm by a complex of Exportin-5 and Ran-GTP6 (Winter et al., 2009). Further processing of the miRNA and assembly of RISC (RNA-induced silencing complex) in the cytoplasm is mediated by the RISC loading complex (RLC). RLC is composed of RNase III Dicer, the double-stranded RNA-binding domain proteins TRBP (Tar RNA binding protein) and PACT (protein activator of PKR), and the core component Argonaute-2 (Ago2) (Winter et al., 2009). The RNase Dicer in complex with TRBP/PACT cleaves the pre-miRNA hairpin to generate a roughly 22-nucleotide long miRNA duplex. After unwinding of the miRNA duplex into a guide and a passenger strand, the guide strand is loaded together with Ago2 proteins into the RISC whereas the passenger strand is degraded. The functional strand guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation (Winter et al., 2009). Recently new non-canonical pathways for miRNA biogenesis have emerged including those that are independent of Drosha or Dicer (Ha and Kim, 2014).

Recognition of the target mRNA happens through complementary binding of the so-called seed sequence in the 5′ region of the miRNA (nucleotides 2–7) with its target sequence (usually in the 3'UTR of the mRNA). Interestingly one miRNA can target many mRNAs and one mRNA can get targeted by many miRNAs. It

has been estimated that more than half of the human protein coding genes could be a target of miRNAs (Bartel, 2009). It is proposed that miRNAs either acts as binary off-switches to repress target protein output for disallowed genes in a particular cell type or they acts as a rheostat (tuning interactions) to maintain optimal level of a functional protein in the cell (Bartel, 2009). Because of these characteristics miRNAs can regulate many cellular processes (Guay et al., 2011).

Genome-wide association studies (GWAS) have identified many genes associated with increased risk of T2D and most of them have been linked to beta cell function (Groop and Lyssenko, 2009). However despite great efforts GWAS studies can so far only explain ~10% of T2D, suggesting that much remains to be discovered (Billings and Florez, 2010). Recently, epigenetics and non-coding RNAs especially miRNAs have been found to play an important role in beta cell development and function (Dayeh et al., 2014, Kameswaran et al., 2014, van de Bunt et al., 2013, Bolmeson et al., 2011, Eliasson and Esguerra, 2014, Bagge et al., 2012). In human pancreatic islets 366 miRNAs have so far been identified using RNA-seq (van de Bunt et al., 2013). Moreover, miRNAs are known to affect many important components of glucose-stimulated insulin secretion such as glucose uptake and metabolism, membrane depolarization, insulin biosynthesis and exocytosis (Esguerra et al., 2014). Therefore miRNAs as important players in gene regulation have a huge potential of identifying new therapeutic approaches against diabetes and associated complications (Eliasson and Esguerra, 2014). Also it has been suggested that circulating miRNAs in blood can be used to evaluate health status and disease progression therefore miRNAs can emerge as novel biomarkers for diabetes (Guay and Regazzi, 2013).

Statins

Obesity is a risk factor for both T2D and cardiovascular disease (CVD). The latter is the leading causes of mortality worldwide. Hypercholesterolemia is one of the major CVD risk factors, therefore it becomes very important to normalize blood cholesterol levels (Brault et al., 2014, Prospective Studies et al., 2007). Statins are used effectively to reduce cholesterol levels and thereby reduce the risk of CVD disease. However, recent studies have shown diabetogenic effects of statins (Ridker et al., 2008, Cederberg et al., 2015).

Cholesterol is an important structural component of all animal cell membranes and it also serves as a precursor for the biosynthesis of certain hormones and vitamins (Berg, 2002). Since cholesterol is essential, each cell can synthesize it through a complex process called the mevalonate pathway where production of mevalonate by the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase is the rate-limiting step (Buhaescu and Izzedine, 2007). Though every cell can produce

cholesterol the rate at which they do so varies by cell type and organ function. The liver is responsible for most of the daily cholesterol production in the body (Berg, 2002). In blood, cholesterol is transported by lipoproteins. There are several types of lipoproteins chylomicrons, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and high-density lipoprotein (HDL) (Berg, 2002). The LDL cholesterol is considered "bad" cholesterol because it contributes to plaque formation in blood vessels which leads to atherosclerosis. The HDL cholesterol is considered "good" cholesterol because it helps remove cholesterol from the arteries and transport it back to the liver for excretion (Elshourbagy et al., 2014).

Statins, also known as HMG-CoA reductase inhibitors, are a family of drugs that are used to lower the cholesterol levels and thereby reduce the risk of CVD. Since their discovery, many different statins has entered the market like lovastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin and most recently rosuvastatin (Abbas et al., 2012). Several clinical trials have justified the role of statins in primary and secondary CVD prevention (Brault et al., 2014).

Statins inhibit the HMG-CoA reductase which leads to decreased *de novo* hepatic cholesterol synthesis (Fig. 7). This results in expression of more LDL receptors on hepatocytes which in turn increase the LDL uptake. The end result is reduced circulating levels of LDL and thereby decreased risk of CVD (Abbas et al., 2012, Brault et al., 2014). Statins in general are considered safe. However, in the "Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin" (JUPITER) study although rosuvastatin was found to prevent cardiovascular events physician reported diabetes among patients given rosuvastatin was significantly increased (Ridker et al., 2008). Another study has also pointed out the diabetogenic effects of statins, which seemed to be dose dependent. Moreover, statin treatment was demonstrated to be associated with both an increased risk of

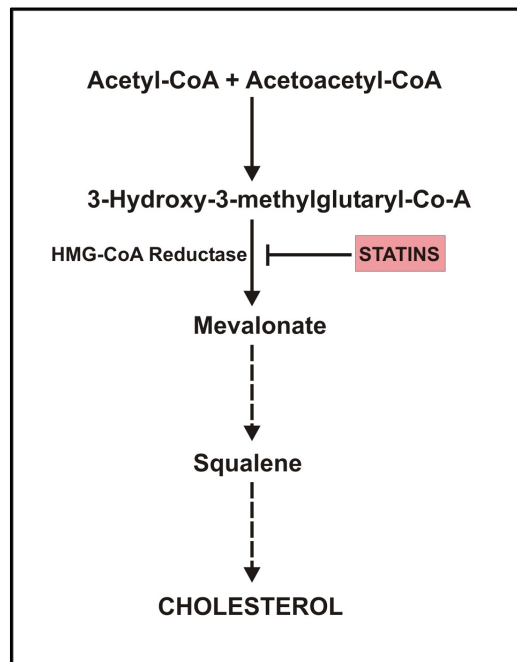

Figure 7. Mevalonate pathway of cholesterol synthesis.

insulin resistance and a reduced insulin secretion (Cederberg et al., 2015). Among the statins, rosuvastatin has been recognized as one of the more diabetogenic (Navarese et al., 2013).

Aims

The general objective of this thesis was to investigate the role of Ca^{2+}, miRNAs and rosuvastatin in the regulation of beta cell function. I have focused mainly to elucidate their effects on ion channels, exocytosis and insulin secretion of beta cells.

The specific aims were to

I. Examine the dynamics of Ca^{2+}-dependent insulin exocytosis with respect to pool depletion and Ca^{2+} current inactivation in INS-1 832/13 cells.

II. Investigate the role of miR-375 in the regulation of voltage-gated Na^{+} channel properties, exocytosis and glucose-stimulated insulin secretion in insulin-secreting beta cells.

III. Investigate whether miR-335 regulates the expression of exocytotic genes and affects exocytosis and insulin secretion in beta cells

IV. Study the effects of rosuvastatin on exocytosis and the stimulus-secretion coupling in the INS-1 832/13 cells.

V. Understand the cellular mechanism by which rosuvastatin influences glucose homeostasis and to investigate the effect on glucose uptake in target tissue and insulin secretion from pancreatic beta cells in high fat diet (HFD) and normal diet (ND) fed mice.

Materials and Methods

Cell culture

Most of the experiments in this thesis are done in the INS-1 832/13 cell line. This clonal cell line was derived by stably transfecting original rat insulinoma INS-1 cells with a plasmid containing the human proinsulin gene (Hohmeier et al., 2000). INS-1 832/13 cells secrete insulin both in a K_{ATP} channel-dependent and -*in*dependent manner in response to glucose concentrations in the physiological range (Hohmeier et al., 2000). These cells are generally stable both in terms of the desired secretion phenotype and ease of maintenance; hence this cell line is a good model for mechanistic studies. Moreover, using cell lines is in accordance with the 3R (replacement, reduction and refinement) principle of animal studies (European Commission, 2015).

Rodent animal models were used in study V to perform *in vivo* experiments. Moreover islets, adipose tissues and isolated primary beta cells from rodents or human cadaver donors have been used whenever necessary for the studies (study II, III and V).

Transfection

Lipofectamine transfection reagent was used for transfecting oligonucleotides into INS-1 832/13 cells with high efficiency (Zhao et al., 2008). Lipofectamine reagent works on the principle of lipofection as it can entrap the oligonucleotides and form liposomes. The phospholipid bilayer nature of liposomes and its net positive charge helps it to merge easily with the negatively charged cell membrane (Felgner et al., 1987).

