Roles of voltage-gated Ca2+ channel subunits in pancreatic β cells

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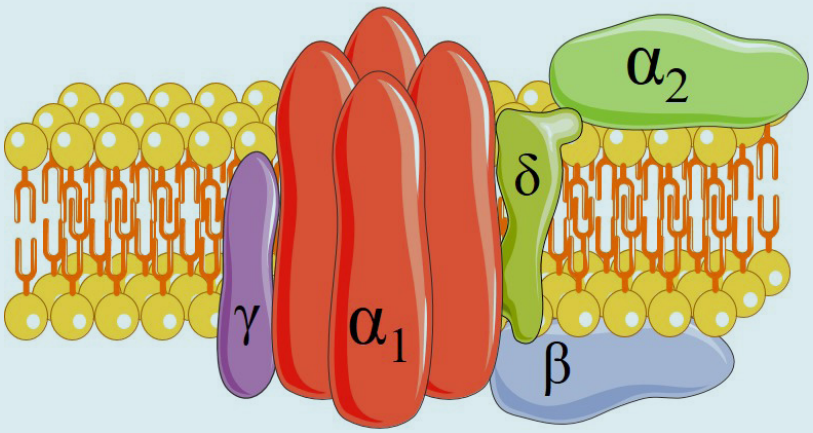

Roles of voltage-gated Ca^{2+} channel subunits in pancreatic β cells

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LUND UNIVERSITY

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Hallmarks of type 2 diabetes (T2D) include elevated blood glucose and free fatty acids (FFAs) as a result of impaired β cell insulin secretion and decreased β cell mass. The glucose-stimulated insulin secretion (GSIS) in β cells is triggered by depolarization-evoked Ca^{2+} entry through voltage-gated Ca^{2+} (Ca_V) channels. The majority of Ca_V channels are believed to reside in cholesterol-rich membrane microdomains called membrane rafts. Ca_V channels consist of the main pore-forming α_1 subunit and three auxiliary subunits, β, $\alpha_2\delta$, and γ. The roles of the Ca_V auxiliary subunits and the membrane rafts in pancreatic β cells are not fully understood, but we have recently shown that the *TCF7L2* gene, associated with the strongest genetic risk factor of T2D, regulates *Cacna2d1* ($\alpha_2\delta_1$).

This thesis aims to elucidate the roles of β_1, β_{2a}, and $\alpha_2\delta_1$ subunits, as well as membrane rafts, in regulating the α_1 subunit and, in turn, insulin secretion and β cell survival. Human islets from donors with T2D contained decreased membrane rafts. A similar phenotype was also observed in the diabetic rat model Goto Kakizaki (GK) rat islets. Cholesterol depletion in healthy human islets by cholesterol oxidase (CO) reduced membrane rafts, resembling islets from donors with T2D. Cholesterol depletion resulted in elevated basal insulin release in both human and rat islets. The reason for this appeared to be the declustering of $Ca_V1.2$, elevation in basal Ca^{2+} oscillations, and an increase in single-Ca_V channel activity as observed in patch-clamp experiments. When suppressing the *Tcf7l2* gene, $\alpha_2\delta_1$ (mRNA and protein) was downregulated and intracellular Ca^{2+} was reduced as measured by confocal microfluorimetry. The decrease in *Cacna2d1* expression resulted in Ca_V channel internalization in the recycling endosomes. This lowered the whole-cell Ca^{2+} current and decreased insulin secretion.

Human gene expression analysis showed that both *Cacnb1* (β_1) and *Cacnb2a* (β_{2a}) genes are abundant in pancreatic islets. When examining the GK rat islets, the expression of both genes was downregulated. Immunoblot experiments showed that high glucose treatment also reduced protein levels of β_1 and β_{2a} in INS-1 832/13 cells. Silencing the β_1 subunit reduced insulin secretion, which may be due to the observed decrease in whole-cell Ca^{2+} currents. By contrast, β_{2a} suppression did not affect insulin release. When comparing the palmitoylation state of β_{2a}, cells overexpressing the non-palmitoylated β_{2a} had a decreased membrane expression of both β_{2a} and α_{1C}. However, overexpression of palmitoylated β_{2a} increased intracellular Ca^{2+}, although without affecting secretion. FFA (palmitate) treatment reduced intracellular Ca^{2+} under stimulatory conditions thus decreasing GSIS. Cells that either lack β_1 or express excess palmitoylated β_{2a} have increased risk of apoptosis. These data reveal novel roles of membrane rafts and β_1, β_{2a}, and $\alpha_2\delta_1$ subunits in regulating Ca_V channel trafficking and activity, thus influencing β cell function and survival.

Key words: Type 2 diabetes, β cell, insulin, voltage-gated Ca^{2+} channel, calcium, membrane raft, auxiliary subunits, TCF7L2, cholesterol.

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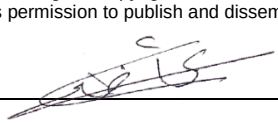
Signature  Date 01-11-2017

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This thesis is warmly dedicated to:

*My mother **Durreya** and my brothers **Huthaifa** and **Orwa***

Table of Content

List of papers included 8

Abbreviations 9

Introduction 12

- Diabetes Mellitus....12
  - Type 2 diabetes....13
  - The pancreatic islet....16
  - The pancreatic β cell....17
  - Membrane rafts....19
  - Ca_V channels....20
  - α_1 subunit....22
  - β subunit....24
  - $\alpha_2\delta$ subunit....26

Aims 30

Materials and Methods 31

- RNAseq and MicroArray 31
  - Microarray 31
  - RNAseq 31
- Protein Quantification 31
  - Immunoblot 32
  - Immunohistochemistry....33
- Ca^{2+} Quantification....33
- Knockdown and Overexpression....34
  - Knockdown....34
  - Overexpression....34

Results and Discussion....35

- Paper I 35
  - Results 35
  - Discussion....37
- Paper II....39
  - Results 39
  - Discussion....40

Paper III....42
Results....42
Discussion....43
Paper IV....43
Results....43
Discussion....45
Conclusion....46
Future Perspectives....47
Attribution....50
Acknowledgements....51
References....54

List of papers included

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Abbreviations

1,4-dihydropyridine	DHP
α-interaction domain	AID
Adenosine triphosphate	ATP
AID-binding pocket	ABP
ATP-sensitive potassium channel	K_{ATP}
β-interaction domain	BID
Bovine serum albumin	BSA
Ca^{2+} modulated protein	Calmodulin
Calmodulin	CaM
Calmodulin kinase	CaMK
Carboxypeptidase E	CPE
Cholesterol oxidase	CO
Detergent-resistant membrane	DRM
Diabetes Mellitus	DM
Endoplasmic reticulum	ER
Enhanced chemiluminescence	ECL
Free fatty acid	FFA
Fasting plasma glucose	FPG
Genome-wide association study	GWAS
Glucose-stimulated insulin secretion	GSIS
Glucose transporter	GLUT
Glycosylated hemoglobin	HbA_{1C}
Glycosylphosphatidylinositol	GPI

Goto Kakizaki rat	GK
Green fluorescent protein	GFP
Guanylate kinase domain	GK
High voltage-gated Ca^{2+} channel	HVGCC
Inositol 1,4,5-trisphosphate	IP_3
Intracellular Ca^{2+} concentration	$[Ca^{2+}]_i$
IP_3 receptor	IP_3R
Low voltage-gated Ca^{2+} channel	LVGCC
Low density lipoprotein	LDL
Methyl β-cyclodextrin	MβCD
Mitochondrial Ca^{2+} uniporter	mCU
Mitochondrial Na^+/Ca^{2+} exchanger	mNCX
Neurogenin 3	Ngn3
Non-esterified fatty acid	NEFA
Oral glucose tolerance test	OGTT
Pancreatic and duodenal homeobox factor 1	PDX1
Pancreatic polypeptide	PP
Paraformaldehyde	PFA
Phosphatase 2A	PP2A
Plasma membrane Ca^{2+}-ATPase	PMCA
Polyacrylamide gel electrophoresis	PAGE
Polyvinylidene difluoride	PVDF
Pore loop	P-loop
Prohormone convertase	PC
Protein kinase A	PKA
Protein kinase C	PKC
Red blood cell	RBC
RNA-induced silencing complex	RISC
Ryanodine receptor	RyR

Sarcoendoplasmic reticulum Ca^{2+}-ATPase pump	SERCA
Signal recognition particle	SRP
Single-nucleotide polymorphism	SNP
Sodium dodecyl sulfate	SDS
Soluble N-ethylmaleimide-sensitive factor attachment protein receptor	SNARE
Src homology 3 domain	SH3
Synaptosomal-associated protein 25	SNAP-25
Synaptotagmin 1	SYT1
Trans-Golgi network	TGN
Transcription factor 7-like 2	TCF7L2
Tricarboxylic acid	TCA
Triglyceride	TG
Type 1 diabetes	T1D
Type 2 diabetes	T2D
United States dollar	USD
V-maf musculoaponeurotic fibrosarcoma oncogene homology A	MafA
V-maf musculoaponeurotic fibrosarcoma oncogene homology B	MafB
Vesicle-associated membrane protein 2	VAMP-2
Voltage-gated Ca^{2+} channel	Ca_V
Voltage-gated K^+ channel	K_V

Introduction

Diabetes Mellitus

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by elevated blood glucose levels, a state referred to as hyperglycemia. During the 5th century BC, DM was first described as 'honey-like urine' [1]. The disease was later termed diabetes and then mellitus in the 2nd and 17th century respectively, followed by the discovery of insulin in the 20th century. Since then, DM has been better understood and thus classified into 3 main types: type 1, type 2, and gestational diabetes.

Type 1 diabetes (T1D), making up 5-10% of diabetic patients, is an autoimmune disease where white blood cells destroy pancreatic β cells causing insulin deprivation [2]. Patients with T1D (also known as insulin-dependent diabetes) are mostly diagnosed as children or adolescents and require regular blood glucose monitoring, a strict diet, and daily insulin injections to maintain normal blood glucose.

Type 2 diabetes (T2D), previously referred to as non-insulin dependent diabetes, is the most common form of the disease accounting for approximately 87-91% of all diabetes cases [3]. In T2D, pancreatic β cells are not destroyed by immune cells but rather lose their function while tissues such as liver, muscle, and fat become resistant to insulin.

A similar phenomenon is also observed in gestational diabetes, a form of diabetes affecting women during pregnancy. Gestational diabetes, occurring in 16% of all pregnancies, increases the risk of pregnancy complications [4]. In addition, patients with gestational diabetes, along with their born child, are more likely to develop T2D later in life. Other types of diabetes also exist, although less common.

In 2015, DM was estimated to have affected 415 million individuals and was responsible for the deaths of 5 million people worldwide [3]. The estimated cost of treatment and prevention of diabetes and its complications was between USD 673-1,197 billion [3, 4].

Type 2 diabetes

Genetic factors

T2D is a chronic metabolic disorder influenced both by environmental and genetic factors. It is characterized by a combination of insulin resistance and reduced insulin output. Insulin is an integral hormone of the glucose-lowering system driven by pancreatic β cells. So far, 90 risk genes have been identified and associated with T2D [5]. Genome-wide association study (GWAS), a widely used method for gene-association, takes advantage of common single-nucleotide polymorphisms (SNPs) that may vary between diabetic and non-diabetic individuals depending on the SNP examined. The SNPs that associate with T2D are labeled genetic risk factors which can then be used to identify susceptible individuals and take preventive measures prior to the onset of the disease. In 2000, the first gene linked to T2D was identified as *CAPN10* (calpain 10) [6]. Following that, many candidate genes were investigated and 3 were found to be associated with the disease, with *TCF7L2* (transcription factor 7-like 2) having the strongest association among all ethnic groups [7]. The transcription factor TCF7L2 is part of the Wnt signaling pathway that is involved in regulating glucose-stimulated insulin secretion (GSIS) and β cell growth and survival [8, 9]. Since 2007, many GWAS studies have been conducted to identify novel T2D genetic risk factors [10]. One interesting discovery was the link between T2D and obesity through the *FTO* (fat mass and obesity-associated protein) gene [11]. This gene is linked to body mass index (BMI) and is responsible for an increase in adiposity and food intake in mice and humans [12, 13]. This is interesting, as patients with T2D have a lipid disorder where cholesterol and non-esterified fatty acids (NEFAs) are elevated in the blood [14-17].