MiRNAs are very short in length and in order to down-regulate them it is necessary to use highly specific and sensitive oligonucleotides. Locked nucleic acid (LNA) based oligonucleotides were used to down-regulate specific miRNAs (Braasch and Corey, 2001). LNAs are a class of high-affinity RNA analogs in which the ribose ring is “locked” in the ideal conformation (N conformation) for Watson-Crick binding. LNA based oligonucleotides show very good thermal

stability when hybridized to a complementary DNA or RNA strand (Exiqon, 2015, Braasch and Corey, 2001). For transient overexpression, chemically-modified doublestranded mature miRNAs were used. These are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs. However, they are not hairpin constructs and should not be confused with pre-miRNAs.

For down-regulation of genes of interest pre-designed siRNA were used against the particular mRNA. siRNAs are small (20-25 base pairs), double-stranded RNA molecules. They inhibit expression of their target gene typically by causing destruction of mRNA molecules of complementary nucleotide sequences through the well-known RNA interference (RNAi) pathway (Agrawal et al., 2003).

Reverse transcription quantitative real-time PCR

In order to robustly detect and quantify gene expression, amplification of the gene transcript is necessary and reverse transcription quantitative real-time PCR (RT-qPCR) technique was used for this purpose. RT-qPCR is a two-step process where the first step is to convert the RNA template into a complementary DNA (cDNA) using a reverse transcriptase and in the second step the resulting cDNA is used as a template for exponential amplification and simultaneous quantification using real-time PCR (Heid et al., 1996). Reverse transcription was performed for mRNA using random primers and for miRNA using sequence-specific stem-loop primers. The stem-loop primer accomplishes two important tasks a) it is specific for only the mature miRNA target and b) it extends the 5’ end of the RT amplicon thus making it amenable to downstream PCR amplification (Chen et al., 2005).

For qPCR, TaqMan assays (5′nuclease assay process) with primers and probes specific for each gene of interest are used (Heid et al., 1996). The TaqMan®MGB probes contains a reporter dye (FAM™ dye) linked to the 5′end of the probe, a minor groove binder (MGB) at the 3′end of the probe and a nonfluorescent quencher (NFQ) at the 3′end of the probe (Applied Biosystems, 2011). When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence but when DNA polymerase cleaves the probes it separates the reporter dye from the quencher dye. This results in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR therefore nonspecific amplification is not detected (Heid et al., 1996, Jensen et al., 2004).

A relative quantitation allows for a quantification of the difference in expression level of a gene between different samples (e.g. treated vs untreated samples). The data is expressed as a fold-change of expression levels of that particular gene. To

obtain accurate relative quantification and to correct for variations (e.g. loading error) it is important to normalize the expression of the gene of interest with the expression of a proper endogenous control. The genes used as an endogenous control are genes whose expression levels does not differ between the investigated samples (e.g. housekeeping genes) (Vandesompele et al., 2002). The comparative C_T method ($\Delta\Delta C_T$) was used for the normalization with endogenous control (Schmittgen and Livak, 2008).

Patch-clamp technique

Electrophysiology is the study of the electrical properties of cells and tissues where patch-clamp is one of the important techniques. The most important step in this technique is to form a tight pipette-membrane seal (resistance of 10-100 gigaohams). This is done by applying slight suction to the pipette where a small patch of membrane is sucked into the pipette to form an omega-shaped semivesicle. The high seal resistance is important to get rid of background noise and to perform high-resolution current measurements (Sakmann and Neher, 1984, Hamill et al., 1981). The firm pipette-membrane seal allows a certain number of configurations to be performed as follows a) cell-attached b) perforated c) Whole-cell c) outside-out d) inside-out

Cell attached configuration is used to measure single ion currents in the membrane patch after formation of the seal. Outside-out and inside-out patch configurations are used to measure single ion channel currents in the small patch once withdrawn from the cell. Perforated patch or standard whole-cell configuration is used to measure the summed currents from all ion channels in the cell. In the latter configurations the amplifier has electrical contact with the cell interior. In perforated patch configuration cytoplasm is intact, whereas in standard whole-cell configuration the cytoplasm is exchanged by the content of the pipette solution. In this thesis I have mainly used the standard whole-cell configuration, which allows for studies of the electrical behaviour of the entire cell. With this configuration we can use voltage-clamp or current-clamp mode to study 1) currents passing through the membrane 2) measurements of membrane potential (action potentials) 3) exocytotic and endocytotic events.

Currents can be measured in the voltage-clamp mode where membrane potentials are controlled by the amplifier. The cell is held at a negative membrane potential (-70 mV) and different depolarization protocols are used to study currents flowing through different voltage-gated ion channels.

Capacitance measurements

Exocytosis can be measured as a function of changes in cell membrane capacitance (C_m). This is based on the fact that biological membranes act as capacitors. A typical capacitor in an electronic circuit contains at least two electrical conductors (plates) separated by a dielectric where the dielectric is largely impermeant to current flow. Opposite charges are attracted to one another across the cell membrane. Capacitance in patch clamp measurements is a measure of the charge separated by the cell membrane. The alignment of charge along the cell membrane is sufficiently uniform for membrane capacitance to be a reliable index of membrane area (Kornreich, 2007). The specific capacitance of biological membrane is 9 fF/µm^2 (Gentet et al., 2000). The C_m is proportional to the membrane surface area (A) and the relationship is given by the equation

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

Where ε is a constant (specific membrane capacitance) and d is the distance between the two layers of phospholipids.

There are several advantages to the technique a) the possibility to conduct single cell experiments b) the total exocytosis in a cell can be monitored in one experiment c) high time resolution (~1 ms) d) access to the cytosol (Rorsman and Renstrom, 2003). On the other hand capacitance changes is calculated on net changes in cell surface area hence it does not discriminate between exocytosis and simultaneous endocytosis and between fusion of insulin granules and fusion of other vesicles such as small GABA-containing synaptic-like microvesicles (SLMVs). However it has been demonstrated that endocytosis is a slower process (Eliasson et al., 1996) and SLMVs are much smaller and do not contribute so much to the total change in capacitance (Braun et al., 2004).

In this thesis two different protocols have been used to investigate exocytosis. In one protocol exocytosis was elicited by ten successive 500 ms long depolarizations from -70 mV to 0 mV applied at 1Hz. This protocol is called train of depolarizations which is used to study release of granules from different pools (RRP and RP). The other protocol used is termed pulse length protocol which is used to study early exocytosis (IRP and RRP). Here, exocytosis was elicited by depolarizations (from -70 mV to 0 mV) at varying pulse durations from 5 ms to 800 ms and with varying interval between the depolarizations to examine exocytosis with respect to pool depletion and/or Ca^{2+} influx. Pulse-length data has been analysed using mixed effect modelling.

Mixed-effects statistical modelling

Common statistical models incorporates fixed effects which are parameters associated with an entire population or random effects which are associated with individual experimental units drawn at random from a population. A model which incorporates both fixed effects and random effects is called a mixed-effects model.

This model is appropriate for studying clustered data, e.g., pulse-length protocol where several depolarizations are applied to the same cell. Pooling of data neglects natural cell heterogeneity but mixed-effects modelling can handle and quantify biological variation and at the same time account for within-cell correlation (Pinheiro and Bates, 2000). Mixed-effects model describes relationships between a response variable and some covariates in the data. For analysing pulse-length data the linear mixed-effects model was used which included treatment group as fixed effect and cell as random effect (Pedersen et al., 2011, Pedersen, 2011).

Results and discussion

Paper I

Calcium current inactivation rather than pool depletion explains reduced exocytotic rate with prolonged stimulation in insulin-secreting INS-1 832/13 cells

T2D has been associated with a reduction in first phase insulin secretion. Previously our group together with others have suggested that first phase insulin secretion is associated with RRP. Several factors have been suggested to impact the size of RRP including cAMP (Rorsman and Renstrom, 2003, Renstrom et al., 1997, Eliasson et al., 2003). However, the main determinant of exocytosis is Ca^{2+} and the influx of Ca^{2+} is reduced during a depolarization due to Ca^{2+} channel inactivation. It is therefore important to study exocytosis as a function of Ca^{2+} influx (measured as Q) rather than pulse duration to determine the pool depletion (Pedersen, 2011) as has not been performed in some previous studies (Gopel et al., 2004, Olofsson et al., 2002, Rorsman et al., 2011). Moreover, single cell responses are often heterogeneous, which is neglected in most studies when only comparing mean values. Mixed-effects modelling can handle biological variation and at the same time considers within-cell correlation (Pinheiro and Bates, 2000).

In paper 1 we wanted to investigate the exocytotic response in INS-1 832/13 cells with respect to pool depletion and Ca^{2+} current inactivation. In order to do so, we applied different depolarization protocols to the cells and used linear mixed-effects modelling of the increase in capacitance (ΔC_m) as a function of Q. We also investigated the Ca^{2+} current sensitivity; with this we mean the sensitivity of exocytosis to Ca^{2+} entry via Ca^{2+}channels.

The overall hypothesis was that only a clear deviation (i.e. concave curve) from a linear relation between the Q and ΔC_m would suggests a pool depletion.

A linear relationship between exocytosis and Ca^{2+} influx was found in INS-1 832/13 cells and the mixed-effect model analysis revealed unaffected IRP and reduced Ca^{2+} current sensitivity in presence of EGTA

Exocytosis measured as capacitance increases was evoked by a standard pulse length protocol with varying pulse durations (5, 10, 20, 40, 80, 160, 320 and 640 ms) to investigate pool depletion under control conditions or in presence of the

calcium-buffer EGTA in the pipette solution to vary the $[Ca^{2+}]_i$. The ΔC_m showed a biphasic relation to the pulse length such that the average rate of exocytosis was higher during shorter than during longer pulses. However, plotting ΔC_m to Q gave a linear relationship.

Using linear mixed-effects model the common intercept for the control and EGTA groups was estimated to be 6.5±1.6 fF. The intercept reflects exocytosis in the limit of zero Ca^{2+} entry; here it corresponds to ~10 granules, which probably represent the IRP in INS-1 832/13 cells. The IRP was not affected by EGTA, but as expected the Ca^{2+} current sensitivity was significantly lower (reduced by 55%) in the EGTA group compare to control. However, there was no deviation from linearity in the EGTA group.

A 50-ms pre-pulse leads to depletion of IRP but not later exocytosis

To investigate pool depletion from another angle we used a double pulse protocol (two 50 ms depolarizations separated by 100 ms). The estimated intercept was different in the first and second pulse, but again it was not influenced by EGTA and therefore the same for the control and EGTA group. For the first pulse, the common intercept for the two groups was estimated to be 6.94±68 fF. In contrast, for the second pulse the common intercept was estimated to be -0.40±1.59 fF, showing that the first pulse depleted the small IRP. As expected, EGTA lowered the Ca^{2+} current sensitivity (by 71%) compared to control. Interestingly, the exocytotic response to Ca^{2+} entry was reduced by EGTA. The reduction was similar for the first and second pulse. This indicates that the first pulse did not deplete the granule pool responsible for later exocytosis.

Double pulse protocol was the subset of the larger protocol where we used varying pulse duration for the second pulse from 50-800 ms. The hypothesis was; if the cell possessed a limited granule pool then a prepulse of 50 ms would lead to changed Ca^{2+} current sensitivity of the following longer pulses. This would be so because the inflowing Ca^{2+} would have fewer granules to act upon because the prepulse would have reduced the pool of available vesicles. Here, linear mixed-effects modelling for the second pulse independent of the pulse duration showed that Ca^{2+} current sensitivity was not reduced either in the control group or in the EGTA group following a 50 ms prepulse. These results speak against the notion of pool depletion causing the biphasic capacitance pattern.

Recovery of Ca^{2+} current was enough to reset the exocytotic response

To study whether pool depletion or Ca^{2+} channel inactivation is the cause of the declined exocytotic response from another perspective, we used the combined prepulse pulse length protocol. Here, two initial depolarizations were followed by a third 500 ms depolarization after a rest interval at -70 mV lasting either 200 ms (protocol I) or 10 seconds (protocol II).

The idea was that if the first two pulses do not deplete the RRP (as shown by previous results) then current recovery would dictate the exocytotic response evoked by the third depolarization. Therefore, in this case one should expect the Ca^{2+} current sensitivity between protocols I and II to be equal. This idea was based on the fact that the Ca^{2+} current recovers much faster (Rorsman et al., 2011) from inactivation than the RRP recovers from depletion (~1 min) (Gromada et al., 1999, Barg et al., 2001).

For smaller second pulses the Ca^{2+} current recovered substantially (mean recovery >75) in a rest interval of just 200 ms. In contrast, following longer second pulses (≥200 ms) the current did not recover much during the 200 ms resting period, but recovered almost completely in 10 s. Mixed-effects modelling for third-pulse data with the longer second depolarization (≥200 ms) showed that Ca^{2+} current sensitivity between protocols I and II was not different. These findings show that the exocytotic response recovers in parallel to Ca^{2+} currents and therefore speak against the notion of pool depletion.

A train of 500 ms depolarizations did not able to empty the RRP

As observed above, short depolarizations were able to deplete IRP. We next wanted to study if more intense stimulation in the form of the widely used train protocol (train of ten 500 ms depolarizations), would deplete the relatively larger pool i.e. RRP. Here by using mixed-effects modelling we related the cumulative increase in membrane capacitance evoked by the depolarizations to the cumulative Ca^{2+} influx. There was no sign of pool depletion and intercept was estimated to be -3.1±15.1 fF and Ca^{2+} current sensitivity was estimated to be 1.81±0.48 fF/pC. However, if analysed only for first three pulses then the data showed an estimated intercept of 29.1±8.6 fF in agreement with the presence of IRP from the data analysed in the previous results.

In summary

a) INS-1 832/13 cells possess an IRP of ~10 granules.

b) Most exocytosis of granules occurs from a large pool. Exocytosis of granules from this pool is attenuated by the calcium-buffer EGTA, while IRP is unaffected by EGTA.

c) Pool depletion plays a minor role in the decline of exocytosis upon prolonged stimulation in INS-1 832/13 cells.

Discussion

Exocytosis in beta cells is operational at $[Ca^{2+}]_i$ of tens of micromolars (Takahashi et al., 1997). These levels can only be reached in the close vicinity of the Ca^{2+} channels. In beta cells calcium channels are organized in clusters (e.g. L-type Ca^{2+} channels) with secretory granules, which makes sure that the exocytotic machinery is exposed to high $[Ca^{2+}]_i$ (Barg et al., 2001, Wiser et al., 1999). In mouse beta

cells, IRP comprises ~10% of RRP. Recovery of IRP and RRP are rate limiting where IRP takes even longer time than RRP to recover (Barg et al., 2001). Here, we demonstrate that the same pools exist in INS-1 832/13 cells but their kinetics is different from primary cells which are illustrated in Fig. 8.

In INS-1 832/13 cells we found a tiny pool of ~10 granules, that could be released by minimal amounts of entered Ca^{2+}. It is therefore likely that this pool is the IRP situated near the Ca^{2+} channels. Importantly, this pool was not affected by EGTA and depletion of this pool did not disturb later exocytosis probably because of its small size. In contrast later exocytosis was strongly affected by EGTA, which suggests that most exocytosis in INS-1 832/13 cells occurs away from Ca^{2+} channels. Also, the large granule pool was not depleted by depolarizations lasting even up to a second. Interestingly in INS-1 832/13 cells even repeated 500-ms pulses did not lead to the exhaustion, if anything late exocytosis was tended to increase when Ca^{2+} entry was taken into account probably due to residual Ca^{2+} from the first pulses. This indicates that either this pool is very large or very rapidly refilled. Therefore, it seems likely that most of the exocytosis seen in our experiments is due to a HCSP (Fig. 8) or due to newcomers as in INS-1 cells (Yang and Gillis, 2004, Zhu et al., 2013).

In mouse and human beta cells there is a clear role of pool depletion contributing to biphasic insulin secretion (Henquin et al., 2002, Henquin et al., 2006). However, in INS-1 832/13 cells using patch-clamp capacitance measurements and mixed-effect modeling we showed that, RRP is not as easily depleted as previously thought and pool depletion plays a negligible role in shaping the decline in the exocytotic response. The observed exocytotic profile is instead mostly determined by the kinetics of Ca^{2+} current inactivation.

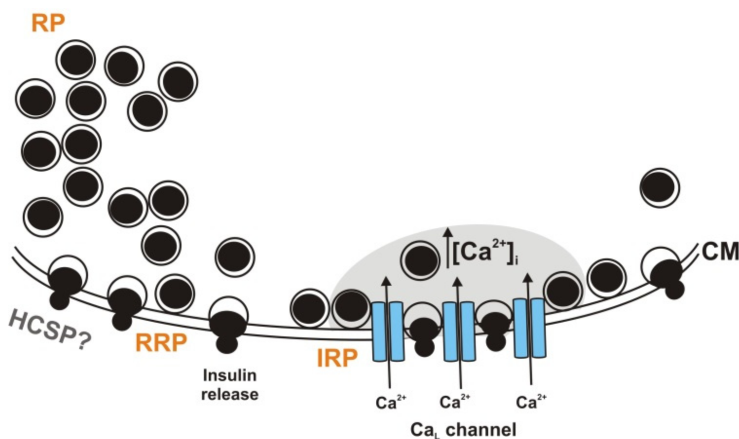

Figure 8. Model figure summarizing important results of paper I.

Paper II

Modulation of microRNA-375 expression alters voltage-gated Na^+ channel properties and exocytosis in insulin-secreting cells

After an initial detailed investigation on how Ca^{2+} affects exocytosis in the beta cells I moved into the studies of how miRNAs influences ion channels, exocytosis and thereby insulin secretion in beta cells. I started with miRNA-375, the most abundant miRNAs found in beta cells (Poy et al., 2004). Earlier studies have shown that modulation of miR-375 levels affect insulin secretion through effects on the exocytotic process (Poy et al., 2004) and miR-375 was found to be involved in glucose regulation of insulin gene expression (El Ouaamari et al., 2008). Moreover, miR-375 has been suggested to play an important role in islet development (Kloosterman et al., 2007). In agreement, a miR-375 knockout (375KO) mouse model showed that miR-375 is crucial for beta cell proliferation and thereby maintenance of normal beta cell mass and glucose homeostasis (Poy et al., 2009). Thus, miR-375 has multiple functions in the beta cells. Nobody though had earlier investigated in detail the effects on voltage-gated ion channels.