Environmental factors

T2D is an interaction between both genetic and environmental factors. Environmental factors, such as low physical activity and excess calorie intake or unhealthy food intake, could lead to the development of some clinical risk factors like high BMI, elevated fasting plasma glucose (FPG), and high serum concentrations of TG and cholesterol. This would contribute to the development of T2D; however, it is important to consider the genetic variation between individuals as it often explains why some have low BMI and still develop the disease while others who have high BMI do not. Nevertheless, clinical risk factors alone are strong predictors of future diabetes [18]. For example, a combination of high levels of NEFAs in the blood, high BMI, and old age could be indicators of the start of insulin resistance and thus enable early detection and intervention.

The prevalence and risk of diabetes also differs depending on ethnicity [19]. An ethnic group normally shares a common gene pool which allows for certain genetic traits to prevail, including diabetes gene variants. For example, Asians have a lower

obesity threshold than Caucasians, and thus the risk of T2D among Asians is higher [20]. Another example is the difference between two Pima Indian populations, one residing in mountains in northwestern Mexico and the other in Arizona, USA [21]. Although both populations share similar genetic makeup, Arizona Pimas have a much higher prevalence of obesity and T2D than Mexican Pimas. The reason behind this is the different lifestyles associated with the two populations, one a 'traditional' while the other an 'abundance' lifestyle.

Insulin resistance

The cause of T2D is insulin resistance followed by impaired insulin secretion. Obesity is the result of overnutrition and it strongly correlates with insulin resistance [22]. Initially, frequent food intake, and in turn incretin hormones, cause a continuous pancreatic release of insulin into the blood. Insulin-target tissues such as liver, skeletal muscle, and fat, become desensitized to insulin, and hence, reduce glucose uptake. This signals the pancreatic β cell to increase output as the current level is insufficient to lower blood glucose.

β cell mass and function

In T2D-susceptible individuals, β cells eventually 'burn out' from increased insulin release, thus losing their function and undergoing apoptosis. Many studies have reported a reduction in β cell mass (24-65%) in patients with T2D [23-28]. This is further supported by studies showing a reduced number and size of pancreatic islets from patients with T2D [29]. Early studies in rat have shown that reduced β cell mass is the main contributor to T2D instead of β cell dysfunction, since lower number of β cells equates to less plasma insulin [30]. However, it has been shown that human donors who underwent pancreatectomy (30-50% pancreas removal) did not develop T2D albeit having impaired glucose tolerance, suggesting that β cell dysfunction is the main cause of the disease rather than β cell mass [27, 31-33]. In fact, several studies have reported that the β cell capacity to release insulin has been reduced between 50-97% in patients with T2D [28, 34-36]. Patients with T2D subjected to bariatric surgery or short-term caloric restriction restore their blood glucose within days, supporting the contribution of β cell function rather than mass to this quick restoration [37, 38].

Toxicity

Excess energy intake along with a decline in insulin sensitivity results in glucotoxicity and lipotoxicity. The chronically elevated levels of glucose, cholesterol, and NEFAs in the blood exert detrimental effects on insulin-target tissues and pancreatic β cells [39]. For example, liver, muscle, and fat cells release glucose, reduce glucose uptake, and secrete fatty acids into the bloodstream, respectively, under glucolipotoxic conditions. In the case of β cells, elevated glucose

hampers the actions of the transcription factors pancreatic and duodenal homeobox factor 1 (PDX1) and v-maf musculoaponeurotic fibrosarcoma oncogene homology A (MafA), in turn reducing insulin gene expression [40]. In addition, elevated glucose levels increase the demand for insulin output, thus putting load on the endoplasmic reticulum (ER) to synthesize more insulin. This induces ER stress due to the accumulation of misfolded proteins, consequently triggering the unfolded protein response (UPR) [41]. Persistent ER stress and UPR will ultimately result in β cell death via apoptosis. It has been shown that NEFAs hinder insulin release, albeit transiently potentiating secretion [14, 42]. Unlike unsaturated FFAs, saturated FFAs such as palmitate induce ER stress, resulting in cell apoptosis and decreased β cell mass [43]. As in the ER stress, an increase in cellular metabolism increases mitochondrial workload yielding more reactive oxygen species (ROS), resulting in oxidative stress. Islets of T2D patients were shown to have increased markers of oxidative stress which correlated with impairment of GSIS [44]. ROS have deleterious effects in β cells such as disrupting insulin synthesis, mitochondrial membrane, and DNA, as well as increasing ER stress, leading to dysfunction and apoptosis [45-48]. Hyperglycemia also causes a reduction in the number, morphology, and function of mitochondria which in turn diminishes ATP production [49]. Disruption of Ca^{2+} homeostasis in the form of elevated levels of mitochondrial Ca^{2+}, ER Ca^{2+} store depletion, reduced Ca^{2+} influx, and chronic increase in intracellular Ca^{2+} will negatively impact β cell function and mass [50]. In addition to glucose, oxidized low density lipoprotein (LDL) can also reduce preproinsulin expression [51].

Diagnosis

Early detection of T2D risk factors is critical for prevention of T2D. The FPG method is used to diagnose diabetic and pre-diabetic individuals. The fasting takes place for ≥ 8 hours followed by a blood glucose measurement [52]. A similar blood-measuring method, termed oral glucose tolerance test (OGTT), requires the individual to drink a glucose load of 75 g 2 hours prior to measuring blood glucose. A third method has recently been adopted which, instead of plasma glucose, measures hemoglobin A_{1C} (HbA_{1C}) [52]. Hemoglobin is a protein found in red blood cells (RBCs) that binds to oxygen. Interestingly, hemoglobin also binds to glucose and transforms into glycosylated hemoglobin, or HbA_{1C}. Hence, HbA_{1C} is an accurate measure for the average levels of plasma glucose during the last months, since it is stable for 8-12 weeks (the lifespan of an RBC). HbA_{1C} reflects the long-term plasma glucose levels, as opposed to FPG and OGTT where the short-term response to high glucose is shown. The criteria for diagnosing diabetes is listed in Table 1.

Table 1 Diabetes and prediabetes diagnosis
Criteria for diagnosing diabetes and prediabetes [53].

Method	Prediabetes	Diabetes
HbA_{1C}	5.7 – 6.4%	≥ 6.5%
FPG	5.6 – 6.9 mmol/l (100 – 125 mg/dl)	≥ 7 mmol/l (≥ 126 mg/dl)
OGTT	7.8 – 11 mmol/l (140 – 199 mg/dl)	≥ 11.1 mmol/l (≥ 200 mg/dl)

Treatment

Metformin is the most common and preferred treatment for T2D. It is usually the first drug given to patients with T2D, along with diet and exercise recommendations. If the treatment strategy is ineffective, another drug is added to the metformin therapy (e.g. sulfonylurea(SU), DPP4 inhibitors, GLP1 analogs, or SGLT2 inhibitors), and if the treatment target is not reached, a third drug is added to the treatment strategy. However, patients with severe kidney or liver problems avoid metformin as part of their treatment. If all treatment plans fail even after an initial success, insulin would be administered for patients with T2D.

Complications

T2D is a metabolic disorder that affects critical tissues such as the heart, nerves, blood vessels, kidneys, and eyes, if poorly managed. These complications are categorized into microvascular and macrovascular diseases [54]. Microvascular disease refers to damage to small blood vessels and could lead to blindness from retinopathy, kidney failure from nephropathy, and diabetic foot from neuropathy. Macrovascular refers to damage to the large blood vessels and could lead to cardiovascular diseases such as stroke and heart attack.

The pancreatic islet

Composition

In the pancreas, the hormone-secreting endocrine cells, making up 1-2% of the pancreas, reside in highly vascular punctate regions called islets of Langerhans [55]. There are ~3.2 million islets in the human pancreas, each consisting mainly of α cells (glucagon), β cells (insulin), δ cells (somatostatin), γ cells (pancreatic polypeptide), and ε cells (ghrelin). The β cell distribution in the human islet appears to be more scattered as opposed to rodent islets in which the β cells are more focused in the center [56].

Development

During development, pancreatic progenitor cells differentiate into the 5 different cells mentioned earlier depending on the expression of certain transcription factors. For example, PDX1 and neurogenin 3 (Ngn3) drive the progenitor cells into β progenitor cells. Further development of the β cell takes place with the help of MafB while MafA is vital for mature β cell function [57, 58]. MafA is only expressed in insulin-positive cells while MafB is found in both glucagon and insulin-positive cells prior to birth in mice. However, after birth, MafB is specifically expressed in α cells.

The pancreatic β cell

Insulin

The *INS* gene expression is regulated by glucose and FFAs via PDX1 and MafA [59]. The gene encodes a 110-amino acid preproinsulin that is targeted to the ER lumen [60]. This takes place when the cytosolic ribonucleoprotein signal recognition particle (SRP) interacts with the hydrophobic signal peptide on the preproinsulin N-terminus, transferring it into the ER lumen via the peptide-conducting channel. The enzyme signal peptidase then removes this hydrophobic end to form proinsulin. With the help of ER chaperone proteins, proinsulin is folded and forms 3 disulfide bonds. Upon reaching the trans-Golgi network (TGN) from the ER, proinsulin is sorted into immature insulin secretory granule (ISG) along with ions such as Ca^{2+}, Zn^{2+}, and H^{+} and various proteins including carboxypeptidase E (CPE) and prohormone convertase (PC) [60, 61]. The ISG matures when Ca^{2+} is abundant and the pH of the lumen drops, activating both CPE and PC to trim proinsulin into a 51-amino acid insulin and C-peptide. Prior to cleavage, the C-peptide, namely C chain, was situated between A and B chains. After removal of the C chain, A and B chains become attached by disulfide bonds giving rise to mature insulin.

GSIS

Pancreatic β cells act as glucose sensors to respond to fluctuating levels of glucose. When plasma glucose is at basal levels, the cell is said to be in a resting state with a membrane potential of around -70 mV. In the resting state, the ATP-sensitive K^{+} channel (K_{ATP}) remains open allowing diffusion of K^{+} out of the cell. This keeps the intracellular environment more negative and the voltage-gated Ca^{2+} (Ca_V) channel remains inactive, and only low levels of insulin are released. When circulating glucose is high, the 6-carbon sugar is taken up by glucose transporters GLUT1, GLUT3, and possibly GLUT2 to start the triggering pathway in GSIS (Fig. 1). GLUT2 (Km 11.2) is believed to be the main glucose transporter in human

pancreatic β cells. While this may be true in rodents, GLUT2 expression levels in human islets have been found to be low [62]. Therefore, it has been suggested that GLUT2 may not be considered the main glucose transporter in human pancreatic β cells [63]. In addition, GLUT1 (Km 6.9) properties are more in agreement with the dose-dependent curve for GSIS (Km 6.5) suggesting that GLUT2 is likely contributing less, if any, to GSIS in human β cells [62, 64, 65]. Although GLUT1 is currently believed to be the primary glucose transporter in human islets, GLUT3 has shown to be equally highly expressed [63]. Once glucose enters, however, it immediately becomes phosphorylated into glucose-6-phosphate and undergoes glycolysis. Two pyruvate molecules are formed from glucose-6-phosphate which then enter the mitochondria where they are converted into acetyl-CoA, enter the tricarboxylic acid (TCA) cycle, and finally yield chemical energies in the form of adenosine triphosphate (ATP). The rise in ATP:ADP ratio blocks the ATP-sensitive K^+ channel (K_{ATP}) causing a more positive membrane potential and thus depolarization [66]. This activates the Ca_V channel permitting the inflow of Ca^{2+}. The rise in intracellular Ca^{2+}, particularly in regions close to the secretory granules, triggers pulsatile insulin exocytosis [67]. In fact, it has been shown that in mouse β cells, the L-type Ca_V channel interacts with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins residing on the insulin granules [61]. SNARE proteins facilitate granular fusion with the plasma membrane. There are around 10,000 insulin-containing granules in a rat pancreatic β cell with an average of 120 mM insulin concentration [68]. Insulin granules are grouped into two pools; the readily releasable pool (RRP; 1-5%) and the reserve pool (RP; 95-99%). Insulin granules in the RRP localize to the plasma membrane ready for exocytosis, whereas the ones in the RP do not. The first spike of insulin release lasts for approximately 10 minutes and is referred to as the 1st phase insulin secretion [68, 69]. By contrast, the 2nd phase insulin secretion occurs gradually over a longer period of time. It is believed that the RRP is responsible for the 1st phase insulin release while the RP accounts for the 2nd phase. This biphasic behavior of insulin secretion allows β cells to immediately respond to a sudden increase in plasma glucose as well as maintaining long-term blood glucose homeostasis.