Different subunits of Na^+ channels were found to be predicted targets of miR-375 and they differed among species (TargetScanHuman, 2015). Interestingly, the role of Na^+ channels in beta cells seems to be species specific. Here we specifically investigated the role of miR-375 on voltage-gated Na^+ channel inactivation properties in beta cells. In addition the effects of modulation of miR-375 levels on exocytosis and insulin secretion were studied.

The studies were performed in INS-1 832/13 cells and beta cells from 375KO mice. To investigate the role of miR-375 in INS-1 832/13 cells we have either down-regulated or overexpressed miR-375 and these cells are called, LNA375 and OE375, respectively. Control cells are called SCR (scrambled).

Effect of miR-375 on insulin secretion, insulin content and exocytosis in INS-1 832/13 cells

Insulin secretion was not changed in OE375 or LNA375 cells. However, insulin content in OE375 cells was reduced by ~20%. Standard whole-cell configuration of the patch-clamp technique was used to study exocytosis. In OE375 cells, exocytosis measured as the sum of the increase in membrane capacitance evoked by the train of ten depolarizations was significantly reduced (by 35%) compared to SCR cells.

Effect of modulation of miR-375 levels on voltage-gated channel activity in INS-1 832/13 cells

Voltage-dependent currents were evoked by membrane depolarizations from -70 mV to voltages between -40 and +40 mV. The peak current (I_p) represents Na^+

current whereas sustained current (I_{sus}) and charge (Q) are related to the Ca^{2+} current. The maximal I_{sus} and Q at 0 mV was significantly reduced (by ~30%) in OE375 cells compare to SCR cells, whereas in LNA375 cells they were not changed. The I_p was not changed in either OE375 or LNA375 cells suggesting that the peak Na^+ current is unaffected by miR-375.

MiR-375 shifts Na^+ channel inactivation properties in INS-1 832/13 cells and mouse primary beta cells

Inactivation properties of voltage-gated Na^+ channels were measured using a two-pulse protocol. Here we used a conditioning pulse from -70 mV to voltages ranging from -130 to 40 mV; subsequent 1-ms resting period at -70 mV was followed by a depolarizing pulse to 0 mV during which the Na^+ current was measured. The half maximal inactivation (V_h) was not different in OE375 cells compare to SCR cells. Interestingly V_h in LNA375 cells was significantly shifted to the left (-66 ± 2 mV) i.e. towards more negative membrane potential compared to SCR cells (-60 ± 2 mV). On the other hand, V_h in beta cells of 375KO mice was significantly shifted to the right (-69 ± 2 mV) i.e. towards more positive membrane potential compared to beta cells of wild type mice (-83 ± 1 mV). Interestingly, the predicted miR-375 targets among Na^+ channels subunits, SCN3A and SCN3B, were considerably down-regulated (~30%) at protein levels in OE375 cells.

In summary (Fig. 9)

a) Overexpression of miR-375 leads to reduced exocytosis in INS-1 832/13 cells.

b) Overexpression of miR-375 leads to decreased voltage-gated Ca^{2+} influx in INS-1 832/13 cells.

c) Down-regulation of miR-375 shifts Na^+ channel inactivation properties in INS-1 832/13 cells towards more negative and in 375KO mice beta cells towards more positive membrane potential.

d) Overexpression miR-375 results in decreased expression of SCN3A and SCN3B at protein levels.

Discussion

Steady-state inactivation controls the number of channels that can be opened at a particular membrane potential. Hence, small changes in Na^+ channel kinetics can cause a considerable change in the number of channels available for generation of action potentials (Van Petegem et al., 2012). In human beta cells voltage-gated Na^+ channels are active at physiologically relevant membrane potentials and they are important in generation of action potential and glucose stimulated insulin secretion. In human beta cells Na^+ channels show a half maximal inactivation at ~-40 mV (Braun et al., 2008). The role of voltage-gated Na^+ channels in rodent beta cells in generation of action potential has been considered less important because

of their more negative half maximal inactivation (at -70 mV or below) (Hiriart & Matteson, 1988). However, in rat beta cells inhibition of voltage-gated Na^+ channels using tetrodotoxin (TTX) reduces glucose induced insulin secretion (Vidaltamayo et al., 2002), indicating that these channels still might play a role.

Interestingly, we saw that voltage-gated Na^+ channels inactivation properties were regulated by miR-375 in both INS-1 832/13 cells and primary 375KO mice beta cells. Though, the shift in inactivation of Na^+ channels could not lead to any detectable change in insulin secretion in INS-1 832/13 or 375KO mice beta cells, the concept can still be of importance for the regulation of insulin secretion. The notion that different Na^+ channel subunits can be differently targeted by miR-375 depending on the species makes it interesting to investigate if miR-375 is involved in regulation of Na^+ channels in human beta cells where these channels play an important role in generation of action potentials.

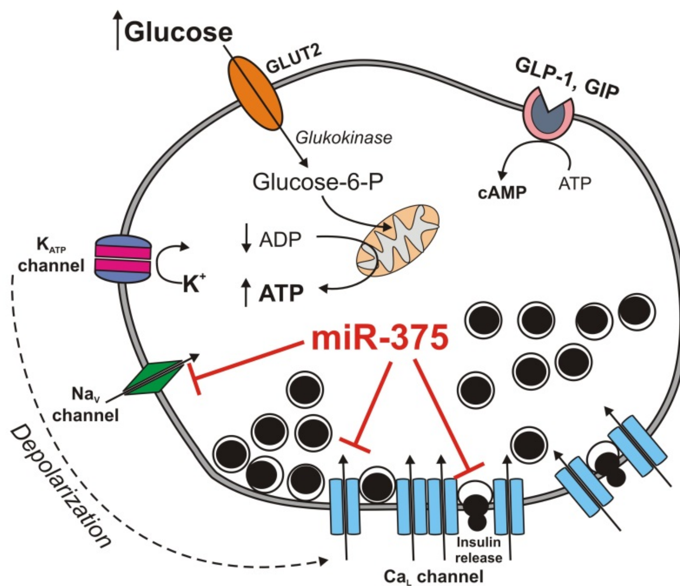

Figure 9. Summary of important results in paper II.

Paper III

MiR-335 over-expression impairs insulin secretion in β-cells through defective priming of insulin vesicles

Another important miRNA in beta cells is miR-335. We have previously demonstrated that this miRNA along with others is upregulated in islets from the GK rat (Esguerra et al., 2011). The GK rat is a well-studied non-obese T2D rodent model characterized by defective insulin secretion (Srinivasan and Ramarao, 2007, Goto et al., 1976). Interestingly, putative targets of upregulated miRNAs in the islets of GK rat are enriched for several beta cell exocytotic proteins (Esguerra et al., 2011). Specifically, miR-335 has been demonstrated to directly target *Stxbp1* by luciferase reporter assay (Esguerra et al., 2011). Interestingly, the expression of many exocytotic genes is down-regulated in islets from the GK rat (Gaisano et al., 2002, Zhang et al., 2002) and human donors with T2D (Andersson et al., 2012, Ostenson et al., 2006).

In paper III we aimed to investigate whether modulation of miR-335 levels regulates the expression of the confirmed exocytotic protein target, STXBP1, as well as the putative targets, SNAP25 and SYT11 (Fig. 10).

We hypothesized that miR-335 overexpression will lead to reduced expression of its target exocytotic proteins and thereby the exocytotic process and insulin secretion will be affected.

To investigate the role of miR-335, we overexpressed the microRNA in INS-1 832/13 cells (OE335) and compared these cells to cells transfected with a scramble control (SCR).

Overexpression of miR-335 leads to reduced glucose- and depolarization induced insulin secretion

The glucose-stimulated insulin secretion at 16.7 mM glucose was reduced (~24%) in OE335 cells compared to SCR cells but insulin content was not significantly different. These findings suggest impaired insulin release. To determine whether the observed defective insulin release is due to a disturbed exocytotic process, insulin secretion was measured athigh concentration of K^+ (50 mM) with a non-stimulatory concentration of glucose (2.8 mM). The K^+-induced insulin secretion was reduced (~23%) in OE335 cells compared to SCR cells.

Overexpression of miR-335 leads to reduced exocytosis

Exocytosis, measured using the patch clamp technique, was significantly reduced (~36%) in OE335 cells as compared to SCR. We observed no significant differences in the size of the Ca^{2+} current (I_{sus}) and charge (Q) in OE335 cells compared to SCR cells at any of the voltages tested.

The effect of elevated levels of miR-335 on the exocytotic process was further studied in detail by TIRF microscopy using the granular marker neuropeptide-Y (NPY)-mEGFP. The total number of exocytotic events in OE335 cells was significantly less than in SCR, but the density of docked granules was not changed. The granule fusion pore lifetime was also not significantly different in OE335 cells compare to SCR. However, the granular content release was significantly faster in OE335 cells compared to SCR.

Overexpression of miR-335 leads to reduced expression of STXBP1, SNAP25 and SYT11

We wanted to investigate whether the impaired insulin secretion and exocytosis observed in OE335 cells were due to down-regulation of some exocytotic prwhich are putative targets of miR-335 (TargetScanHuman, 2015).

The protein levels of three miR-335 targets SNAP25 (~50%), STXBP1 (~25%) and SYT11 (~50%) were significantly reduced in OE335 cells compared to SCR cells (Fig. 10).

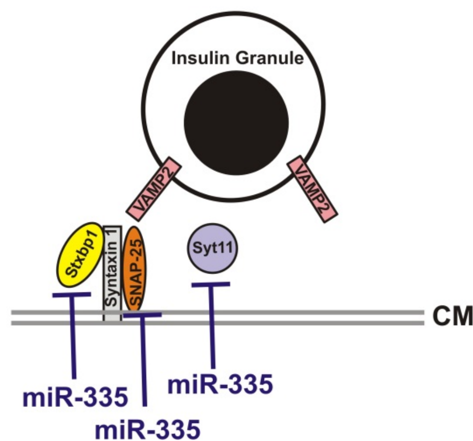

Figure 10. Inhibition of key exocytotic proteins by miR-335.

Knock-down of SYT11 leads to disturbed basal insulin secretion and impaired exocytosis

We down-regulated SYT11 (~65% at mRNA level) in INS-1 832/13 cells (siSYT11) and investigated the response as compared to control (siSCR) cells. Insulin secretion at 16.7 mM glucose was not changed but basal insulin secretion at 2.8 mM glucose was significantly increased (~2 fold) in siSYT11 cells.

Exocytosis was studied as increases in membrane capacitance elicited by a train of ten depolarizations. The capacitance evoked by the first depolarization was significantly lower (~60%) in siSYT11 cells as compared to siSCR cells. We observed no significant differences in the Ca^{2+} current in siSYT11 cells compared to siSCR cells.

In summary

a) Overexpression of miR-335 leads to reduced glucose-stimulated insulin secretion and exocytosis without affecting insulin content, density of docked granules and Ca^{2+} influx.

b) OE335 cells shows reduced expression of the exocytotic proteins STXBP1, SNAP25 and SYT11.

c) Knock-down of SYT11 does not change glucose-stimulated insulin secretion but disturbs basal insulin secretion and impairs rapid exocytosis.

Discussion

It is known that in beta cells of the GK rat, reduced expression of exocytotic proteins plays a major role in defective insulin secretory response (Zhang et al., 2002) and in these cells upregulated miRNAs putatively target several exocytotic proteins (Esguerra et al., 2011). However, it was unclear if dysregulated expression of specific miRNAs can affect the exocytotic process in these beta cells.

Whole-cell patch clamp technique revealed that overexpression of miR-335 leads to a substantial reduction in exocytosis. This reduction in exocytosis could be because of impaired docking and/or priming of the insulin granules. Using TIRF microscopy it was found that miR-335 affects exocytosis through impaired priming or post priming processes such as granule fusion but the docking process was not affected. This interpretation is based on the fact that in OE335 cells the density of docked granules was not changed but the number of exocytotic events was significantly reduced. Moreover, insulin content was also not changed in OE335 cells suggesting that the regulatory effect of miR-335 is mainly on the end stage in the exocytotic process. Indeed, predicted targets among the exocytotic genes where significantly reduced on protein level.

STXBP1 is known to be essential for granular docking (Tomas et al., 2008, Toonen and Verhage, 2007, Voets et al., 2001). Interestingly, although this protein was down-regulated in the OE335 cells the density of docked granules was not affected. One of the possible reasons for this could be that a ~25% reduction of STXBP1 was not sufficient to have noticeable effects on docking. However, it is also important to consider that it is difficult to compare the effects of modulation of miRNA levels with knock-down of single specific protein.

Knock-down of SYT11 leads to disturbed basal insulin secretion and reduced exocytosis without affecting Ca^{2+} influx, suggesting a direct role of SYT11 in the exocytotic process and in basal insulin secretion. This finding is very interesting as Syts are known to act as a clamp to prevent premature triggering of exocytotic events (Martin et al., 1995, Chicka et al., 2008). It is possible that the observed increased basal insulin secretion in siSYT11 cells is due to a failure to prevent premature insulin release because of the down-regulation of SYT11. Importantly, increased basal insulin secretion is vital characteristics of T2D (Del Prato et al., 2002) and expression levels of SYT11 were found to be down-regulated in the islets of T2D human donors (Andersson et al., 2012). SYT11 does not bind to Ca^{2+} (Milochau et al., 2014), and its actual mechanism of action is currently unclear.

In conclusion, this study suggests that modulating the levels of miR-335 may provide a novel approach to restoring insulin secretory functions in diseased pancreatic beta cells.

Paper IV

Rosuvastatin treatment affects both basal and glucose-induced insulin secretion in INS-1 832/13 cells

Statins are consider safe and well-tolerated and used for their ability to lower cholesterol levels and thereby reduce cardiovascular events (Armitage, 2007). However, there are studies suggesting diabetogenic effects of statins including rosuvastatin (Ridker et al., 2008). Our group has earlier demonstrated that cholesterol is an important factor for exocytosis in beta cells (Vikman et al., 2009). Hence, to investigate if the diabetogenic effect of rosuvastatin is due to its cholesterol lowering ability we explored the impact of rosuvastatin on insulin secretion and exocytosis.

Rosuvastatin is one of the most potent inhibitors of HMG-CoA reductase (White, 2002). It is hydrophilic in nature and transported actively into hepatocytes by special transporters e.g. Organic Anion Transporting Polypeptides (OATP) (Kitamura et al., 2008), hence considered safe for non-hepatic tissue (White, 2002). However, functional OATP1B3 has recently been found in pancreatic beta cells (Meyer Zu Schwabedissen et al., 2014) possibly making these cells more vulnerable for the effect of rosuvastatin.

Here we have investigated the effects of short term incubations (24-48 hr) with rosuvastatin on exocytosis and the stimulus-secretion coupling of the INS-1 832/13 cells.

Rosuvastatin decrease both glucose- and K^{+}-induced insulin secretion and increase basal insulin secretion

We first studied the effect of different concentrations of rosuvastatin (20 nM to 20 µM) on basal (2.8 mM glucose) and glucose-stimulated (16.7 mM glucose) insulin secretion. Low concentration (20 nM) of rosuvastatin had no effect on insulin secretion but at concentrations ≥200 nM rosuvastatin reduced glucose-stimulated insulin secretion by ~25% compare to control. Interestingly, 2 µM and 20 µM rosuvastatin markedly increased basal insulin secretion compare to control by ~65% and ~165%, respectively. Basal insulin secretion in cells treated with 20 µM rosuvastatin was not significantly different in the presence or absence of K_{ATP} channel opener diazoxide. K^+-induced (50 mM) insulin secretion in the presence of 2.8 mM glucose was significantly reduced in cells treated with 20 µM rosuvastatin.

High dose of rosuvastatin leads to decreased exocytosis and Ca^{2+} influx through the voltage-gated Ca^{2+} channels

Exocytosis measured, using the patch clamp technique, as the total increase in membrane capacitance elicited by a train of ten membrane depolarizations was significantly reduced (~34 %) in cells treated with 20 µM rosuvastatin compare to control. In these cells there was no change in Ca^{2+} sensitivity. In this study Ca^{2+} was crudely measured by comparing the exocytotic response to the first depolarizing pulse in the train with the Ca^{2+} current elicited by the same pulse. The Ca^{2+} current was significantly reduced (~45%) in cells treated with 20 µM rosuvastatin.

Mevalonate but not squalene can rescue reduced insulin secretion caused by rosuvastatin

Mevalonate is present upstream in the mevalonate pathway whereas squalene is found further downstream in this pathway in one of the arms that exclusively leads to the formation of cholesterol (Fig. 7). In order to study if the observed effects of rosuvastatin on insulin secretion are via the cholesterol synthesis pathway we added mevalonate or squalene to cells treated with 20 µM rosuvastatin.

Interestingly, we found that mevalonate, but not squalene could rescue both glucose- and K^+-induced insulin secretion defects in cells treated with rosuvastatin.

In Summary (Fig. 11)

a) Rosuvastatin dose dependently reduce glucose-induced insulin secretion and increases basal insulin secretion.

b) High dose of rosuvastatin (20 µM) leads to reduced K^+-induced insulin secretion.

c) Rosuvastatin (20 µM) leads to reduced Ca^{2+} influx and exocytosis.

d) Mevalonate can rescue reduction in glucose-induced insulin secretion caused by rosuvastatin.

Discussion

Cholesterol is an important component of the animal cell structure and it is required for the cell to function normally (Berg, 2002). Hence it was interesting to study if rosuvastatin affects the function of beta cells and whether that effect is through the cholesterol lowering ability of rosuvastatin.