In addition to the effect of Ca^{2+} on exocytosis, it also upregulates the insulin gene, *INS* [70]. This occurs through a separate pathway in GSIS which involves cyclic adenosine monophosphate (cAMP) [71, 72]. The production of cAMP is stimulated by Ca^{2+}, ATP, and/or gut hormones called incretins [73, 74]. Once upregulated, cAMP initiates a downstream signaling pathway activating protein kinase A (PKA) and Epac2A, which stimulate insulin secretion [72, 75]. PKA is believed to activate cAMP responsive element binding protein (CREB) promoting the insulin gene *INS* for further hormone synthesis [76, 77]. Moreover, in the amplifying pathway, cAMP/PKA signaling may also facilitate the transport of glutamate, a product of glucose metabolism, into insulin granules and stimulate insulin secretion [72].

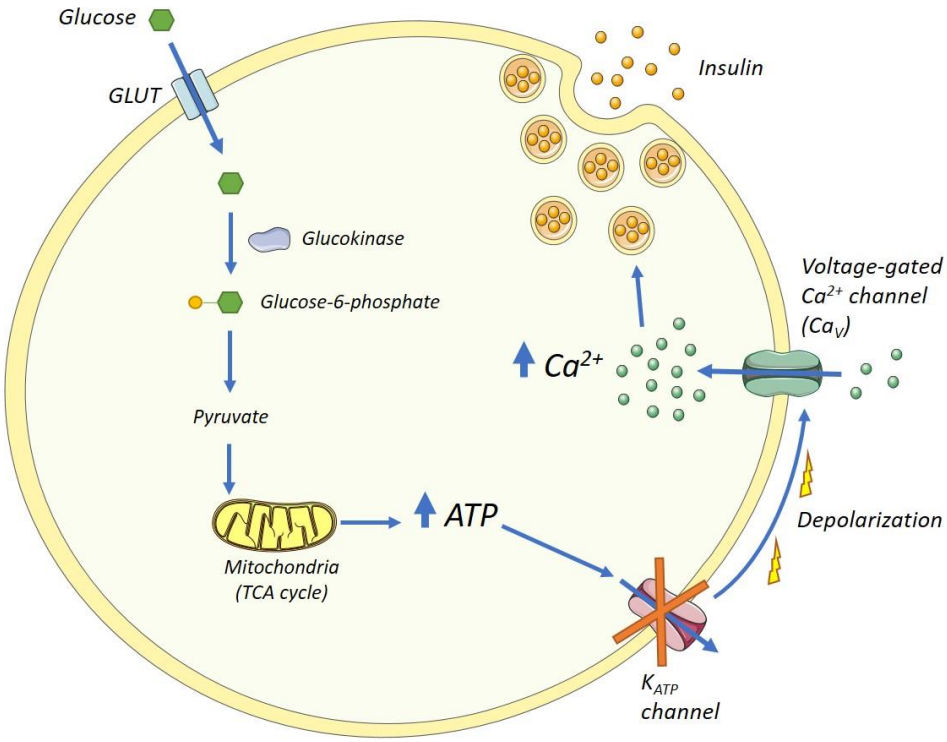

Figure 1 Glucose-stimulated insulin secretion
A schematic of glucose-stimulated insulin secretion and amplifying processes.

Membrane rafts

The cell membrane is made of two layers of phospholipids that join to form an inner hydrophobic lipid part surrounded by hydrophilic phosphate heads. The fluidity of the membrane is affected by the number of cholesterol molecules embedded in it; the more cholesterol, the less fluid the membrane is and thus less permeable [78]. The more permeable the membrane becomes, the less control it has over cellular content. The membrane also consists of cholesterol- and sphingolipid-enriched microdomains that are resistant to detergents and hence were termed detergent-resistant membranes (DRMs) or membrane rafts.

Membrane rafts serve as docking platforms for transport proteins and channels, while also aiding in protein interaction and stability [79]. During exocytosis, SNARE proteins form SNARE fusion complexes that depend on membrane rafts to interact and facilitate the fusion of mature secretory granules to release insulin [80]. In addition, (GPI)-anchoring requires membrane rafts to localize and stabilize proteins to the plasma membrane [81]. To identify the functions of membrane rafts,

two cholesterol-targeting agents are widely used, methyl β-cyclodextrin (MβCD) and cholesterol oxidase (CO). When pancreatic β cells were treated with MβCD, the voltage-gated K^+ channel, $K_V2.1$, resulted in reduced K^+ amplitude and channel activity [82].

Ca_V channels

The nomenclature of Ca_V channels has traditionally differed in different fields. Electrophysiologists used a naming system depending on the Ca_V channel's biophysical and pharmacological properties (L, P/Q, N, R, T), and biochemists adopted Greek letters to distinguish between the different subunits (α_1, β, $\alpha_2\delta$, γ) [83, 84]. Molecular biologists concurrently used alphabetical letters to name Ca_V channel genes (*CACNA1A-I*, *CACNA1S*) [85]. In 2000, Ca_V channels were categorized into 3 families, Ca_V1, Ca_V2, and Ca_V3, based on gene sequence analysis [86]. The nomenclature of the 10 $Ca_V\alpha_1$ genes and proteins are listed in Table 2. In human pancreatic β cells, the Ca_V channels largely contributing to GSIS are the L-type ($Ca_V1.2$ and $Ca_V1.3$) and P/Q type (Ca_V 2.1), whereas other types such as T-type ($Ca_V3.2$) contribute to a lesser extent [87, 88].

Table 2 Ca_V channel nomenclature
Nomenclature for the 10 Ca_V channels.

Type	α_1 (*gene*)	Ca_V	Channel Gating
L-type	α_{1S} (*CACNA1S*) α_{1C} (*CACNA1C*) α_{1D} (*CACNA1D*) α_{1F} (*CACNA1F*)	$Ca_V1.1$ $Ca_V1.2$ $Ca_V1.3$ $Ca_V1.4$	HVA
P/Q-type	α_{1A} (*CACNA1A*)	$Ca_V2.1$	HVA
N-type	α_{1B} (*CACNA1B*)	$Ca_V2.2$	HVA
R-type	α_{1E} (*CACNA1E*)	$Ca_V2.3$	HVA
T-type	α_{1G} (*CACNA1G*) α_{1H} (*CACNA1H*) α_{1I} (*CACNA1I*)	$Ca_V3.1$ $Ca_V3.2$ $Ca_V3.3$	LVA

HVA, high voltage activated; LVA, low voltage activated.

Voltage-gated Ca^{2+} channels (VGCCs) are expressed in the plasma membranes of excitable cells such as nerves, myocytes, retinal cells, and endocrine cells to quickly relay biological and electrical signals such as Ca^{2+} and membrane depolarization. Upon depolarization, the VGCC undergoes a conformational change which either increases or decreases the affinity for extracellular Ca^{2+} [89, 90]. The VGCCs are heteromeric complexes composed of the main α_1 subunit and auxiliary β, $\alpha_2\delta$, and γ subunits that work together to transport Ca^{2+} into the cell (Fig. 2). The VGCCs can be inhibited by channel type-specific Ca^{2+} channel inhibitors, for example, L-type

(1,4-dihydropyridines (DHPs); isradipine) and N-type (ω-conotoxins) Ca_V channel blockers.

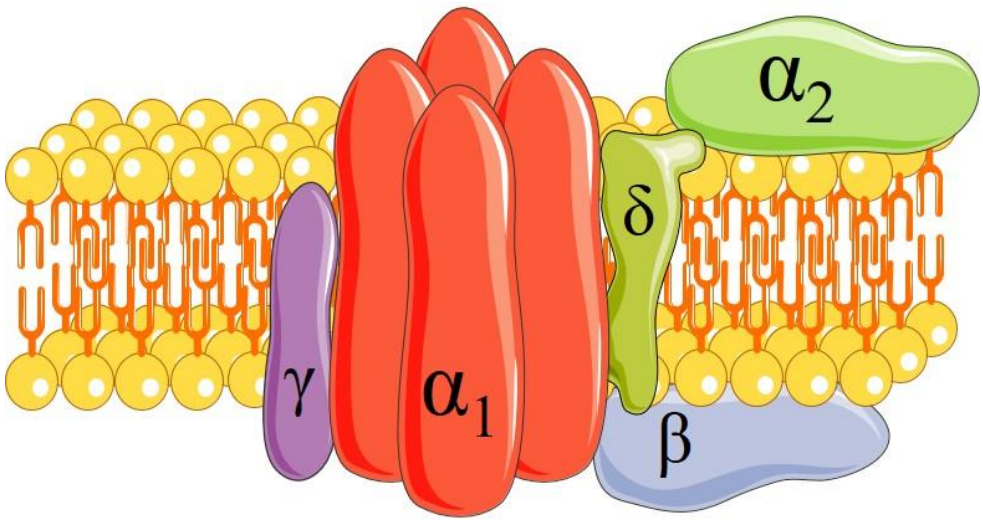

Figure 2 The Ca_V channel
A schematic illustration of the Ca_V channel with all its subunits.

Other Ca^{2+} channels

Other Ca^{2+} channels also exist in β cells that are voltage-independent and contribute to maintaining intracellular Ca^{2+} homeostasis. These include ryanodine receptor (RyR), sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, and inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) which are found in the ER membrane and regulate Ca^{2+} transport [91]. Similar to VGCC localization, transient receptor potential (TRP) channel and plasma membrane Ca^{2+}-ATPase (PMCA) are also situated in the plasma membrane to control Ca^{2+} transport. The mitochondrial Ca^{2+} uniporter (mCU) and mitochondrial Na^+/Ca^{2+} exchanger (mNCX) reside in the inner mitochondrial membrane to facilitate the transport of Ca^{2+} across the mitochondrial membrane.

Additional effects of Ca^{2+}

Aside from promoting insulin maturation and secretion, Ca^{2+} also activates protein kinase C (PKC) which in turn promotes insulin exocytosis in β cells [92, 93]. Ca^{2+} can also drive protein phosphorylation through binding to Ca^{2+} modulated protein (calmodulin (CaM)), forming a complex that regulates CaM kinases (CaMK).

Moreover, Ca^{2+} entry in some cardiac muscle cells can stimulate the release of Ca^{2+} from sarcoendoplasmic reticulum stores [94].