We found that rosuvastatin dose dependently affects insulin secretion. However, the reduced glucose-stimulated insulin secretion in INS-1 832/13 cells is in contrast with what was reported in MIN-6 cells using other statins (Ishikawa et al., 2006). Interestingly, in this study (Ishikawa et al., 2006) they observed no significant effect on glucose-stimulated insulin secretion but importantly they also observed disturbed basal insulin secretion after treatment with different statins as found here in our study. These diverse results could be due to use of different cell line or it could be due to a difference between the statins with respect to how they affect beta cell function. However, the disturbed basal insulin secretion seems to be the more consistent effect of statins which is important to notice because increased basal insulin secretion is a vital characteristics of T2D (Del Prato et al., 2002).

Interestingly, 200 nM rosuvastatin reduces glucose-stimulated insulin secretion without affecting exocytosis and 20 µM rosuvastatin reduces both glucose-stimulated insulin secretion and exocytosis. This indicates that processes both upstreams and downstreams of the K_{ATP} channels are dose affected by rosuvastatin depending on the dose. Diazoxide was not able to rescue the increased basal insulin secretion in rosuvastatin treated cells. It shows that increased basal insulin secretion is not due to premature closure of the K_{ATP} channels.

Mevalonate rescued glucose-stimulated insulin secretion and there was a tendency to a reduced basal insulin secretion as well. Squalene could neither rescue glucose-stimulated insulin secretion nor reduce basal insulin secretion. This is an important finding which shows that the effect of rosuvastatin in INS-1 832/13 cells is not related to the cholesterol lowering effects of the rosuvastatin, but rather side effects of its effective blockage of the mevalonate pathway.

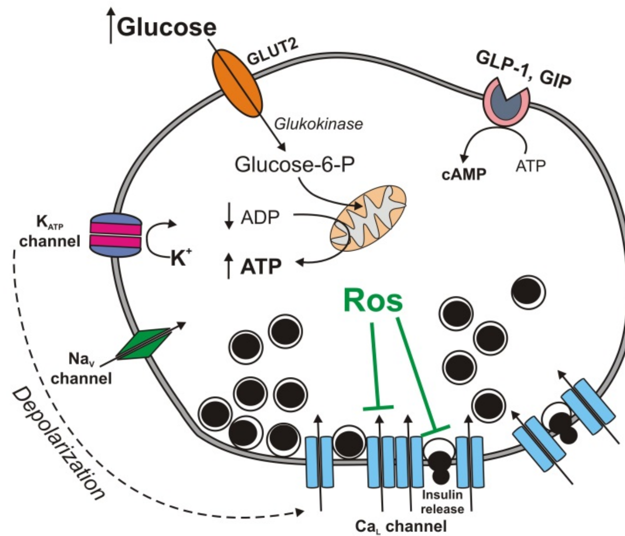

Figure 11. Summary of important results in paper IV.

Paper V

Dual effect of rosuvastatin on glucose homeostasis through improved insulin sensitivity and reduced insulin secretion

After studying the effect of rosuvastatin on insulin secretion and exocytosis in INS-1 832/13 cells, I wanted to investigate its role *in vivo* on glucose homeostasis in mice. We used C57BL/6 mice fed on HFD and compared them with mice fed on ND. The total study period was 12 weeks and these mice were treated with rosuvastatin (~0.2 mg/mice/day) for last 8 weeks.

Here we studied a) effects of rosuvastatin on glucose homeostasis, insulin resistance and beta cell function b) differences in the effect of rosuvastatin in mice on HFD and ND.

The HFD fed mouse is a widely used model to study insulin resistance and factors associated with the metabolic syndrome (Winzell and Ahren, 2004). The HFD fed mice are obese, have elevated blood cholesterol, slightly increased blood glucose and much increased blood insulin levels (Winzell and Ahren, 2004).

Rosuvastatin treatment resulted in improved insulin sensitivity in ND mice

Decreased blood glucose levels were observed during the first 30 min in an oral glucose tolerance test (OGTT) performed in ND mice after 4 weeks of

rosuvastatin treatment. This was reflected by an increased insulin sensitivity in these mice. Moreover, *in vitro* basal glucose uptake was also found to be increased in isolated adipocytes from the same mice.

Rosuvastatin reduce *in vitro* insulin secretion, insulin content and affects Ca^{2+} oscillation in the islets of ND mice

The *in vitro* insulin secretion from isolated islets, performed by using different glucose concentrations in addition with 50 mM K^+ and GLP-1, was significantly reduced in ND mice. Insulin content was also reduced (~25%) and there was no significant difference in the insulin secretion if normalized to insulin content. Intracellular Ca^{2+} oscillations were measured and interestingly several parameters in the Ca^{2+} response were changed. The changed parameters include reduced uptake in to ER (A_d) and a significant delay in first phase Ca^{2+} response to glucose (D_0).

Rosuvastatin reduce insulin secretion *in vivo* but improves glucose uptake and insulin secretion *in vitro* in HFD mice

The acute insulin response in OGTT performed on HFD mice after 8 weeks of rosuvastatin treatment was significantly lower than in the controls. Insulin sensitivity *in vivo* was not significantly different between the groups but there was a tendency towards improved insulin sensitivity in the rosuvastatin group. Indeed, *in vitro* basal and insulin stimulated glucose uptake was increased in isolated adipocytes in the HFD rosuvastatin group. Interestingly, insulin secretion normalized to insulin content *in vitro* was significantly improved in the HFD mice that were on rosuvastatin compared to the control group.

Expression of *CHOP* is reduce by rosuvastatin in islets from ND mice

In order to understand impaired Ca^{2+} response in rosuvastatin mice, we investigated the expression of the ER-related genes *SERCA2, SERCA3, Sel1l* and *CHOP* together with *Calb1*. Interestingly, all 5 genes were significantly reduced in islets of HFD mice compare to ND mice, suggesting a tremendous effect of HFD on expression levels. Expression of *CHOP* was reduced in ND rosuvastatin group compare to ND but no reduction was found in the HFD rosuvastatin group.

In summary

a) In ND mice rosuvastatin treatment resulted in improved *in vivo* insulin sensitivity. *In vitro* it reduces insulin secretion, insulin content and affects Ca^{2+} oscillation but improved glucose uptake in isolated adipocytes.

b) In HFD mice rosuvastatin treatment resulted in reduced insulin secretion *in vivo* but improved insulin secretion *in vitro* and improved glucose uptake in isolated adipocytes.

c) In ND mice islets expression of ER-stress marker *CHOP* was reduced by rosuvastatin treatment.

Discussion

In our *in vivo* experiments rosuvastatin clearly improves insulin sensitivity in the mice on ND and we could observe a tendency of improved insulin sensitivity also in the HFD mice. These findings are supported by the *in vitro* measurements showing increased basal and insulin-dependent glucose uptake in these mice after rosuvastatin treatment. Hence, our study is in agreement with work in support of improved insulin sensitivity after rosuvastatin treatment (Guclu et al., 2004, Okada et al., 2005, Paolisso et al., 1991, Sonmez et al., 2003). In adipose and muscle cells, glucose uptake is facilitated through insulin-regulated glucose-transporter GLUT4 (Stenkula et al., 2010). It would be interesting to examine whether rosuvastatin improves glucose uptake by modulating the activity of GLUT4.

Our data also suggest that rosuvastatin cause impaired beta-cell function and reduced insulin secretion, which is in agreement with the recent METSIM study (Cederberg et al., 2015) and study IV of this thesis. The observed impaired insulin secretion could be a result of reduced insulin content and/or disturbed Ca^{2+} signaling after rosuvastatin treatment. Study IV in this thesis suggests effects on the Ca^{2+} currents, and this study shows changes in Ca^{2+} handling and reduced insulin content. In addition, the observation that rosuvastatin reduced expression of the ER-stress marker *CHOP* in ND mice, suggest a protective effect against ER-stress. Altogether, this might lead to long-term effects on the beta cell Ca^{2+}-signaling and insulin secretion.

This study shows that rosuvastatin has an overall positive effect on glucose homeostasis, although observed deleterious effects on the beta cell function in the long run might lead to hyperglycemia.

Concluding Remarks

In this thesis I have investigated factors that can affect beta cell function, especially exocytosis and insulin secretion. The exocytotic process is crucial in the beta cells and dysfunctional exocytosis plays an important role in the development of diabetes. Here, I have demonstrated (Fig. 12) that Ca^{2+} influx and Ca^{2+} channel inactivation plays a central role in observed exocytotic profile in INS-1 832/13 cells. Dysregulated expression of miRNAs affects voltage-gated Ca^{2+} and Na^{+} channel properties, they also affects exocytosis and insulin secretion by regulating the expression of key exocytotic proteins. Rosuvastatin impairs Ca^{2+} oscillation and high dose rosuvastatin affects Ca^{2+} influx, exocytosis and insulin secretion.

In this thesis the following specific conclusions were reached

I. Pool depletion plays a minor role in the observed exocytotic profile of INS-1 832/13 cells instead it is mostly determined by the kinetics of Ca^{2+} current inactivation. The INS-1 832/13 cells possess a small IRP and most exocytosis occurs from a large pool of granules.

II. Overexpression of miR-375 leads to reduced expression of Na^{+} channel subunits as well as reduced, Ca^{2+} influx and exocytosis in INS-1 832/13 cells. Down-regulation of miR-375 affects Na^{+} channel inactivation properties in INS-1 832/13 cells and 375KO mice beta cells.

III. Overexpression of miR-335 leads to impaired exocytosis and thereby reduced insulin secretion through decreased expression of STXBP1, SNAP25 and SYT11. Down-regulation of SYT11 leads to disturbed basal insulin secretion and impaired exocytosis.

IV. In INS-1 832/13 cells rosuvastatin dose dependently affects Ca^{2+} influx, exocytosis, basal and glucose-induced insulin secretion. This effect is not related to the cholesterol lowering ability of the rosuvastatin, but rather side effects of its blockage of the mevalonate pathway.

V. Rosuvastatin has an overall positive effect on glucose homeostasis in mice. Rosuvastatin treatment resulted in improved *in vivo* insulin sensitivity and improved *in vitro* glucose uptake in isolated adipocytes. In ND mice rosuvastatin treatment *in vitro* reduces insulin secretion, insulin content and affects Ca^{2+} oscillation. In HFD mice rosuvastatin treatment *in vivo* resulted in reduced insulin secretion.

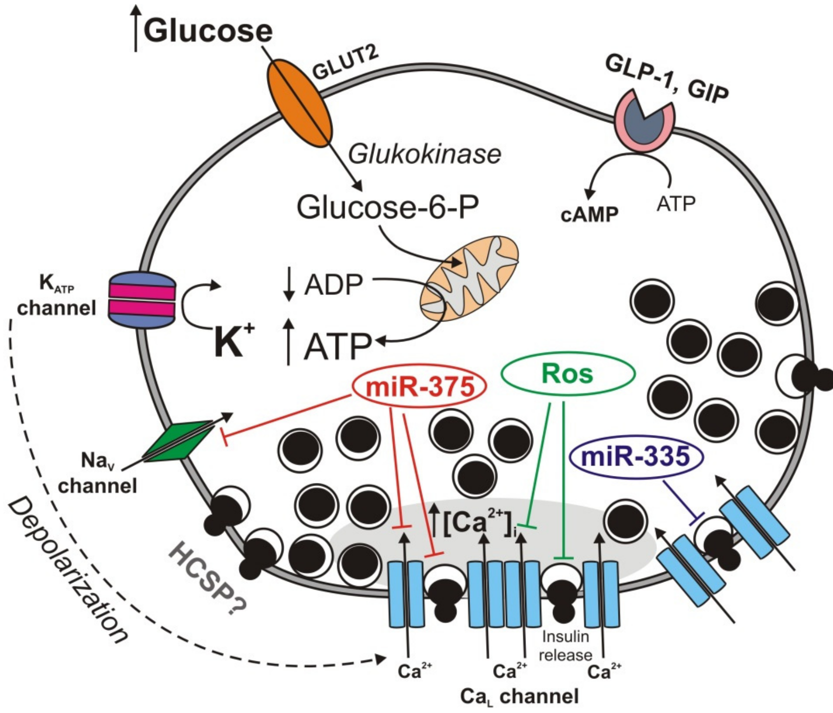

Figure 12. Summary of important results in this thesis showing effects on beta cell function.

Future perspectives

Impaired beta cell function plays an important role in the pathophysiology of T2D. Dysfunctional exocytosis is one of the major factors affecting beta cell function. Understanding the detailed cellular mechanism of exocytosis and factors regulating the exocytotic process could prove important in finding innovative targets for therapeutic intervention in T2D.

Exocytosis in beta cells is dependent on the availability of insulin granules and Ca^{2+} influx. The biphasic exocytotic pattern seen in beta cells is primarily attributed to granule pool depletion. However, reduction in Ca^{2+} influx due to inactivation of Ca^{2+}-channels can also be responsible for observed biphasic exocytotic pattern. We investigated pool depletion in beta cells by using pulse-length protocol and mixed-effect modelling. We found in study I that the exocytotic pattern is mostly determined by the kinetics of Ca^{2+} current inactivation rather than pool depletion in INS-1 832/13 cells. Mixed-effect modelling used in this study takes care of cellular heterogeneity, which needs to be considered more in future analysis of these of type of clustered data to gain more detailed information. Granular pool regulation is central in beta cell exocytosis and defects in this process can very well impair exocytosis and thereby insulin secretion. This study would set a good example in order to investigate pool depletion in rodent and human beta cells in the future.

Beta cell function can be influenced by miRNAs and they have a huge therapeutic potential due to their intrinsic ability to function as master regulators. We investigated miRNAs in this thesis; miR-375 and miR-335. MiR-375 was found to regulate Na^{+} channels inactivation properties. Inactivation properties are important as they can influence the availability of the channels for generation of action potentials. Therefore modulating the levels of miR-375 in human beta cells would lead to better understanding of the importance of regulation of enigmatic Na^{+} channels. This would also further elucidate the role of miRNAs in generation of electrical activity in beta cells. Mir-335 was found to affect the exocytotic process and thereby insulin secretion by regulating the levels of exocytotic proteins (STXBP1, SNAP25 and SYT11). This is an interesting finding making it evident that a dysregulated miRNA can influence the beta cell function through exocytosis. Another interesting finding is that of SYT11, as its downregulation increase the basal insulin secretion and decrease rapid exocytosis from beta cells; both of these phenomena are known to occure in T2D. Therefore, these findings

may be of great significance in T2D pathophysiology and worth investigating in human beta cells. If these roles of miR-335 and SYT11 are proven in human beta cells then they would stand a chance to emerge as an efficient biomarker or therapeutic target in T2D.

Rosuvastatin, a drug used to reduce blood cholesterol and thereby CVD, has been suggested to be diabetogenic. Therefore, we investigated the role of rosuvastatin in insulin secretion and glucose homeostasis. The adverse effects of rosuvastatin were found to be dose dependent and not related to its cholesterol lowering ability. Although rosuvastatin has an overall positive effect on glucose homeostasis it impairs beta cell function. These findings emphasize the need of investigating the exact cellular mechanism by which rosuvastatin influences beta cell function. Understanding these mechansims would allow us to counteract adverse effects of rosuvastatin and thereby render the clearly lifesaving statins even safer to use.

Our studies contribute to improved understanding of the cellular mechanisms of the beta cells, especially regulation of exocytosis and insulin secretion. I believe that, the above mentioned contribution of this thesis work is one small step forward towards solving the puzzle of diabetes.

Populärvetenskaplig sammanfattning

När vi äter ökar nivån av blodsocker (blodglukos) i blodet. Glukos forslas i blodet till målvävnader, såsom fett och skelettmuskel, där det används som energi. Men för att glukos ska kunna tas upp av målvävnaden krävs insulin. Insulin är ett hormon som produceras av beta-celler i bukspottkörtelns Langerhanska öar, vars sekretion ut i blodet regleras av blodglukos. Då denna process misslyckas förmår inte kroppen att sänka blodglukos tillräckligt. Detta leder till utveckling av typ 2 diabetes, som är en kombination av beta-cellernas oförmåga att frisätta tillräcklig mängd insulin och målvävnadens oförmåga att ta upp glukos.
I denna avhandling har jag undersökt mekanismer som påverkar insulinsekretion och då framförallt en process som benämns exocytos. I beta-cellen fylls små runda blåsor, sk granula med insulin, då insulinet väl har producerats. Exocytos är den process varmed dessa granula smälter samman med det omgivande membranet på beta-cellen för att insulinet ska komma ut i blodet. Ökad koncentration av glukos är en grundläggande faktor för att exocytos ska ske och insulin utsöndras. Jag har specifikt fokuserat på tre faktorer som kan påverka exocytos-processen i beta-cellen och därmed också insulinsekretionen och nivån av glukos i blodet. Dessa är calcium, microRNA och rosuvastatin.
Kalcium är den molekyl som initierar fusionen av granula med det omgivande membranet. Jag har i min avhandling på detaljerad cellulär fysiologisk nivå med hjälp av en matematisk modell studerat detaljer i förhållandet mellan kalcium och fusionen av granula, som har medfört ökad förståelse för den här processen. En viktig del i denna studie är att jag tagit tillvara att enskilda celler är heterogena, något som inte tidigare gjorts i liknande studier.
MicroRNA är små molekyler som reglerar mängden av protein i cellen. Då en cell har mycket av ett microRNA så minskar mängden av protein som detta microRNA specifikt reglerar. Dessa molekyler har föreslagits kunna fungerar som biomarkörer då halten av specifika microRNA förändras vid uppkomst av sjukdom. MicroRNA är ofta vävnads-specifika och därför har reglering av mängden microRNA också förslagits kunna användas som läkemedel. Jag har studerat två microRNA, miR-375 och miR-335, och hur de reglerar mängden specifika protein, som påverkar sekretionen av insulin och exocytos-processen. I min avhandling fann jag att miR-375 reglerar beteendet hos en spänningskänslig jonkanal som forslar natrium in i cellen. Vidare fann jag att överuttryck av miR-335 påverkar mängden av tre olika protein som medverkar i exocytos-processen. Det sistnämnda är extra intressant eftersom det tidigare har visats att mängden