α_1 subunit

Structure and function

Molecular cloning detected 10 genes in humans that encode the pore-forming $Ca_V\alpha_1$ (Table 2). The α_1 subunit is a ~170-240 kDa membrane protein with 24 transmembrane segments (S1-6) grouped into 4 homologous transmembrane domains (I-IV) (Fig. 3) [95, 96]. In addition, the subunit has 3 intracellular loops, each linking S6 and S1 of 2 transmembrane domains. Interestingly, loop I-II has the α_1-interaction domain (AID), the site where the β subunit binds $Ca_V\alpha_1$ for trafficking. Furthermore, the SNARE proteins syntaxin 1A, synaptosomal-associated protein 25 (SNAP-25), and synaptotagmin 1 (SYT1) associate with the α_1 subunit at the II-III loop, connecting it to the insulin granules [97]. Intriguingly, the α_1 subunit pore consists of 4 membrane-embedded pore loops (P-loops), each containing a glutamic acid residue that is responsible for Ca^{2+} entry [98]. During depolarization, voltage sensors such as cationic arginine or lysine residues at S4 cause a conformational change resulting in opening of the α_1 pore [90]. Then, upon binding of Ca^{2+} to the extracellular end of the α_1 pore, a Ca^{2+} bound to the intracellular end is repelled into the cytosol and replaced with the new extracellular Ca^{2+} [99].

In recent years, regions at the N- and C-termini of the α_1 subunit have been demonstrated to mediate important functions. Two domains at the C-terminus, proximal and distal C-terminus regulatory domain (PCRD and DCRD), were shown to be involved in channel inactivation [100-102]. In addition, a fragment of the C-terminus was found to regulate Ca_V channel transcription by translocating to the nucleus [103, 104]. The N-terminus is also involved in channel inactivation as it serves as a CaM-binding site in $Ca_V1.2$ and $Ca_V1.3$ channels [102, 105].

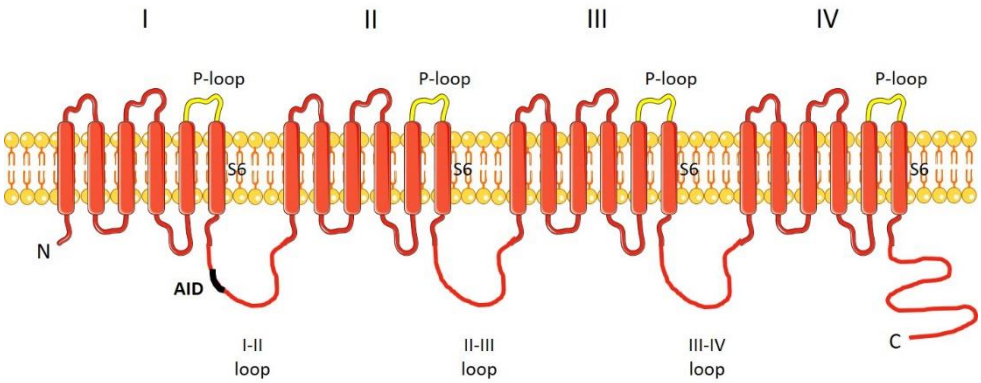

Figure 3 The α_1 subunit structure
An illustration of the $Ca_V\alpha_1$ structure in the plasma membrane.

Regulation

Studies on rabbit and rat identified phosphorylation and glycosylation sites in $Ca_V\alpha_1$, specifically $Ca_V1.1$, $Ca_V1.2$, and $Ca_V2.1$ [96, 106-111]. Phosphorylation was carried out by cAMP-activated PKA and PKC, suggesting an indirect cAMP regulation of $Ca_V\alpha_1$. Although no glycosylation was observed in $Ca_V1.1$ and $Ca_V1.2$, a short form of $Ca_V2.1$ (95 kDa) was found to be glycosylated. Another Ca_V channel regulatory factor is Ca^{2+}. It achieves channel inactivation by forming a complex with CaM which binds to the C-terminus of the Ca_V channel and regulates channel gating.

Activation and inactivation

Gating is an important property of the Ca_V channel that is regulated by processes of activation and inactivation. Activation occurs when the membrane depolarizes, resulting in opening of the channel. Inactivation, on the other hand, is when the Ca_V channel becomes less permeable. There are two types of inactivation, Ca^{2+}-dependent inactivation (CDI) and voltage-dependent inactivation (VDI) [95, 102].

CDI takes place when Ca^{2+} binds channel-tethered CaM, causing it to undergo a conformational change and channel inactivation [89]. The C-terminus-bound Ca^{2+}/CaM complex changes structure to bind the N-terminus of the channel, thus blocking Ca^{2+} entry. Therefore, an increase in Ca^{2+} influx increases CDI, reaching ~65% upon full channel activation [112]. The degree of CDI, however, varies depending on the type of Ca_V channel. L-type channels have strong CDI whereas R-type channels have weak CDI.

The other type of inactivation, VDI, depends on the difference in charge across the membrane which is determined by ions like K^+, Na^+, Cl^-, and Ca^{2+}.

β subunit

Structure

The human β subunit is encoded by 4 different genes (*CACNB1-4*) that are translated by free ribosomes [96]. Crystal structures of the subunit identified 5 domains, 2 highly conserved and 3 highly variable (Fig. 4) [113-117]. The highly conserved Src-homology (SH3) and guanylate kinase (GK) domains play an important role in $Ca_V\alpha_1$ trafficking. In yeast, the GK domain has an active catalytic site [118]. This site is replaced with a hydrophobic AID binding pocket (ABP; also referred to as β-interaction domain or BID) in mammalian GK domain [114-116]. The AID-ABP interaction positions the β subunit near the intracellular end of $Ca_V\alpha_1$ pore. This positioning of the β subunit allows it to regulate channel inactivation, since the AID N-terminus is very close to the IS6 segment of $Ca_V\alpha_1$ [113]. The SH3 domain is required for protein-protein interaction. To achieve this, a β sheet blocked by the HOOK domain is exposed via a conformational change in the SH3 domain. Interestingly, studies on *Xenopus* oocytes show that both SH3 and GK domains interact intramolecularly and disruption of such connection hinders $Ca_V\beta$ from $Ca_V\alpha_1$ trafficking and gating [119].

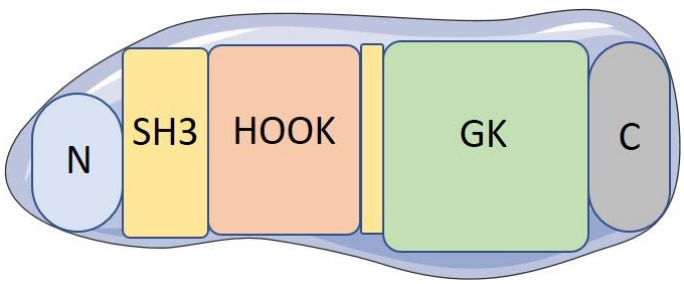

Figure 4 The β subunit domains
An illustration of $Ca_V\beta$ structural domains.

Alternative splicing

Alternative splicing is found in all human Ca_V subunits, including the β subunit. Each of the 4 β subunit genes (*CACNB1-4*) exhibits at least 2 alternative splicings, thus increasing subunit variation. Splicing takes place at the exons of the highly variable N-terminus, C-terminus, and HOOK domain. Interestingly, these splice variants differ in distribution. While β_{2b} in rats is found in the brain, heart, and aorta, β_{2d} is explicitly expressed in the heart [120, 121]. Splice variants also vary in expression during development. The expression of β_{1b} in rat brain increases 3-fold during development while that of β_{2c} decreases [120-122].

Alternative splicing gives rise to $Ca_V\beta$ variants that exert unique functions independent of VGCC. The chicken β_{4c}, for instance, was found to lack 90% of the

AID-harboring GK domain and the whole C-terminus [123]. This permits β_{4c} to interact with the nuclear protein heterochromatin protein 1 (HP1) and localize to the nucleus, suggesting its involvement in transcriptional regulation. In fact, *in vitro* studies in *Xenopus* oocytes have shown that full-length β subunits are capable of interacting with Pax6(S), a transcription factor required for the development of the eye and nervous system [124]. The interaction translocates the β_3 subunit from the cytoplasm to the nucleus and reduces Pax6(S) activity without affecting VGCC properties. Furthermore, β_{4a} forms a complex with B56δ, a nuclear regulatory subunit of phosphatase 2A (PP2A), and translocates to the nucleus to regulate histone dephosphorylation [113, 125].

Localization and function

In the absence of the α_1 subunit, most β subunits, except for β_{2a} and β_{2e}, localize to the cytosol [126, 127]. Although the reason behind β_{2e} localization is unknown, localization of β_{2a} is due to it being palmitoylated and anchored to the plasma membrane [128, 129]. The main role of β subunits in VGCC is trafficking, regulating, and increasing surface expression of Ca_V channels. Regulating the Ca_V channel involves influencing its activation and inactivation state. Inactivation of the Ca_V channel is enhanced by the variable HOOK region in the β subunit [114-116]. Because each β subunit has a different HOOK domain, they differ in degree of inactivation of Ca_V channels.

In contrast to the general role of β subunits, the β_3 subunit surprisingly acts as a brake on insulin secretion [130]. A study showed that β cells from β_3 knockout mice had enhanced Ca^{2+} oscillations and improved insulin exocytosis. In addition, these β cells had elevated intracellular Ca^{2+} due to increased release from intracellular stores via enhanced IP_3 formation.

Palmitoylation of β_{2a}

Palmitate is a 16-carbon fully saturated fatty acid that, although deleterious to the β cell when elevated, is important post-transationally. Palmitoylation is a post-translational modification involving the addition of a palmitoyl group onto a protein. Of all the β subunits, β2a is unique in that it contains two cysteine groups in its N-terminal region which can undergo palmitoylation. In general, palmitoylation is a post-translational modification where the fatty acid palmitate attaches to one or more accessible cysteine residues in a protein structure. There are 3 types of palmitoylation: S-, N-, and O-palmitoylation [131, 132]. In S-palmitoylation, palmitate links to cysteine in a reversible manner with the help of palmitoyl acyl transferases (PATs) [133]. N- and O-palmitoylations form amide and oxyester linkages to N-terminus cysteine and serine residues, respectively [132, 134]. Unlike N-palmitoylation, O-palmitoylation involves a monounsaturated palmitate (palmitoleic acid) and is believed to be reversible [134-137]. Depalmitoylation

involves the removal of palmitate from a protein in a reaction catalyzed by thioesterases [138].

It has been reported that at least 10% of human proteins are subjected to palmitoylation [139]. These proteins are involved in signaling, transcription, and in forming ion channels and receptors [140]. One of the important functions of palmitoylation comes from its hydrophobicity, allowing proteins to dock on the inner leaflet of the phospholipid bilayer. As an example, due to the switch between palmitoylation and depalmitoylation, two small GTPases, NRas and HRas, are capable of alternating between the Golgi membrane and the plasma membrane [140-142]. Additional functions of protein palmitoylation include membrane raft-targeting, protein conformational change, and protein-protein interaction [140].

$\alpha_2\delta$ subunit

Structure

Like the β subunit, the $\alpha_2\delta$ auxiliary subunit (~175 kDa) is also encoded by 4 different genes (*CACNA2D1-4*) in humans, but unlike the β subunit, they are translated by ER ribosomes [84, 96, 143]. At first, the structure of $\alpha_2\delta$ was determined biochemically and was thought to consist of two different proteins linked by a disulfide bond [144]. However, upon cloning of *CACNA2D*, it became clear that both proteins emerged from the same gene. The $\alpha_2\delta$ subunit is synthesized as a continuous polypeptide chain. During processing in the ER and Golgi apparatus, it acquires a disulfide bond between the α_2 and the δ parts and undergoes glycosylation at several amino acid residues (Fig. 5) [145]. However, post-translational cleavage by proteases splits the protein into α_2 and δ, keeping them connected via the disulfide bond [146]. The $\alpha_2\delta$ subunit consists of 5 domains: N-terminus, C-terminus, von Willebrand factor A (VWA), and 2 chemosensory-like domains (CSDs; or Cache domain) (Fig. 5) [147, 148].

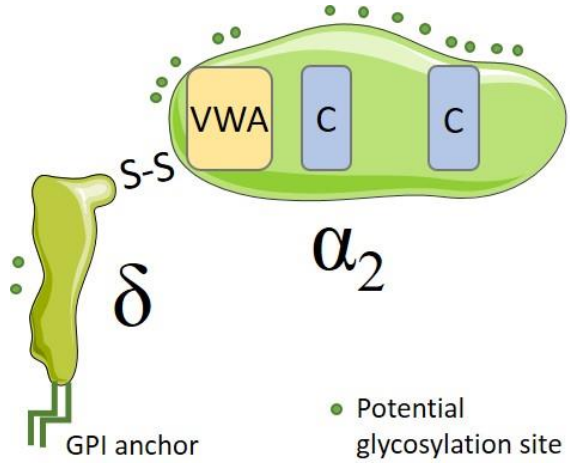

Figure 5 The mature $\alpha_2\delta$ subunit domains
An illustration of $Ca_V\alpha_2\delta$ structural domains.

The N-terminus has a signal sequence that guides the newly synthesized $\alpha_2\delta$ into the ER lumen [149]. The C-terminus is hydrophobic and is thought to be a transmembrane domain, although a predicted small sequence of this domain is in the intracellular environment [150, 151]. Proteomic prediction analysis shows that the $\alpha_2\delta$ subunit can be anchored to membrane rafts by glycosylphosphatidylinositol (GPI) which was also confirmed by many biochemical studies [152, 153]. Interestingly, the VWA domain, with the help of its metal ion-dependent adhesion site (MIDAS) motif, is involved in protein-protein interaction with extracellular matrix proteins and cell-adhesion proteins [154]. The MIDAS motif binds a divalent cation such as Ca^{2+} or Mg^{2+} causing a structural change in the subunit and allowing it to interact with other proteins. Although the VWA is an $\alpha_2\delta$ subunit domain, it has been found in other proteins that require protein-protein interaction, for example in integrins. Lastly, the CSDs were also discovered in bacteria and serve as multiple nutrient sensors [147].

Localization

The $\alpha_2\delta$ subunit, similar to the β subunit, is expressed in excitable tissues like skeletal and cardiac muscles, brain, endocrine tissue, and retina [155, 156]. In human and mouse pancreatic islets, the predominant *CACNA2D* is the *CACNA2D1* [157-159].

Table 3 $Ca_V\alpha_2\delta$ tissue distribution
Tissue distribution of $\alpha_2\delta$ subunit.

Subunit	Human		Mouse	
	mRNA	Protein	mRNA	Protein
$\alpha_2\delta_1$	Brain Heart Skeletal muscle Pancreas	Brain Heart Kidney Spleen Testis	Brain Heart Skeletal muscle Pancreas	Many including Brain Heart Lung Pancreas
$\alpha_2\delta_2$	Brain Heart Skeletal muscle Pancreas	Brain Heart Testis	Brain Heart Skeletal muscle	Brain Heart Kidney Lung
$\alpha_2\delta_3$	Brain Heart Skeletal muscle Kidney	Brain	Brain	Brain
$\alpha_2\delta_4$	Brain Heart Skeletal muscle Kidney Liver Lung Pancreas	Brain Small intestine Liver Adrenal gland Pituitary gland	Brain Muscle Lung Retina	Retina

Function

The study of the auxiliary subunit $\alpha_2\delta$ in pancreatic islets is minimal compared to in neurons and muscles. In general, the subunit facilitates Ca_V channel surface expression and turnover, decreases the opening time of the α_1 subunit (i.e. increases inactivation), and increases Ca_V current with the aid of the MIDAS motif [160-165]. Knockout of $\alpha_2\delta_1$ subunit in mice, via reduction of L-, P/Q-, N-, & R-type Ca_V channel currents, hindered the first and second phase insulin secretion, as well as decreased β cell mass [166]. The effect was sex-dependent as male mice developed diabetes while female mice solely had a higher risk of the disease [167]. This was due to the increase in basal insulin release in female mice, which had a preventive effect on diabetes development. A recent study showed that trafficking and activity of neuronal $Ca_V2.2$ appeared to be dependent on the post-translational cleavage of the $\alpha_2\delta_1$ subunit [168]. Surprisingly, this modification is unnecessary for $\alpha_2\delta_1$ transport to the plasma membrane.

Additionally, because of the $\alpha_2\delta$ subunit 3 arginine (RRR) motif near the VWA domain (Fig. 5), the $\alpha_2\delta$ subunit is capable of interacting with gabapentin, an anti-epileptic drug [169, 170]. *In vivo* studies on rat brain neurons showed that the binding of gabapentin to the $\alpha_2\delta_1$ subunit lowered Ca^{2+} currents. The $\alpha_2\delta$ subunit can also block and enhance the actions of ω-conotoxins (painkillers) and DHP

antagonists, respectively [165, 171]. In case of ω-conotoxins, the role of the subunit, specifically the α_2 part, is believed to involve blocking the drug binding site on the Ca_V channel [171, 172]. As for the DHP antagonists, the $\alpha_2\delta$ subunit increases channel inactivation and since DHP antagonists generally prefer binding to inactivated channels, the affinity of antagonist binding to channels associated with $\alpha_2\delta$ subunit increases [173].

Aims

Ca_V channel auxiliary subunits are an important element in trafficking and regulating the main α_1 subunit. However, their role in pancreatic β cells remains unclear. This thesis investigates the roles of β_1, β_{2a}, and $\alpha_2\delta_1$ subunits in pancreatic β cells. The thesis also addresses the role of membrane rafts in Ca_V channel function.

The specific aims are as follows:

1. To explore the role of membrane rafts in regulating voltage-gated Ca^{2+} channel (Ca_V) activity and insulin release in β cells.
2. To examine the role of *Tcf7l2* in regulating the expression of $\alpha_2\delta_1$ subunit and the subsequent effect on Ca_V channel trafficking and activity in β cells.
3. To investigate the role of the β_1 subunit in regulating Ca_V channel activity and the resulting effect on insulin secretion and β cell survival.
4. To study the role of palmitoylation of the β_{2a} subunit in regulating Ca_V channel activity and the ensuing effect on insulin secretion and β cell survival.

Materials and Methods

RNAseq and MicroArray

Microarray

Microarray technique is used to detect gene expressions among a library of transcripts. This method of gene expression analysis has the advantage of being quick, robust, and reliable. It is currently cheaper than RNA sequencing (RNAseq). However, it requires prior knowledge of the desired transcript and, thus, it is not ideal for finding novel genes, structural variations, or isoforms. The data produced by a microarray method only indicates relative expression and as such should not be used for quantification purposes.

RNAseq

RNAseq technique is also used to detect gene expressions, however, it extracts data from the transcriptome pool and thus does not prior knowledge of a sequence. This is of particular importance as it allows discovering novel genes, isoforms, structural variations, or transcripts. This relatively new method has the advantage of being highly sensitive compared to microarray, however, it has a higher cost. It also has the advantage of providing absolute quantifications instead of relative expressions. This, however, demands more time for data analysis and larger storage space. Because of it being relatively new, there is no standard RNAseq protocol, and therefore, data are harder to compare.

Protein Quantification

Immunoblotting and immunohistochemistry methods use antibodies specific to a desired protein to semi-quantify and visualize the protein, respectively.

Immunoblot

Immunoblot (or western blot) is composed of 3 stages: running, transfer, and detection.

Running

Briefly, this stage involves loading an amount of protein onto a sodium dodecyl sulfate-polyacrylamide gel to perform electrophoresis (SDS-PAGE) after being denatured with DTT and heat. The denaturing step is important for migration of the protein as it breaks the sulfide bonds from secondary and tertiary structures, making the protein linear. The proteins will migrate, due to their negative charge, from the cathode (negative) to the anode (positive) ends (top of gel to bottom) with the help of ions in the running buffer. Depending on the molecular weight of the protein, it will migrate at a certain speed on the gel. This is because SDS-PAGE gels contain a percentage of polyacrylamide which gives the gel structure a certain sized 'holes' through which proteins migrate. The more acrylamide, the more rigid the gel is and the smaller these 'holes' are. This means that only smaller sized proteins will reach faster towards the bottom of the gel while the larger ones will get held back. Using a gradient gel, such as 4-15%, is often useful to capture different sized proteins.

Transfer

The gel is then placed, along with a polyvinylidene difluoride (PVDF) membrane, in a sandwich cassette such that the gel is closer to the cathode while the PVDF membrane is closer to the anode. This is to ensure the transfer of proteins from the gel onto the PVDF membrane i.e. from negative end to the positive end. Current is then applied to the sandwich cassette submerged in Tris-based transfer buffer, allowing the proteins to migrate onto the membrane. This is called wet transfer as opposed to dry or semi-dry transfers where the setup is slightly changed. One of the advantages of a PVDF membrane over a nitrocellulose membrane is the ability to prevent proteins from passing through the membrane (overtransfer). This is especially useful if studying two or more proteins with vastly different sizes as smaller proteins transfer at a faster rate than larger ones. The transfer stage is vulnerable because a mere tiny bubble can render the membrane useless.

Detection

After transfer, the membrane is incubated with a blocking agent containing around 5% protein such as skimmed milk or bovine serum albumin (BSA). The 5% protein in milk binds non-specifically to spaces on the PVDF membrane that are left unbound. This reduces background noise during detection as these spaces, if unblocked, may bind to antibodies during incubation. Next, the membrane is incubated first with primary and then with secondary antibodies that will

specifically bind to the desired protein. After that, the protein bands are visualized under ultra violet light using enhanced chemiluminescence (ECL) and analyzed using appropriate softwares.

Immunohistochemistry

Immunohistochemistry uses a similar concept to immunoblot. The Detection stage is similar while Running and Transfer are replaced with Fixation. Although this method is not considered quantifiable, it is useful for detecting protein localization.

Fixation

Cells are fixed with 4% paraformaldehyde (PFA) and permeabilized with a detergent such as saponin. Permeabilization of the plasma membrane is required for antibody entry and binding of intracellular proteins.

The following stage will be similar to immunoblot Detection stage where a blocking agent, primary and secondary antibodies are used. The fixed cells are then visualized under a confocal microscope.

Ca^{2+} Quantification

Ratiometric vs non-ratiometric

Ca^{2+} is an essential contributor to GSIS and thus quantifying it is of great interest. Ca^{2+} quantification is achieved with two main methods: ratiometric and non-ratiometric.

Ratiometric

This technique measures free intracellular Ca^{2+} ions using a ratiometric fluorescent dye called aminopolycarboxylic acid or Fura-2. The fluorescence from the dye can be used to quantify Ca^{2+}, since upon excitation at 340 nm and 380 nm, the ratio of emission at these wavelengths is directly proportional to the amount of free Ca^{2+} bound to Fura-2. The advantage of this ratiometric technique is the elimination of confounding factors such as dye concentration, bleaching, change in focus, variations in laser intensity, and cell thickness. However, this technique is more difficult in measuring and processing data as it requires specific settings that are only available with some microscopes.

Non-ratiometric

This technique is used to detect free intracellular Ca^{2+} in a non-quantifiable way using a fluorophore such as Fluo-5F. The excitation and emission are at 494 nm and 516 nm wavelengths, respectively. The fluorescence intensity may reflect the amount of free intracellular Ca^{2+}. However, the fluorescence can be influenced by other factors such as change in focus, variations in laser intensity, and dye concentration.

Knockdown and Overexpression

One of the most common methods to determine protein function is to attempt to either eliminate the protein or over-produce it. This is attained by knockdown and overexpression techniques.