miR-335 är förhöjd i diabetiska djurmodeller och mängden av flera proteiner som medverkar vid exocytos-processen är reducerad i Langerhanska öar vid typ 2 diabetes.
Rosuvastatin är en statin som används för behandling av kardiovaskulära sjukdomar för att sänka kolesterolnivån. På senare tid har det via flera studier framkommit att medicinering med statiner medför en ökad risk att få diabetes. För att undersöka detta närmare har jag i min avhandling undersökt hur rosuvastatin påverkar exocytos-processen, sekretion av insulin och blodglukosnivåer. Jag fann att rosuvastatin minskade insulinsekretionen och påverkade nivåerna av kalcium inne i cellen. Vidare hade rosuvastatin en positiv effekt på upptaget av glukos i målvävnad, och åtminstone under den period som vår studie pågick förändrades inte blodglukosnivåerna. Den minskade insulinsekretionen kan dock på längre sikt komma att öka glukoshalten i blodet. Detta får framtida studier visa.
Sammanfattningsvis visar jag i min avhandling vikten av en fungerade exocytos-process för fungerande sekretion av insulin. Genom denna kunskap har ytterligare en pusselbit lagts till det pussel som behöver läggas för att vi ska få en ökad förståelse av hur insulin utsöndras och hur defekter i denna process leder till typ 2 diabetes. Nödvändigt för en framtida förbättrad behandling av sjukdomen.

लोकप्रिय विज्ञान गोषवारा

आपण जे काही खातो त्या मुळे रक्ता तील साखरे (ग्लुकोस) चे प्रमाण वाढते. शरीराला उपयुक्त अशा या साखरेचे परिवहन शरीराच्या विभिन्न भागात केले जाते; उदाहरणार्थ: चरबी, विविध स्नायू इत्यादी., जिथे तीचा वापर उर्जा स्त्रोत म्हणून केला जातो. परंतु ही साखर शरीरा मध्ये शोषून घेण्या साठी इन्सुलिन नावाच्या संप्रेरकाची गरज भासते. इन्सुलिन नावाचे हे संप्रेरक स्वादुपिंडा तिल बीटा पेशी द्वारे तयार केले जाते. इन्सुलिन चा रक्तातील प्रादुर्भाव हा शरीरातील साखरे च्या प्रमाणा वर अवलंबून असतो. मानवी शरीरातील हि एक सर्व सामान्य प्रक्रिया आहे, या प्रक्रिये मध्ये काही बिघाड झाल्यास, शरीर रक्तातील साखरेचे प्रमाण नियंत्रित करू शकत नाही. पुढे जाऊन याचे रुपांतर मधुमेह-प्रकार-२ नावाच्या आजारात होते. या आजारा मध्ये स्वादुपिंडा तील बीटा पेशी इन्सुलिन तयार करण्यास असमर्थ असतातच त्याच बरोबर शरीरातील स्नायू सुद्धा इन्सुलिन शोषून घेण्यास असमर्थ होऊन जातात.

प्रस्तुत शोधनिबंधा मध्ये; इन्सुलिन बनविण्याच्या सर्व सामान्य प्रक्रियेवर बाधा आणणारे घटक आणि विशेषत: एक्सोसायटोसीस (शरीरातील एक प्रक्रिया: ज्या द्वारे पेशी मधील घटक द्रव्य काही कारणास्तव पेशी बाहेर काढले जातात); या विषया वर मी संशोधन केले. बीटा पेशी मध्ये तयार झालेले इन्सुलिन, छोट्या फुग्या सारख्या आकारामध्ये साठवले जाते; त्याला इन्सुलिन चे गोळे असेही म्हणतात. त्या नन्तर एक्सोसायटोसीस च्या प्रेक्रीये द्वारे इन्सुलिन चे गोळे बीटा पेशी च्या बाह्य आवरणा तून पेशी च्या बाहेर रक्ता मध्ये सोडले जातात. शरीरा मध्ये अवास्तव वाढलेली साखर हे एक्सोसायटोसीस प्रक्रियेला चालना देणार आणि इन्सुलिन च्या निर्मितीला भाग पाडणार मूळ कारण आहे. माझ्या संशोधना मध्ये मी प्रामुख्याने तीन घटकांचा अभ्यास केला ते म्हणजे; कॅल्शियम, लघु-आर.एन.ए., रोसुवास्तातीन. कारण की या तीनही घटकांमध्ये; एक्सोसायटोसीस प्रक्रिया-त्यावर अवलंबून असणारी इन्सुलिन निर्मिती प्रक्रिया आणि रक्तातील साखरे च प्रमाण; या महत्वाच्या गोष्टी वर परिणाम करण्याची क्षमता आहे.

कॅल्शियम या पदार्था मुळे वेग-वेगळे गोळे सभोवतालच्या आवरणास चीकटन्यास मदत होते. मी गणिती पद्धतीने अभ्यास करून कॅल्शियम चे प्रमाण आणि इन्सुलिन चे गोळे यांच्या तील परस्परसंबंध विस्तृत पणे तपासला. विशेष म्हणजे या अभ्यासा मध्ये "प्रत्येक पेशी ही विभिन्न प्रकार ची असु शकते" हि गोष्ट विचारात घेण्यात आली. आमच्या माहिती प्रमाणे अशाप्रकारचा प्रयोग आम्हीच सर्व प्रथम करत आहोत.

लघु आर.एन.ए.: हे लहान असे पदार्थ पेशी मधील एखाद्या ठराविक प्रथिना चे प्रमाण कमी-जास्त करू शकतात. जर एखाद्या पेशी मध्ये एखाद्या ठराविक प्रथिन चे जास्त लघु आर.एन.ए. असतील तर ते त्या ठराविक प्रथिना ची संख्या कमी करतात. त्याच मुळे विशिष्ट आजाराशी संलग्न असलेल्या लघु आर.एन.ए चा वापर त्या विशिष्ट आजाराचे निदान करण्या साठी होऊ शकतो. लघु आर.एन.ए. सहसा विशिष्ट स्नायू साठीच कार्यरत

असतात, म्हणूनच लघु आर.एन.ए. च्या प्रमाणा वर नियंत्रण मिळवून आपण त्याचा उपचार करण्या साठी वापर करू शकतो. मी; मीर-३७५ व मीर-३३५ हे दोन लघु आर.एन.ए. तपासले, हे दोघे एखाद्या प्रथिना च्या निर्मिती मध्ये कसा व्यवहार करतात? ज्याच्या मुळे इन्सुलिन च्या एक्सोसायटोसीस प्रक्रिये वर परिणाम होत असावा. या संशोधना मध्ये मला असे आढळून आले कि मीर-३७५ हा विधुतदाबाला संवेदनशील असणाऱ्या सोडीअम आयन चानेल चे नियमन करतो. अधिक संशोधना मध्ये मला असेही आढळून आले कि मीर-३३५ हा एक्सोसायटोसीस प्रक्रिये शी संलग्न असलेल्या तीन प्रथिनांचे नियमन करतो. आधी च्या प्रयोगा मध्ये शास्त्रद्नानांनी दाखवले आहे कि प्रयोग शाळेतील मधुमेहाच्या मोडेल प्राण्यान मध्ये मीर-३३५ चे प्रमाण हे वाढलेलच असते. हा निकाल माझ्या संशोधनाला पूरकच म्हणता येईल.

रोसुवास्तातीन : हे एक हृदयविकार च्या उपचारासाठी वापरण्यात येणार औषध आहे. बाकीच्या शास्त्राद्नांनी आधी केलेल्या अभ्यासात आढळून आलय कि या औषधा च्या वापरामुळे मधुमेह-प्रकार-२ होऊ शकतो. माझ्या अभ्यासात, रोसुवास्तातीन कशा प्रकारे एक्सोसायटोसीस प्रक्रिया- त्यावर अवलंबून असणारी इन्सुलिन निर्मिती प्रक्रिया आणि रक्तातील साखरेच प्रमाण इत्यादी वर कशा प्रकारे परिणाम कारक आहे हे पाहण्यात आले. रोसुवास्तातीन मुळे पेशी मधील इन्सुलिन निर्मिती कमी झाली आणि कॅल्शियम चे प्रमाण सुद्धा बदलले. त्या शिवाय रोसुवास्तातीन मुळे विशिष्ट स्नायू मधला साखरेच प्रमाण हि वाढल आणि टिकवून राहील. निष्कर्ष: माझ्या अभ्यासातून मी; एक्सोसायटोसीस चे इन्सुलिन निर्मिती मध्ये असणारे महत्व दाखवून दिले. विस्तारितस्वरूपामध्ये केलेल्या या अभ्यासातून इन्सुलिन निर्मिती प्रक्रिया, त्या मध्ये येणार्या अडचणी मुळे मधुमेह-प्रकार-२ होण्याची शक्यता इत्यादी गोष्टी लक्षात आल्या. हा अभ्यास भविष्यामध्ये मधुमेहा वरिल उपचार पद्धती विकसित करण्यास निश्चितच उपयुक्त ठरेल.

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