Knockdown

The protein expression can be reduced (knocked down) by up to 90-95% but not completely eliminated with standard cell manipulation techniques (a complete elimination will be termed knockout and is achieved by DNA-editing techniques). Protein knockdown takes place at the mRNA level where a small interference RNA (siRNA) targets the desired mRNA and activates the RNA-induced silencing complex (RISC) machinery to break down the mRNA, preventing it from being translated. The results are compared to the control siRNA which does not correspond to any known RNA sequence and theoretically should pose no change to cellular physiology.

Overexpression

With this technique, the protein is overexpressed using a plasmid. This plasmid can either carry a tag-attached-protein or the protein sequence alone. A popular tag used for detection is green fluorescence protein (GFP). The plasmid-encoded protein is expressed using the cell's transcription and translation machineries.

Results and Discussion

Paper I

Results

Type 2 diabetic islets show decreased plasma membrane cholesterol content and membrane rafts

In type 2 diabetes (T2D), the aberrant blood lipid profile has been suggested to contribute to pancreatic β cell dysfunction. To investigate the importance of cholesterol-enriched membrane rafts in pancreatic islets, the sphingolipid dye ATTO-SM and the cholesterol dye filipin were used to stain healthy Wistar and diabetic GK rat islets (Fig. 1a-e in Paper I). GK rat islets showed a marked decrease in filipin and ATTO-SM stainings (~40% and ~60% respectively) compared to healthy islets. This suggests a depletion in cholesterol-enriched membrane rafts in rat islets under diabetic conditions ($p < 0.1$, $n = 3$ rats/condition; Fig. 1b & 1d in Paper I).

Are the membrane rafts expressed differently between α and β cells? To elucidate this, we stained dispersed Wistar islets with ATTO-SM and found that, in comparison with α cells, β cells showed approximately 300% higher membrane raft intensity ($p < 0.5$, $n = 3$ rats/group; Fig. 1e in Paper I).

To explore whether these results in rat translated to the human situation, human islets from donors with T2D were stained and found to display a similar membrane raft phenotype as that of GK rat islets. A significant decrease (~50%) in ATTO-SM staining was observed in islets from donors with T2D as oppose to healthy islets ($p < 0.1$, $n = 3$ donors/condition; Fig. 1f-g in Paper I). A reduction in membrane rafts was also observed in healthy human islets treated with the cholesterol-depleting enzyme cholesterol oxidase (CO), which resulted in ~40% reduced ATTO-SM intensity ($p < 0.1$, $n = 3$ donors/condition; Fig. 1f-g in Paper I). CO dosage and treatment time were optimized for activity and cell toxicity on INS-1 832/13 cells prior its use on islets (Suppl. Fig. 1 in Paper I).

Disruption of membrane rafts causes increased basal insulin secretion

The main functions of β cells are to sense changes in blood glucose and secrete insulin in order to maintain euglycemia, i.e. normal blood glucose. To evaluate the effect of CO on β cell function, we measured glucose stimulated insulin secretion (GSIS) in human and rodent islets as well as in INS-1 832/13 cells following CO treatment. For comparison, the effects of the widely used cholesterol-depleting agent MβCD were studied in parallel. First, optimal time was determined using a time-dependent experiment where INS-1 832/13 cells were treated with CO for 0.5, 1, and 2 hours. A 1 hour CO treatment time was selected as the enzyme influenced both basal (2.8 mM) and stimulated (16.7 mM) insulin secretions at that time point (Fig. 2a). Next, INS-1 832/13 cells were pretreated with either MβCD or CO and insulin release was measured. Under both conditions stimulated secretion was slightly increased. However, the main finding was that basal secretion was greatly elevated compared to the control condition (Fig. 2b in Paper I). As a result, the stimulatory effect of glucose, expressed as fold-change between 16.7 mM and 2.8 mM glucose, was decreased by half compared to control. In agreement with the cell line data, human and rat islets subjected to CO treatment showed elevation in basal insulin secretion (8-fold in human and 3-fold in rat) compared to control (Fig. 2c-d in Paper I). MβCD treatment, on the other hand, showed little effect on basal secretion in human and rat islets.

Since membrane-clustering of the SNARE protein syntaxin 1A facilitates insulin secretory granule exocytosis, the importance of membrane rafts for this process was assessed. Indeed, INS-1 832/13 cells treated with CO showed a scattered syntaxin 1A localization as opposed to its native membrane association. The ratio of syntaxin 1A membrane expression to the intracellular level was greatly reduced from 3.2 ± 0.8 to 1.0 ± 0.2 in CO-treated cells compared to control (Suppl. Fig. 2 in Paper I).

Activation of $[Ca^{2+}]_i$ oscillations in CO-treated cells under depolarizing and resting conditions

After membrane depolarization, voltage-gated Ca^{2+} (Ca_V) channels trigger insulin release by allowing Ca^{2+} entry. This causes a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) stimulating exocytosis. Due to the importance of this step, Ca^{2+} entry into INS-1 832/13 cells and depolarization-evoked increases in $[Ca^{2+}]_i$ were tested with or without membrane raft dispersion. To examine the Ca^{2+} signaling, cells were incubated with the Ca^{2+} fluorophore Fluo-5F, and depolarized with 70 mM K^+ to trigger Ca^{2+} influx and rise in $[Ca^{2+}]_i$. Compared to control cells, the cells treated with CO had higher $[Ca^{2+}]_i$ after K^+ stimulation ($p < 0.001$, $n = 3$; Fig. 3a-c in Paper I). When investigating Ca^{2+} oscillations under resting conditions, the Ca^{2+} spikes were 85% more frequent in CO-treated cells compared to control cells under resting condition ($p < 0.5$, $n = 3$; Fig. 3d-e in Paper I). To determine whether Ca^{2+} stores contribute to this rise, thapsigargin (TG) was used. TG is an inhibitor of the

sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, thus preventing Ca^{2+} entry into the ER. The effect of CO on Ca^{2+} oscillations was also observed in resting TG-treated cells (Fig. 3d & 3f in Paper I).

To check whether the increased $[Ca^{2+}]_i$ is due to altered localization, clustering, or expression of $Ca_V1.2$, INS-1 832/13 cells were transfected with EGFP-$Ca_V1.2$ and subjected, or not, to CO treatment. Interestingly, CO treatment caused disruption of $Ca_V1.2$ clustering on the plasma membrane, but neither localization nor expression were affected ($p < 0.5$, $n = 3$; Fig. 3g-i in Paper I).

CO treatment increases Ca^{2+} influx via Ca_V channels

Ca_V channel activity can either be measured as whole-cell or single-channel currents. The whole-cell current measures the activity of all Ca_V channels in the cell, whereas the single-channel current measures the activity of a single Ca_V channel. To assess the effect of CO on whole-cell Ca^{2+} currents, whole-cell patch-clamp recordings were performed in INS-1 832/13 cells. An increase in Ca^{2+} current was observed with membrane cholesterol oxidation (Fig. 4a-b in Paper I). Next, single-channel recordings were performed to determine whether the increase in whole-cell Ca^{2+} current is due to an increase in number or activity of single Ca_V channels. We confirmed that the latter suggested mechanism was indeed responsible for the CO-induced Ca^{2+} influx, as single-channel activity was upregulated when INS-1 832/13 cells were treated with CO (Fig. 4c in Paper I).

A prolonged elevated glucose disperses membrane rafts

Glucotoxicity is considered a major contributor to T2D pathogenesis. In order to observe the effect of glucose on membrane rafts, INS-1 832/13 cells were subjected to glucose treatment and stained with ATTO-SM. Like T2D islets, the rat β cell line displayed, in a time-dependent manner, a severe reduction in membrane rafts under glucotoxic conditions (Fig. 6 in Paper I).

Discussion

Pancreatic β cells secrete insulin following rapid Ca^{2+} influx by voltage-gated Ca^{2+} (Ca_V) channels. The composition of cholesterol-rich membrane rafts is essential for the function and localization of many proteins, including Ca_V channels. Sphingolipids are constituents of membrane rafts that are synthesized from palmitate and serine. Palmitate treatment has effects on insulin secretion, including increasing basal insulin release [174, 175]. However, palmitate has many effects in the β cell and the increase in basal secretion may be by other means than by disrupted membrane rafts.

Healthy human islets show normal membrane raft expression (Fig. 1). However, once the healthy islets are depleted of cholesterol, they exhibit dispersed membrane rafts. Could high blood glucose influence membrane raft integrity? Indeed, islets from donors with T2D display much lower membrane raft expression when compared to healthy islets (Fig. 1 in Paper I). A study showed that INS-1 cells exposed to high glucose displayed a reduction in membrane cholesterol content and dismantled membrane rafts, further supporting our findings [176].

To understand the role of these rafts in pancreatic β cells, we have disrupted the microdomains using the enzyme cholesterol oxidase (CO). This enzyme selectively oxidizes membrane cholesterol to 4-cholesten-3-one thus dispersing membrane rafts [177, 178]. Despite the numerous studies reporting that insulin secretion is affected by changes in membrane cholesterol content, they are inconsistent. One study has shown that MβCD treatment in rodents promoted insulin release [80]. On the contrary, another study has shown that MβCD treatment lowered insulin exocytosis in mouse β cells [82]. Furthermore, a report demonstrated reduced insulin secretion under conditions of excess cholesterol [179]. Interestingly, MβCD-mediated cholesterol depletion restored insulin secretion under that condition. A possible overarching explanation that would reconcile these divergent observations would be that the relation between cholesterol content and insulin secretion is bell-shaped. When cholesterol is at normal levels, β cells secrete insulin normally. If, however, cholesterol levels were increased or depleted, secretion will be compromised. To further clarify an area with such conflicting results, an alternative tool also used for manipulating membrane cholesterol composition, CO, was utilized. Unlike data from MβCD treatment, CO data are more consistent between human/rat islets and INS-1 832/13 cells.

Basal insulin secretion rises in human/rat islets and β cells when treated with CO (Fig. 2 in Paper I). A similar effect was observed in mouse islets after silencing caveolin-1 (Cav-1), a protein component of specialized rafts called caveolae. Dispersion of membrane rafts was discovered to also increase basal glucagon secretion in α cells. These data further emphasize the importance of membrane rafts in controlling basal insulin exocytosis in β cells. Moreover, exocytotic SNARE proteins require intact membrane rafts to function. We have shown that dispersing the rafts with CO indeed delocalizes the SNARE protein syntaxin 1A from the plasma membrane to the endosomes, at least in INS-1 832/13 cells (Suppl. Fig. 2 in Paper I).

The mechanism behind the effect of membrane raft dispersion on basal insulin secretion is only partially elucidated. We show that Ca^{2+} oscillations under both resting and stimulatory conditions are elevated in cells depleted of membrane cholesterol (Fig. 3d-f in Paper I). Consequently, depolarization-evoked intracellular Ca^{2+} levels are also increased (Fig. 3a-c in Paper I). The reason for this could be an

increase in either the number of active Ca_V channels or an increased activity in every single Ca_V channel. In fact, we found that the increase in Ca^{2+} influx is driven by the longer opening time of each single channel (Fig. 4c in Paper I). This is in line with a study showing that the gating of Kir channels and the current density of N-type Ca_V channels are affected by membrane cholesterol composition [180-182]. Membrane raft dispersion also promotes $Ca_V1.2$ declustering (Fig. 3g-h in Paper I). A way to better understand this is if one regards the SNARE protein complex as a gate-keeper for insulin exocytosis and as clustering agents for Ca_V channels. Hence, a rise in blood glucose could induce membrane raft dismantling leading to a reduced membrane expression of SNARE proteins. In turn, this would result in Ca_V channel declustering and elevated basal Ca^{2+} spikes leading to dysregulated basal insulin exocytosis. In support of this theory, a study in mouse MIN6B1 cells showed that a rise in basal insulin secretion was associated with an elevated cytosolic Ca^{2+} [183]. To conclude, the work presented in this paper has added important insight into the mechanism behind elevated basal insulin secretion upon membrane raft dispersion in pancreatic β cells and islets.

Paper II

Results

TCF7L2 controls expression of Cacna2d1/$\alpha_2\delta_1$

An intronic genomic region close to the *TCF7L2* gene has been identified as the strongest genetic risk factor for T2D. We have previously shown that this transcription factor regulates multiple genes including the Ca_V auxiliary subunit gene *Cacna2d1* [184]. To expand our understanding of this link, we investigated the effect of *Tcf7l2* gene expression on *Cacna2d1*. Rat INS-1 832/13 cells and islets were transfected with siRNA targeting *Tcf7l2*. Indeed, both $\alpha_2\delta_1$ gene expression and protein levels were downregulated in INS-1 832/13 cells and rat islets following *Tcf7l2* silencing (Fig. 1 in Paper II).

Silencing Cacna2d1 prevents trafficking of $Ca_V1.2$ to the plasma membrane

The $\alpha_2\delta_1$ subunit is suggested to be involved in Ca_V channel trafficking and regulation. To study the effect of silencing *Cacna2d1* on $Ca_V1.2$ in insulin-secreting cells, we suppressed *Cacna2d1* and quantified the gene expression and protein levels of $Ca_V1.2$. Interestingly, the reduced levels of $\alpha_2\delta_1$ in rat β cells resulted in reduction of $Ca_V1.2$ surface expression by ~50% while the total amount of $Ca_V1.2$ remained unchanged (Fig. 2 in Paper II). Since one of the roles of $\alpha_2\delta_1$ is Ca_V channel trafficking, $Ca_V1.2$ can be envisioned to be entrapped in the ER after protein

translation, and we therefore investigated the localization of $Ca_V1.2$ in cells lacking $\alpha_2\delta_1$ subunits. However, we found that $Ca_V1.2$ was retained in recycling endosomes, suggesting that the $\alpha_2\delta_1$ subunit mainly acts to translocate the channel from the recycling endosome back to cell surface.

Silencing Cacna2d1 affects Ca^{2+} influx and exocytosis

Regulating Ca_V channel activity is crucial for normal β cell function. To test the effect of *Cacna2d1* suppression on depolarization-evoked Ca^{2+} current, INS-1 832/13 cells with knocked-down *Cacna2d1* gene expression were incubated with the Ca^{2+} fluorophore Fluo-5F and depolarized with 70 mM K^+ to trigger Ca^{2+} influx and consequent rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Silencing *Cacna2d1* or *Tcf7l2* in clonal β cells lowered Ca^{2+} entry leading to a fall in $[Ca^{2+}]_i$. The Ca^{2+}-lowering effect was also observed in cells treated with the anti-epileptic drug gabapentin (Fig. 4A-D in Paper II); this drug is believed to inhibit $\alpha_2\delta_1$ subunit function. To follow the Ca^{2+}-lowering effect further, we voltage-clamped mouse β cells from a -70 mV holding potential to membrane potentials between -50 and 10 mV. Cells that were either *Cacna2d1*-silenced or gabapentin-treated showed a significant reduction (~33%) in whole-cell Ca^{2+} current (Fig. 4E in Paper II). To explore the implication of this on β cell function, we tested insulin secretory function. Upon silencing *Cacna2d1*, INS-1 832/13 cells secreted less insulin under both resting and stimulated conditions (Fig. 4F in Paper II).

Discussion

TCF7L2 is a transcription factor involved in the Wnt/β-catenin signaling pathway and regulates several genes involved in pancreas development, including *ISL1*, *MAFA*, and *PDX1*. It has been reported that TCF7L2 affects insulin secretion in rodent and human pancreatic islets [184]. Moreover, silencing *Tcf7l2* reduces β cell survival and impairs GSIS [185]. We have recently shown that the *Cacna2d1* ($\alpha_2\delta_1$) gene is also regulated by TCF7L2 [184]. The auxiliary subunit $\alpha_2\delta$ is involved in voltage-gated calcium (Ca_V) channel trafficking and gating. For this reason, we hypothesize that $\alpha_2\delta_1$ subunit may contribute to the proper execution of GSIS in pancreatic β cells.

In this paper, we show that $\alpha_2\delta_1$ gene expression and protein levels are downregulated upon *Tcf7l2* silencing (Fig. 1 in Paper II). Since the $\alpha_2\delta$ subunit aids in Ca_V channel trafficking, we explored the effect of $\alpha_2\delta_1$ subunit removal on $Ca_V1.2$ localization. Knocking down *Cacna2d1* in INS-1 832/13 and mouse β cells resulted in decreased $Ca_V1.2$ plasma membrane expression caused by retention of the channels in recycling endosomes (Fig. 2C-E & 3 in Paper II). Our data is in line with work presented in HEK293 cells using a co-expression system [186].

The $\alpha_2\delta$ subunit carries a three arginine (RRR) motif at the von Willebrand factor A (VWA) domain allowing it to bind to the anti-epileptic drug gabapentin. The drug structurally resembles the neurotransmitter GABA but does not bind to GABA receptors. Instead, chronic exposure to gabapentin resulted in reduced surface expression of $\alpha_2\delta_1$ and α_1 subunits leading to lower Ca^{2+} current in mouse neurons [169, 187]. In consonance with this, our data confirmed the inhibitory effect of gabapentin on Ca^{2+} activity and consequently reduced intracellular Ca^{2+} in rodent pancreatic β cells (Fig. 4 in Paper II). The degree of inhibition on Ca^{2+} influx due to gabapentin was similar to that observed in the $\alpha_2\delta_1$ knockdown group (Fig. 4E in Paper II).

The consequence of $\alpha_2\delta_1$ gene expression silencing is a significant reduction in insulin secretion (Fig.4F in Paper II). However, gabapentin treatment had no influence on secretion (data not shown). The reason behind the failure of gabapentin to affect secretion remains unclear. A possible explanation could be the involvement of gabapentin in pathways such as GABA production, that may compensate for the decrease in insulin secretion driven by the loss of $\alpha_2\delta_1$ subunit. GABA may have an autocrine effect on pancreatic β cells by binding to $GABA_A$ receptor which in turn promotes insulin release [188-191]. Seemingly contrasting this notion, we observed a decrease in Ca^{2+} currents when cells were treated with gabapentin. This, however, is due to the nature of the patch-clamp technique where the membrane potential of the cell is controlled by the experimenter, preventing any glucose- and K^+-stimulated depolarizations from taking place. In the case of the insulin secretion experiments, however, the membrane potential of the cell was not voltage-clamped. This means that any effect involving increased production of GABA and an autocrine action via $GABA_A$ receptors should become manifest. In the central nervous system (CNS), $GABA_A$ receptors are considered inhibitory, i.e. they hyperpolarize the post-synaptic neuron. In the β cell, the situation is more complex as the cell is reported to exhibit relatively high intracellular Cl^- concentration ($[Cl^-]_i$), even in the range of 40 - 70 mM. This will result in V_{EQ} ~ -20 - -40 mV i.e. a mild-robust depolarization [192]. Consequently, insulin secretion may be unaffected or even enhanced, in spite of the inhibitory effect of gabapentin on $\alpha_2\delta_1$ subunits. In favor of this view, gabapentin was reported to induce severe hypoglycemia in six patients (diabetic and non-diabetic), suggesting it having insulinotropic effects [193]. In conclusion, TCF7L2 is important for maintaining proper β cell function as it regulates $\alpha_2\delta_1$ subunit, and in turn influences Ca_V channel trafficking and activity.

Paper III

Results

Gene expression of $Ca_V\beta_1$ in rat islets

There are four auxiliary β subunits in the human genome. The expression of these β subunits may vary depending on the tissue being explored. Gene expression analysis confirmed the expression of all β subunits in human islets (Fig. 1B in Paper III). In order to understand the role of the β_1 subunit in pancreatic β cells, we examined the expression level of *Cacnb1* in Wistar and GK rat islets. Islets from the diabetic model showed more than 40% reduction in *Cacnb1* expression (Fig. 1C in Paper III). The results were reproduced by Western blot when rat-derived INS-1 832/13 cells and rat islets were treated with 20 mM glucose or 1 mM palmitate, respectively (Fig. 1D-E in Paper III).

Insulin secretion is reduced in beta cells with reduced $Ca_V\beta_1$ expression

To better understand whether the expression of the β_1 subunit in rat INS-1 832/13 cells influences insulin secretion, we tested the effect of β_1 suppression on β cell secretory function. GSIS was markedly reduced in cells lacking β_1 subunits (Fig. 2 in Paper III).

$Ca_V\beta_1$ is required for maintaining healthy intracellular Ca^{2+} levels

The β subunits have been suggested to regulate Ca_V channel activity, as well as their translocation to the plasma membrane. To find the reason for the disrupted insulin secretion upon β_1 silencing, we measured intracellular Ca^{2+} levels by Ca^{2+} imaging by using the Ca^{2+} fluorophore Fluo-5F. Cells were incubated with Fluo-5F and stimulated with 70 mM K^+ to allow observation of depolarization-evoked intracellular Ca^{2+} elevations by confocal microfluorimetry. We also measured whole-cell Ca^{2+} currents using the patch-clamp technique. Interestingly, Ca^{2+} influx was significantly lowered in the absence of β_1 subunit (Fig. 3C in Paper III). This also resulted in a drastic reduction in K^+-stimulated intracellular Ca^{2+} levels by ~66% in rat β cells (Fig. 3A-B in Paper III). Because β cell function was compromised, β cell survival in Ca^{2+}-depleted environments was explored. When silencing *Cacnb1*, β cells suffered apoptosis which suggests an important role of the β_1 subunit in β cell survival (Fig. 4 in Paper III).

Discussion

The β auxiliary subunit is believed to regulate Ca_V channel trafficking and gating in excitable tissues. However, the mechanism behind this remains unclear. Our gene expression data have shown that *CACNB1* encoding the β_1 subunit is abundant in human pancreatic islets (Fig. 1A-B in Paper III). The expression of *Cacnb1* in GK rat compared to Wistar rat, however, was greatly reduced (Fig. 1C in Paper III). This decrease in gene expression was also observed at the protein level in INS-1 832/13 cells and rat islets following chronic exposure to high glucose and palmitate, respectively (Fig. 1D-E in Paper III). Taken together, these results indicate an intriguing role of the β_1 subunit as a target of detrimental environmental factors that may lead to T2D.

Insulin secretion and β cell survival were both explored after silencing the β_1 subunit. Indeed, the data show a reduction in insulin secretion by ~75% in *Cacnb1*-silenced cells while an increase in apoptosis was observed (Fig. 2 and 4 in Paper III). False elevation of insulin secretion could take place under apoptotic conditions due to cell rupture and leakage. Normally, this is evident as strongly increased basal insulin secretion and loss of regulated insulin release. However, to further minimize the risk of this confounding factor, an additional washing step was added and then insulin secretion was normalized to total protein. Further investigation was made to identify the reason for β cell dysfunction and toxicity. Intriguingly, the patch-clamp and Ca^{2+}-imaging experiments demonstrate that β cells lacking the β_1 subunit had significantly lower Ca^{2+} influx and depolarization-evoked cytosolic Ca^{2+} levels (Fig. 3 in Paper III). This argues against Ca^{2+}-induced cell toxicity, but underscores that Ca^{2+} homeostasis is essential for β cell function and survival. It can be speculated that under diabetic conditions such as hyperglycemia, the β_1 subunit expression decreases in β cells, leading to intracellular Ca^{2+} starvation. In an attempt to restore Ca^{2+} homeostasis, Ca^{2+} from the ER and mitochondria are released into the cytosol. However, in doing so, these compartments deplete their Ca^{2+} stores and trigger stress elements causing apoptosis [194]. In support of this view, depletion of the ER Ca^{2+} stores was shown to trigger ER stress and programmed cell death [195].

Paper IV

Results

Gene expression of $Ca_V\beta_{2a}$ in human and rat islets

Gene expression data showed that among the β_2 isoforms expressed, the levels of the β_{2a} isoform were the highest in human pancreatic islets (Fig. 1A & C in Paper

IV). A comparison in expression levels of β_{2a} between healthy Wistar and diabetic GK rat islets was performed. Since diabetic islets showed reduced β_1 expression, we speculated that the β_{2a} subunit expression would reveal a similar outcome. In support of our hypothesis, GK rat islets proved to express much less *Cacnb2a* compared to Wistar rat islets (Fig. 1B in Paper IV). To examine the effect of high glucose on β_{2a} expression, we performed Western blot on INS-1 832/13 cells following chronic glucose exposure, and could conclude that chronic glucose exposure leads to a decrease in β_2 subunit levels (Fig. 2C in Paper IV).

Palmitoylation is required for $Ca_V\beta_{2a}$ tethering to the plasma membrane

The β_{2a} subunit is post-translationally modified by palmitoylation. We tested whether the state of palmitoylation is essential for β_{2a} function. INS-1 832/13 cells were made to overexpress either wildtype palmitoylatable (β_{2a}; WT) or non-palmitoylatable ($\beta_{2a}^{C\Delta S}$; mutant), GFP-tagged β_{2a} subunit. Whereas the WT β_{2a} was inserted in the plasma membrane, non-palmitoylatable $\beta_{2a}^{C\Delta S}$ was largely localized to the cytosol (Fig. 2A in Paper IV). We then followed this aberrant localization by investigating the cellular localization of the Ca_V channel α_{1C} subunit ($Ca_V1.2$). To this end, we used COS-1 cells, which lack endogenous Ca_V channels. The Ca_V-free COS-1 cells were co-transfected with both α_{1C} and β_{2a} or $\beta_{2a}^{C\Delta S}$ GFP-tagged plasmids. The $Ca_V1.2$ channel was incapable of tethering to the plasma membrane in COS-1 cells expressing the mutant β_{2a} (Fig. 2B in Paper IV). This demonstrates the importance of β_{2a} palmitoylation in regulating Ca_V channel trafficking to the plasma membrane. We further investigated the effect of palmitoylation state of β_{2a} on insulin secretion and β cell death. INS-1 832/13 cells overexpressing WT β_{2a} secreted normal amounts of insulin with respect to glucose. However, overexpressing WT β_{2a} also induced apoptosis, an effect not observed using the $\beta_{2a}^{C\Delta S}$ isoform (Fig. 3E-F and 4F-G in Paper IV).

Overexpression of $Ca_V\beta_{2a}$ upregulates basal intracellular Ca^{2+} concentration

Since β_{2a} palmitoylation state affected $Ca_V1.2$ localization, we further investigated the effect of β_{2a} palmitoylation state on intracellular Ca^{2+} levels. Cells were incubated with Fluo-5F and stimulated with 70 mM K^+ to induce membrane depolarization and Ca^{2+} influx. Using confocal microscopy, we observed a more than 100% increase in basal intracellular Ca^{2+} concentration in cells overexpressing WT palmitoylatable β_{2a}, but not the mutant (Fig. 3A-D in Paper IV). The high K^+-stimulated $[Ca^{2+}]_i$ reveals an important role for β_{2a} subunit in Ca^{2+} homeostasis and β cell survival.

Discussion

T2D is a metabolic disease characterized by high levels of glucose, cholesterol, TG, and palmitate in the blood. Palmitate is a fully saturated free fatty acid (FFA) that has many biological functions such as being a precursor for sphingolipid synthesis and necessary for palmitoylation of proteins. Furthermore, this study demonstrates the capacity of WT palmitoylated β_{2a}, but not the mutant, for proper trafficking of both β_{2a} and pore-forming α_{1C} Ca_V channel subunits to the plasma membrane (Fig. 2A-B in Paper IV). Interestingly, excess of palmitoylated β_{2a} also elevates cytoplasmic Ca^{2+} and induces cellular apoptosis (Fig. 3 in Paper IV). This rise in intracellular Ca^{2+}, however, did not stimulate GSIS (4F-G in Paper IV). A possible explanation for the paradoxical absence of an effect of β_{2a} overexpression on insulin secretion could be due to a downregulated expression of other β subunit isoforms that are required for secretion. This decreased expression could, therefore, counteract the potential increase in insulin release. However, excess membrane expression of palmitoylated β_{2a} upregulates intracellular Ca^{2+} levels, leading to apparent Ca^{2+} overload and toxicity (Fig. 3 in Paper IV). Therefore, the possibility of a counteracting effect on insulin secretion by other β subunits cannot explain this secretion-ineffective rise in cytosolic Ca^{2+}.

Another possible explanation for the failure of β_{2a}-mediated increases in intracellular Ca^{2+}-signals to stimulate insulin secretion is a β_{2a} interaction with dynamin. Dynamin is suggested to be important for normal endocytosis and exocytosis, and thereby insulin secretion [196]. It has been shown that the conserved SH3 domain in β subunits could bind to dynamin [197]. It could be proposed that palmitoylated β_{2a} interacts with dynamin leading to either a partial inhibitory effect on insulin exocytosis or a 'kiss-and-run' where the insulin granule alternates between exo- and endocytosis without fully releasing its insulin content.

Interestingly, it has been reported that ER stressors such as cytokines and thapsigargin could increase cytosolic Ca^{2+} while having minimal or no effect on insulin secretion [198]. On the other hand, metabolic stressors such as FFAs and high glucose are able to increase both intracellular Ca^{2+} and insulin secretion. One could speculate that excess palmitoylated β_{2a} could trigger ER stressors via a mechanism involving the palmitoylated state of β_{2a} since $\beta_{2a}^{C\Delta S}$ had no effect on cytosolic Ca^{2+}. What we can conclude with certainty is that the palmitoylation state of β_{2a} can influence intracellular Ca^{2+} levels and excess of the palmitoylated subunit induces β cell death.

Conclusion

1. The work presented in Paper I has partly explained the mechanism behind elevated basal insulin secretion upon membrane raft dispersion in pancreatic β cells, a phenomenon that appears relevant for the increased basal insulin secretion observed in T2D. Membrane rafts are required for proper clustering of $Ca_V1.2$ and surface expression of the SNARE protein syntaxin 1A. Declustering of $Ca_V1.2$ is suggested to lead to dysregulated basal Ca^{2+} oscillations and increased single-channel activity and hence a rise in basal insulin release.
2. The work presented in Paper II revealed the importance of TCF7L2 in maintaining proper β cell function as it regulates $\alpha_2\delta_1$ subunit expression, and in turn influences Ca_V channel trafficking and activity. Cells lacking $\alpha_2\delta_1$ subunit show reduced surface expression of $Ca_V1.2$ and decreased Ca^{2+} currents, leading to reduced insulin secretion.
3. The work presented in Paper III concluded that β_1 subunit expression is required for Ca^{2+} homeostasis. Cells with low β_1 subunit expression exhibit not only low Ca_V channel activity and impaired insulin secretion, but also apoptosis. Thus, the expression of β_1 subunit is essential for β cell function and survival.
4. The work presented in Paper III showed that the palmitoylation state of β_{2a} subunit is important for proper Ca_V channel trafficking and activity. Elevated expression of palmitoylated β_{2a}, however, can induce cell death via elevated cytosolic Ca^{2+}. Therefore, the palmitoylation state of β_{2a} may also affect β cell survival.

Future Perspectives

Introduction

Ca_V channels play a crucial role in regulating basal and stimulated insulin secretion in β cells. The α_1 pore-forming subunit of Ca_V channels is believed to depend on auxiliary subunits not only for membrane trafficking but also for channel activity. In this thesis, novel roles for the auxiliary subunits $\alpha_2\delta_1$, β_1, and β_{2a} in controlling β cell Ca^{2+} homeostasis, insulin secretion, and survival were identified.

Membrane rafts and exocytosis

To better understand the mechanism behind the elevation in basal secretion following membrane raft dispersion, the members of exocytotic machinery should be further investigated. The expression, localization, and clustering of SNARE proteins such as syntaxin 4, SNAP-25, and synaptotagmin-7 may be explored by implementing qPCR, immunohistochemistry, and immunoblotting. Furthermore, using other cholesterol- or sphingolipid-depleting agents such as nystatin and myriocin to disrupt these rafts might help in better understanding the role of membrane raft composition in regulating β cell function.

Ca_V channel regulation by $\alpha_2\delta_1$

More studies are needed to further elucidate the role of $\alpha_2\delta_1$ subunit in regulating Ca_V channels. For example, the $\alpha_2\delta$ subunits are believed to anchor on the cell surface via GPI-anchoring in membrane rafts. It would be interesting to explore the role of membrane rafts in regulating $\alpha_2\delta_1$ subunit and the implications on β cell function. In addition, the $\alpha_2\delta_1$ subunit affects whole-cell Ca^{2+} currents. However, to further explore this effect single-channel and capacitance measurements would be considered.

The data in Paper II are based on reducing *Tcf7l2* or *Cacna2d1* expression. It would be of interest to investigate the effect of overexpressing *Tcf7l2* on *Cacna2d1* and in turn the effect on Ca_V channel trafficking and activity. Since TCF7L2 is associated with T2D, it would be interesting to investigate the role of elevated glucose and FFAs on the expression and localization of $\alpha_2\delta_1$ subunit.

β_1 subunit and insulin secretion

To further explore the effect of silencing β_1 subunit on Ca^{2+} signaling, it would be interesting to dissect the cause for the decrease in whole-cell Ca^{2+} currents by using single-channel measurements as in Paper I. Moreover, the role of the β_1 subunit in Ca_V channel trafficking could also help elucidate the Ca^{2+} signaling effects. If one would follow the effect of β_1 subunit on β cell survival, exploring cellular stress pathways such as ER and mitochondria stress may be considered. It is possible that *TCF7L2* not only regulates $\alpha_2\delta_1$ expression but also the expression of the β subunits. Lastly, β_1 overexpression could be applied in order to identify a potential rescue effect, developing our understanding of the role of β_1 subunit in β cells.

β_{2a} and Ca^{2+} overload

To explore the effect of β_{2a} on Ca^{2+} signaling, patch-clamp techniques could be utilized to determine the whole-cell and single-channel activity. Moreover, the absence of effect of β_{2a} on insulin secretion could be further investigated via capacitance measurements that would reflect exocytotic activity. These experiments would explain whether the rise in intracellular Ca^{2+} is due to the number or activity of single Ca_V channels, and whether exocytosis is compromised or not. Lastly, the hypotheses presented earlier, dynamin and ER stressors, are also worth investigating to better understand the role of β_{2a} and its palmitoylation state in β cell function and survival. It is also important to confirm the results from Paper I-IV using animal models and human islets to better relate to our physiology.

TCF7L2 and membrane rafts

The transcription factor TCF7L2 is harbors the strongest genetic risk factor for T2D, and hence, investigating this transcription factor is key to understanding the disease. TCF7L2 is regulated by the Wnt/β-catenin signaling pathway and a decrease in this signaling could potentially lower insulin secretion and cause β cell death. Interestingly, disruption of the membrane raft results in decreased Wnt/β-catenin signaling in HEK293 cells [199]. Indeed, as shown in Paper I, dispersion of membrane rafts increases basal insulin release, which is an indication of dysregulated granular exocytosis of a type that bears resemblance with the phenotype observed in T2D. Therefore, further studies are encouraged to better understand the link between membrane rafts, Wnt/β-catenin signaling, and TCF7L2.

Final remarks

In conclusion, this thesis has demonstrated that Ca_V channel auxiliary subunits are targets of environmental stressors relevant to T2D. Their altered expression leads to impaired β cell function and consequently increases the risk of T2D. Gene auxiliary subunits, namely $\alpha_2\delta_1$, are also targets for genetic risk factors of T2D. Taken

together, understanding the role of Ca_V subunits in T2D can ultimately lead to better preventive strategies or improved T2D treatment.

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